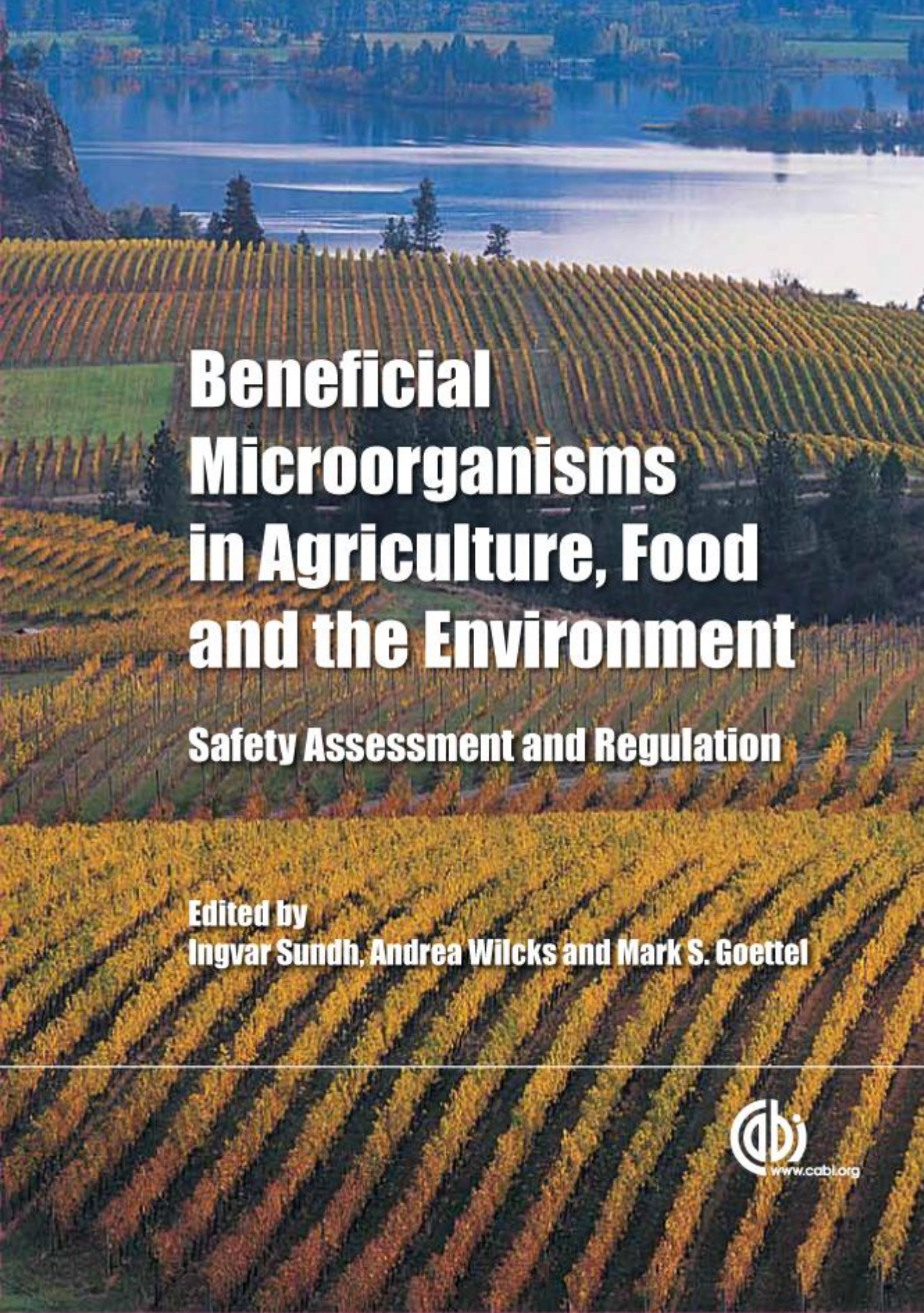
Beneficial Microorganisms in Agriculture, Food and the Environment
Safety Assessment and Regulation
Edited by
Ingvar Sundh, Andrea Wilcks and Mark S. Goettel
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Beneficial Microorganisms in Agriculture, Food and the Environment: Safety Assessment and Regulation

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Preface

Microorganisms have been vital in the evolution of life on earth and they play paramount roles in the environment as well as for humankind. The earliest examples of active human exploitation of their activities are microbes that were 'domesticated' thousands of years ago for use in bread, alcohol fermentations and dairy products. Alongside societal development and strong growth in our understanding of the nature and properties of microorganisms, and the ability to culture single microbial isolates, several additional fields of application have surfaced within the agricultural, environmental and biotechnological sectors. However, with the realization during the last two centuries that many serious diseases are due to microorganisms causing infections or producing toxic compounds, it has become obvious that large-scale culture, marketing and utilization of single isolates of living microorganisms necessitate a careful safety assessment. Thus, a microorganism cannot be considered truly 'beneficial' until a certain level of safety has been established. As a consequence, many types of applications with microorganisms are presently subject to various regulatory measures.

The introduction of regulatory systems can have consequences for the inclination of research entrepreneurs and industry to venture into new projects with beneficial microorganisms. Long and costly registration procedures that are not commensurate with actual risks can unnecessarily hamper the development of useful microbiological products. Conversely, lack of appropriate regulation could potentially result in serious negative consequences for public health or for the environment. Many different types of legislation are applicable to the marketing and use of microbiological products. General acts of legislation – e.g. regarding consumer, occupational or environmental safety – cover the marketing and use of beneficial microorganisms, and state that the producer or manufacturer of a product is responsible for an adequate safety assessment. In other specific cases, the submission of a data dossier followed by authorization is a prerequisite for market introduction.

The idea for this book emerged as a sequel to the conference '*Microbes and the Law – Setting the Limits for Practical Use*' (http://www.mistra.org/program/dom/home/pressroom/newsarchive/news/microbesandthelawsettingthelimitsforpracticaluse.5.6b38234911d6cedb125800039217.html; accessed 12 January 2011), which was arranged by the research programme DOM (Domestication of Microorganisms: http://www.mistra.org/dom; accessed 12 January 2012) and was held at SLU (Swedish University of Agricultural Sciences) in Uppsala, Sweden, on 5–9 October 2008. Experts from science and industry, as well as from authorities responsible for the regulatory oversight of microorganisms, made presentations on recent

developments in legislation and policy making as well as on new strategies for safety assessments of individual microbial isolates. A specific goal of the meeting was to create a forum for exchange of ideas and expertise across different scientific disciplines and fields of applications with microorganisms, because, to the best of our knowledge, that had never been done before.

The book does not aim to present a detailed account of regulations and data requirements or authorization processes for the many possible ways of utilizing microbes. As the main impetus for regulating microorganisms is to reach an acceptable level of safety with respect to human health and environment, the main aim of the book is instead to critically examine the safety principles that have governed the development of regulations in representative countries, and to what extent these correctly mirror actual hazards and risks. After an introductory chapter by the editors, the book starts with chapters treating regulatory systems for typical application areas in representative jurisdictions. These are followed by others discussing various approaches for assessing the safety of microbes and their utilization, as well as new methodologies to determine pathogenicity, virulence and toxicity. Special chapters examine how international initiatives and agreements as well as public 'risk perception' can influence the implementation of microbial solutions to environmental or agricultural problems. We hope and believe that taking this generic approach will make this book useful to anyone interested in these topics, in any part of the world. The book is aimed at researchers (in academia as well as industry), postgraduate university students, regulators in governmental authorities responsible for risk assessment and authorization of microorganisms, personnel responsible for safety in microbiological laboratories, and non-governmental organizations within the agricultural, food and biotechnological sectors.

This book could not have materialized without input from several different scientific and regulatory disciplines. Such a cross-disciplinary work is, by necessity, dependent on contributions from experts within a broad range of topics. Our sincere thanks are extended to all the authors who have contributed to this publication, which is the first in-depth treatment of safety assessment and current regulations and policies for microorganisms across the major types of their application. It is our hope that this cross-application approach will lead to the employment of more relevant, efficient and harmonized systems for safety assessment and regulation of microbial products, thereby further facilitating safe utilization of microorganisms to the good of humankind as well as the environment.

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January 2012

1 Microbes and the Law – Safety Assessment and Regulation of Beneficial Microorganisms

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1.1 Microorganisms are Vital in Evolution and the Main Drivers of Environmental Resilience

1.1.1 Some facts and figures

Microorganisms have been vital in the evolution of life on earth. The earliest life forms were all unicellular, prokaryotic microorganisms; eukaryotic multicellular organisms appeared relatively late in the course of evolution. Modern phylogenies based on gene sequences reveal that microbial life forms make up the majority of the known biodiversity on earth (Woese *et al*., 1990; Pace, 2009). A predominant part of these life forms are known only from sequences obtained from 'environmental' DNA and have never been cultured (Rappé and Giovannoni, 2003; Alain and Querellou, 2009). Numerous examples illustrate that microorganisms colonize any available habitats of the biosphere. For example, 1 ml of lake water and 1 g of topsoil typically harbour 10^6–10^7 and 10^8–10^9 prokaryotic cells (bacteria and archaea), respectively (Paul and Clark, 1989; Sundh and Bell, 1992). It has been estimated that, globally, the standing stock of prokaryotes (dominated by open ocean, soil and marine and terrestrial subsurfaces) constitute 60–100% of the total carbon in plants on earth and about tenfold more of phosphorus and nitrogen than do plants

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(Whitman *et al.*, 1998). Fungi and protists are not even included in these direct estimates of standing biomass and nutrient pools, but are generally considered to contribute less than prokaryotes in most ecosystems (Fierer *et al.*, 2009). Aboveground plant compartments are substrates for epiphytic and endophytic microorganisms (Beattie and Lindow, 1999; Bayman, 2006; Whipps *et al.*, 2008), and invertebrate animals harbour large numbers of microorganisms in their gastrointestinal tracts (König, 2006; Brinkman *et al.*, 2008).

Collectively, microorganisms stand for an extremely wide metabolic versatility and are the main drivers of indispensable biogeochemical processes and transformations (Falkowski *et al.*, 2008). For instance, microbes are essential for the degradation of organic matter in soils and other ecosystems and, thereby, for nutrient recycling. Photosynthetic cyanobacteria and eukaryotic micro-algae provide the majority of the primary production of organic matter from CO_2 in the upper water horizons of oceans and lakes, and in so doing have a major role in the global carbon budget (Chavez *et al.*, 2011).

1.1.2 Human life is intertwined with the lives of microbes

Human life is intimately connected with the activities of microorganisms. Without any need for active intervention, we constantly live with microorganisms and by the services they provide. Not only is humanity dependent on the way that microbes shape and maintain essential functions in the environment, including agricultural production systems (i.e. they provide what has been coined 'ecosystem services'; Ducklow, 2008), but also on the direct contributions of microbes within our bodies. This is because, in common with other vertebrate as well as invertebrate animals, our well-being depends on establishment of a balanced and functioning microbiota in the gastrointestinal tract (Eckburg *et al.*, 2005). The intestinal tract of an adult human contains a factor of 10–100× more bacterial cells than the total number of body cells and 150× more microbial genes than there are in the total human genome (Zhu *et al.*, 2010). Human skin and mucous membranes are also inhabited by microorganisms, normally without any adverse effects on health (Cogen *et al.*, 2008; Grice and Segre, 2011).

1.1.3 Actively taking advantage of microbes

That humanity actively takes advantage of specific microorganisms and the beneficial products of their metabolism is not new. The earliest documented examples come from thousands of years ago, when yeasts and lactic acid bacteria were used in the preparation of bread, alcoholic beverages, and dairy products such as cheese (Caplice and Fitzgerald, 1999; Fox and McSweeney, 2004). By specific treatment and storage conditions of the raw materials, spontaneous development of particular microbial consortia could be stimulated, giving the food special, desired properties. In these early times, little was known about the nature of microorganisms and single strains could not be cultivated in pure form and could thus not be added for specific purposes as such.

The invention of the microscope by Antonie van Leeuwenhoek by the end of the 17th century and the substantial progress in understanding of the biology of microorganisms during the 18th and particularly the 19th century made it possible to obtain, cultivate and study single isolates. Subsequent development of knowledge of the ecology, metabolic diversity and molecular biology of microorganisms during the 20th century paved the way for uncovering the great range of possibilities of utilizing microbes for specific purposes. By this time, it was possible not only to stimulate spontaneously occurring microbial communities and consortia, but also to take single, well-characterized isolates into consideration. For instance, microorganisms can be used for many types of beneficial applications, as listed in Box 1.1 and exemplified in Figs. 1.1–1.4.

1.2 Identifying the Potential Hazards of Microorganisms

In spite of the indispensable roles of microorganisms in the maintenance of ecosystem functioning and human life, microbes can

Box 1.1. Examples of various areas of beneficial applications with microorganisms, each with a few references for further reading.

1. Food and feed, including probiotics for humans and animals (example in Fig. 1.1)
(Havenaar and Huis in't Veld, 1992; Weinberg and Muck, 1996; Caplice and Fitzgerald, 1999; Fox and McSweeney, 2004).
2. Antagonistic or pathogenic microbes for biological control of pests and diseases (examples in Figs 1.2–1.3)
(Evans, 2000; Inglis *et al.* 2001; Haas and Defago, 2005).
3. Addition of microorganisms as bioprophylactics to minimize the emission of chemical pollutants or for bioremediation of soils or sediments
(Bouwer and Zehnder, 1993; Alexander, 1999; de Lorenzo, 2008).
4. Plant growth-promoting microbial agents for, e.g. strengthening plant stress resistance or nutrient uptake
(Kloepper *et al.*, 1980; Glick, 1995; Moulin *et al.*, 2001; Preston, 2004).
5. Production of biofuels by microbial degradation of various waste fractions
(Ahring, 2003; Hahn-Hägerdal *et al.*, 2006; Karakashev *et al.*, 2007; Weber *et al.*, 2010).
6. Biotechnological use of microbes for production of specific metabolites, enzymes, etc. (example in Fig. 1.4)
(Lee *et al.* 2004; Chou, 2007; Ruiz *et al.*, 2010).
7. Fermentation of microbes for obtaining bulk biomass for use as, e.g. animal feed or production of biofuel
(Kiessling and Askbrandt, 1993; Alper and Stephanopoulos, 2009; Romarheim *et al.*, 2011; Shi *et al.*, 2011).
8. Medical use of microorganisms, e.g. for treatment of serious intestinal disorders
(Tvede and Rask-Madsen, 1989; Cain and Karpa, 2011; Landy *et al.*, 2011).

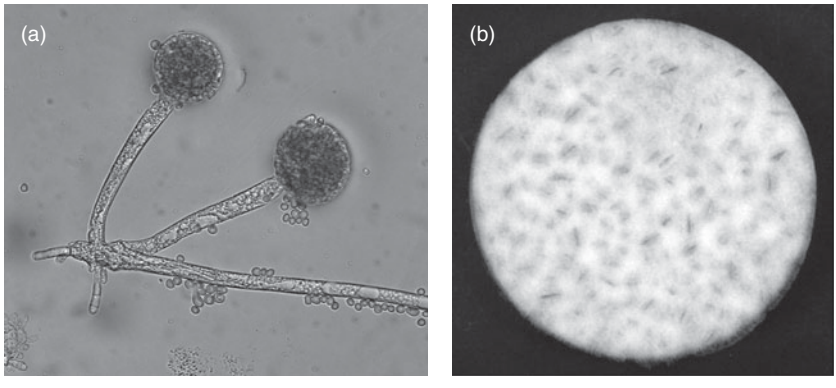

Fig. 1.1. The micrograph (a) shows sporangiophores and sporangiospores of the filamentous fungus *Rhizopus oligosporus*, which is used for preparation of the traditional Indonesian meal tempeh from fermented soybeans. To the right (b) is a petri dish containing barley tempeh made by the inoculation of barley with *Rhizopus oligosporus*. The fungus has completely covered the barley kernels. Photos: Xin-Mei Feng.

also have strong negative impacts. For instance, it has been estimated that 5–10% of the world's food production is destroyed by the growth of spoilage fungi and/or bacteria (Pitt and Hocking, 1999; Gram *et al.*, 2002), with substantially higher losses in less technically developed countries. Besides causing a general loss of food and feed commodities, a variety of spoilage microbes produce toxic compounds. Mycotoxin production by fungi (Richard, 2007) is highly significant in this respect, e.g. the carcinogenic aflatoxins and ochratoxins produced by some *Aspergillus* and *Penicillium* moulds. Other spoilage

Fig. 1.2. Barley seed treated with the biological control product Cedomon® at a seed treatment facility in Sweden. Cedomon contains the bacterium *Pseudomonas chlororaphis* and controls soil-borne fungal diseases in the growing barley.

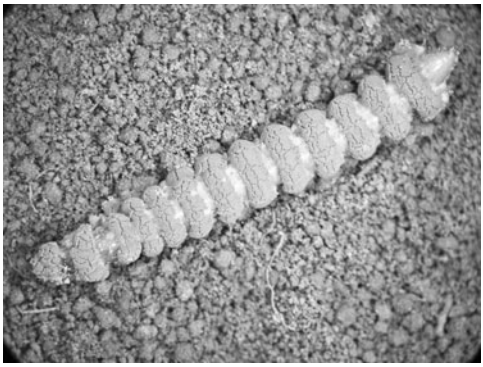

Fig. 1.3. A wireworm (order Coleoptera, species *Agriotes obscurus*) infected with the insect pathogenic fungus *Metarhizium anisopliae*. This fungus is the active agent in biological insect control products in different parts of the world, e.g. North America, Latin America, Australia and Ukraine. Photo: Todd Kabaluk.

microbes can cause food-borne disease, e.g. *Salmonella* spp. (Velge *et al*., 2005) and *Bacillus cereus* (Schoeni and Wong, 2005). Additionally, the risk of microbial spoilage of food and feed products make special, energy-demanding, arrangements such as drying or storage under cool or frozen conditions necessary. Mould growth can also contribute to the deterioration of many other types of processed materials, e.g. in buildings, and thereby to fungal infestation of houses and the exposure of people to mycotoxins (Gravesen *et al*., 1999).

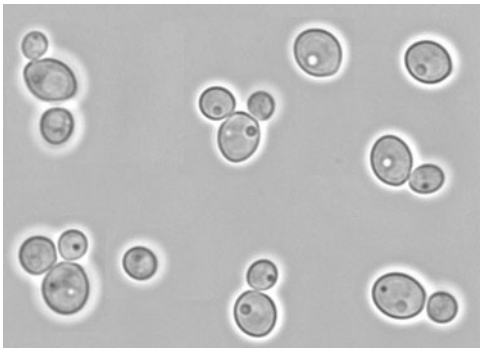

Fig. 1.4. Vegetative cells of the domesticated yeast *Pichia anomala*. This yeast is used for the production of phytase. Additionally, some isolates have antifungal activities and have shown promise for the postharvest biocontrol of spoilage fungi on cereals and fruit. Photo: Inger Ohlsson.

The advancement in the understanding of the nature of microorganisms during the 19th century came in close parallel to the awareness that microbes were the agents that caused many devastating diseases. Pathogenicity in a microbe is not in itself a factor driving adaptation and evolution, and a current view is that it is not possible to define a specific 'pathogenic' lifestyle (Falkow, 2008). Rather, the wide ecological and metabolic diversity in microbes, their relatively short generation times and their ability to rapidly adapt to and colonize highly specific niches, including specific compartments of animals, humans and plants, allow some microorganisms to cause disease. More specifically, examples of attributes that may confer pathogenicity on a microorganism are those that allow entry into the host, the attainment of a unique niche, avoidance of host defence mechanisms, relatively high rate of multiplication and ability to exit the host and survive outside it (Falkow, 2008). Although less obviously than for bacteria and fungi, these general principles also apply to endosymbiotic protozoans, of which some are parasitic and cause serious human diseases. Examples of the latter are the Apicomplexa, species of which cause malaria (*Plasmodium palcifarum*), toxoplasmosis and cryptosporidiosis. These organisms often have complex life cycles, including additional hosts, and stages that

survive and/or proliferate in environmental compartments.

The fundamental hazards that need to be considered for the utilization of any new microorganism are often similar for different taxonomic groups, and are to some extent independent of the intended type of application. With respect to adverse effects on humans, microorganisms can cause infectious disease, toxicity and/or allergenicity. Lately, the risk that the use of probiotic microorganisms will contribute to further spread of resistance to antibiotics used in human or veterinary medicine has also been emphasized (EFSA, 2008). In natural environments and agricultural production systems, microbes can cause infection or toxicity in non-target organisms, and this, in turn, can lead to competitive exclusion and changed community species composition that have effects on biodiversity and ecosystem function (Cook *et al*., 1996; Winding *et al*., 2004; Mensink and Scheepmaker, 2007).

1.3 Assessing the potential risks of microbial applications

In the context of safety assessments of microorganisms, 'hazard' stands for a fundamental, specific adverse effect, while 'risk' is a quantitative measure of the magnitude of the adverse effect. In risk assessment, the risk is estimated as the product of the probability and the severity of a hazard. A full risk analysis additionally includes risk management (how to mitigate the risks) and risk communication (how to inform others) (Byrd and Cothern, 2000). Before the large-scale cultivation and utilization of microorganisms, it must be ensured that they do not have unacceptable side effects on people (during the whole chain from production to handling and distribution to end use) or in natural or managed ecosystems.

Most discoveries of potentially useful properties in microorganisms are made for species not belonging to known human pathogens or toxin producers. Hence, in contrast to the fields of biosafety and biosecurity (see Knutsson *et al*., 2010 for a discussion of terms and definitions), which concentrate on measures for protection against harmful organisms, for beneficial microorganisms, the issue is approached from another angle: how can it be demonstrated that the organism is *not* harmful? For example, within the food sector, 'microbiological risk assessment' deals with risks that microbial pathogens may contaminate food and thereby affect human populations (Forsythe, 2002). In this case, the hazard is distinctly defined and the approach for risk assessment is fairly straightforward in the sense that it can be based on the capacity for spread and the growth properties of the pathogen. However, this approach for risk assessment cannot be easily practised on new isolates showing antagonism against pathogens which therefore have potential use as, e.g. human probiotics or biocontrol agents against plant pathogens, owing to the fact that the potential hazards are not easily identified.

Clearly, to conduct a relevant risk assessment of a specific way of utilizing a certain microbial isolate, a thorough identification of the isolate at least to species level is imperative. This must be followed by a characterization of the organisms' basic biological and ecological properties. Another factor that should not be overlooked is that many beneficial microbes exert their activities in target habitats housing resident microbial communities. Thus, to assess the *added* risk to humans or other non-target organisms from exposure to the microbe during production and use, knowledge is also required of the background exposure to other microorganisms in that particular environment (including any identical or similar strains).

1.4 Regulating the Use of Beneficial Microorganisms

1.4.1 Why are some beneficial microorganisms regulated?

From a societal view, the objective of regulating the use of microorganisms is to provide protection for humans and the environment from potential harm. It is essential that regulations are tuned to the magnitude of the potential risks involved. On the one hand, insufficient attention to

the potential risks can result in harm to humans, natural environments and agricultural production systems. Over-regulation, on the other hand, can unnecessarily stifle the implementation of new, microbial solutions to various health and environmental problems. Of the various acts of legislation that cover the utilization of microorganisms, the majority have their fundament in the way that the organisms are used, rather than in the organisms' phylogeny or biological and ecological properties. This is reflected in the structural outline of this book, which has separate chapters for various fields of application. Exceptions to this are national 'quarantine' legislations, which attempt to prevent the import and spread of known human, animal or plant pathogens (or goods with which they can be inadvertently transported). Such measures can possibly prevent the export/import of beneficial microorganisms across national boundaries, and were among the first regulations that potentially affected the utilization of microorganisms. The earliest example we have been able to find of registration of a microorganism for use in a specific application is that of the insect pathogenic bacterium *Paenibacillus popilliae* (formerly *Bacillus popilliae*) for controlling populations of the Japanese beetle (*Popillia japonica*). This was registered as a pesticide in the USA under FIFRA (the Federal Insecticide, Fungicide and Rodenticide Act) in 1948 (Cook, 1996).

1.4.2 Different types of regulation affect the opportunities for utilization of microbes

Different regulatory systems can have implications for the potential use of beneficial microorganisms:

1. Various acts of general legislation regarding consumer, occupational or environmental protection apply to the production, marketing and use of many types of products, including microorganisms.
2. Different quarantine legislations aim to restrict the export/import of microbial cultures or of goods which may contain pathogenic microbes.
3. For some areas of application, regulations for a specific group of products encompass living microorganisms along with other types of substances, e.g. the marketing of microbial biocontrol agents is regulated, as is marketing of chemical pesticides.
4. There are international conventions and agreements regarding proprietary issues and the exchange of biological resources or the fair and equitable sharing of the benefits arising from their exploitation.

Some regulations generally dictate that producers and manufacturers are responsible for determining that adequate safety assessments have been performed (mostly point one above), while others stipulate that premarket authorization (point three) or approval of the import/export (points two and four) of new microbial agents and products is required. As a rule, the chain from basic research on microbe and product development to marketing, and thence to the final utilization of a new microbiological product, will involve several of these different types of regulatory systems.

1.5 Safety Assessment and Regulation of Beneficial Microorganisms: an Overview

This book covers numerous aspects associated with the safety and regulation of beneficial microorganisms. In Part I, Chapters 2–5 treat the use of microbial cultures in food and feed. Over time, the approach to utilization of microbiological processes in the preparation and storage of food has been one of trial and error, and based on previous experience. Lately, live microorganisms have been formulated as probiotics, given in feed to domesticated animals and consumed by humans. Chapter 2 gives an outline of how new systems were introduced during the second half of the 20th century for the regulatory oversight of microbial products within food or feed, while Chapter 3 contains an analysis of the potential risks connected with the use of microorganisms in food or feed – including their use as probiotics – and a discussion of to what extent current regulations meet

these safety requirements. In this food and feed context, the European QPS (Qualified Presumption of Safety) system is considered and compared with the GRAS (Generally Recognized As Safe) system in the USA. These systems are intended to make regulation easier, but are there things that are overlooked and can the systems be extrapolated to other areas, e.g. in evaluations of microbial plant protection agents? Chapter 4 treats the difficult and debated issue that the use of probiotic microbes might increase the risk that antibiotic resistance elements are transferred to the indigenous microbiota of the gut, and subsequently to pathogenic bacteria. Finally in Part I, Chapter 5 gives an overview of safety issues in the commercialization of microbial post-harvest biocontrol agents for food or feedstuffs, and presents in greater detail the challenges posed by the regulatory requirements for their registration.

For this first part of the book on food and feed, an outline structure also employed in other parts is introduced, whereby an introductory chapter first gives an overview of the topic and the relevant regulatory systems. The following chapters then treat specific safety issues in connection with the particular types of applications or the potential of certain approaches and methodologies used in safety assessment, and discuss to what extent current legislation reflects actual risks or whether new methodology can aid in safety assessment and satisfy current regulatory demands.

Microbial pesticides have an important role to play in sustainable protection against pest organisms and diseases in agriculture and forestry, and this is the subject of Part II. Most countries have implemented strict regulations for microbial biocontrol agents, following similar principles to those used for chemical pesticides. Chapter 6 introduces the regulatory systems for microorganisms used to manage pest populations and plant diseases. However, risk assessments of microbial biocontrol agents cannot solely rely on the rationales used for the risk assessment of chemical pesticides, because microorganisms are living creatures. Following this introduction, Chapters 7–9 outline the specific challenges posed in the safety assessment and registration of microorganisms for the control of invertebrate pests, plant diseases and weeds. These chapters also present and discuss safety aspects in relation to the different basic approaches of biological control, i.e. classical, inoculation, inundation and conservation biocontrol. Unlike microbial agents for the control of plant diseases, there have been few specific regulations in place for plant growth promoting microsymbionts and plant-associated bacteria or fungi, e.g. N-fixating root nodule bacteria, vesicular arbuscular mycorrhizal fungi or root rhizosphere competent bacteria. Chapter 10 evaluates the potential risks from the use of these types of microorganisms, and to what extent general regulations concerning spread of plant diseases or protection of biodiversity affect their utilization.

Collectively, microorganisms possess a staggering ability for the degradation of organic compounds, including various xenobiotic pollutants, and for the biological treatment of different types of organic waste, e.g. in sewage water treatment or anaerobic digestion for biogas production. Although specific regulation of these applications with microbes is rare, Chapter 11 (the sole chapter in Part III) gives an overview of general regulations that may apply to microbes to be used for biodegradation and other industrial uses, e.g. regarding the environmental safety of new substances or organisms.

How can the risk that a novel microbial isolate may cause unacceptable harm to humans or the environment in a wide sense be determined? This is the subject of Part IV of the book. Chapter 12 features an overview of the most urgent potential safety issues, general approaches for addressing these in evaluations of particular strains, and general regulations addressing the use of wild-type as well as genetically modified microorganisms (GMMs) in contained laboratory settings or for environmental release. Determining the absence or presence of certain genes encoding virulence factors can be a tool in the safety assessment of beneficial microorganisms. The molecular approach to microbial pathogenesis has resulted in a huge amount of data on bacterial virulence genes. Chapter 13 summarizes the changing concept of bacterial virulence and the detection of and identification strategies for the recognition of potential

virulence genes in bacterial genera used as probiotics. What are the potentials and limitations of using the presence of virulence genes in predictions of pathogenicity? Workers and operators might become exposed to high numbers of these organisms when producing and manufacturing beneficial microorganisms. Chapter 14 summarizes studies that have investigated the exposure to beneficial microbes under realistic use (e.g. microbial plant protection agents) and presents different risk classification systems for microorganisms. What is the risk of this exposure compared with the total exposure to microorganisms in the studied environments?

Bioassays utilizing various biosensor organisms have a role to play in determination of the toxicity, infectivity and sensitizing properties of microorganisms, and new approaches with invertebrates, cell lines or other microorganisms can potentially replace studies using mammals. This is the subject of Part V. Chapter 15 gives an overview of the advantages and limitations of these approaches for testing microorganisms, and whether they could fulfil the regulatory requirements for new microbial products. In the following Chapter 16, there is a thorough discussion of nematode and insect models currently used for interpreting microbial infectivity and virulence. Many virulence factors required for pathogenicity in mammals are also important for bacterial and fungal survival when they interact with invertebrates, and the host innate immune responses are broadly conserved across many phyla. What is the potential of nematode and insect models in becoming predictive tools in safety assessments of beneficial microorganisms? Apart from virulence and potential pathogenicity, evaluation of the potential acute cytotoxicity is often part of the data requirements for the approval of new microbial products. Chapter 17 discusses the possibilities of employing standardized toxicity tests utilizing arthropods or protozoans as biosensors for screening microbial strains for toxin production. Which biosensor organisms and test strategies would be suitable and could they satisfy regulatory data requirements? Some data requirements also address determinations of the potential genotoxic, sensitization and irritant properties of microorganisms. No specific methodologies for determining these in microorganisms are available, and Chapters 18 and 19 present a critical discussion of whether *in vitro* or *in vivo* approaches for specific compounds, or the use of crude cell extracts, could be useful for testing microorganisms for genotoxic and allergenic potential, respectively.

Many international initiatives and collaborative efforts at the political, industrial, governmental or non-governmental levels (e.g. the United Nations Convention on Biological Diversity) may have an impact on opportunities for international trade and the subsequent use of beneficial microorganisms, and these are covered in Part VI. Chapter 20 investigates whether this situation is warranted from the standpoints of the fair and equitable sharing of the benefits from biological resources, and how the issue can be addressed in the future. Conversely, other international efforts are ongoing to harmonize the way in which microbial pest control agents are regulated in different parts of the world. The Organisation for Economic Co-operation and Development (OECD) has established the BioPesticide Steering Group (BPSG) to address this subject, and Chapter 21 gives an overview of the current international discussion regarding microbial pest control agents, and how the work by BPSG can facilitate registration processes.

The registration of beneficial microorganisms presents a great challenge to regulatory bodies in these times of strong risk aversion in the general public. Chapter 22 outlines recent studies on the perception by the general public of the risk from microorganisms and biotechnology in broad terms, taking both human safety and environmental protection into account. What steps can governments and regulatory agencies take to avoid the fact that unfounded perception of risk unnecessarily hampers the implementation of new products with beneficial microbes?

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2 Safety and Regulation of Microorganisms Added to the Food and Feed Chains, Including Probiotics – Introduction and Overview

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2.1 Introduction

Whether beneficial microorganisms ever reach the consumer is determined by a few – but very decisive – factors. Probably the single most decisive of these is how the appropriate authorities regulate the microorganisms. This chapter will show how the way of regulating these microorganisms has developed to where it is today. The chapter has its primary focus on the European Union (EU), while developments in the USA and in Singapore are also elucidated. Although other regions of the world are not dealt with, it goes without saying that regulatory decisions made for the enormous food and feed markets in the EU and the USA inevitably have an impact on decisions made for food and feed markets elsewhere in the world.

2.2 History from Slop to Pure Culture

Fermentation of foods is, indeed, an ancient process that can be traced back at least 8000 years, whether it was the fermentation of grapes, olives or cereal mash, or of goats' or sheep's milk (Walker, 2004; Fox and

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McSweeney, 2004). Originally, these foods were fermented by the bacteria and fungi that were naturally present on the raw food or in the environment where they were left to ferment, i.e. fermentation by the autochthonous, or adventitious, microbiota. At least over the past century, however, the use of inoculation material containing the fermenting microorganisms has been a known practice (Mogensen *et al*., 2002). Interestingly, after the existence of bacteria became known, and they were connected with disease, the general public has been reluctant to acknowledge the need to add bacterial cultures to food. According to Rodgers (2008), even the Russian scientist Elie Metchnikoff, who systematized the use of probiotic bacteria in food, was aware of popular resistance to this concept. In his book *The Prolongation of Life*, Metchnikoff wrote, 'A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes, as the general belief is that microbes are all harmful. This belief, however, is erroneous' (cited in Rodgers, 2008). Likewise, in his treatise, Conn (1915), who was one of the founders of the American Society for Microbiology, noted that 'Their [bacteria's] presence is entirely consistent with the most perfect health, and, indeed, there are some reasons for believing that they are sometimes directly beneficial to health'. Conn (1915) also remarks on the unfortunate disrepute of bacteria, which does not seem to have changed significantly over the past century: 'To most people the very word bacteria is almost equivalent to disease, and the thought of swallowing microbes in drinking water or milk is decidedly repugnant and alarming. In the public mind it is only necessary to demonstrate that an article holds bacteria to throw it under condemnation'. It is the observation of the present author (SW) that, at least in Denmark, to this day the belief that all microorganisms are harmful is still very prevalent.

Systematic industrial use of starter cultures to ferment foods has only been practised since the middle of the 20th century (Mogensen *et al*., 2002). After the pasteurization of raw foods became usual practice, it was necessary to inoculate these foods with a culture in order to initiate the desired fermentation. Then the use of starter cultures became common practice. At first, starter cultures were isolates from previous fermentations that were maintained and propagated at the site of production, in a process graphically termed 'back slopping' (Brennan *et al*., 2002). As well as the safety aspects of this process, quality was also hard to maintain after inoculation with the undefined, multi-strain cultures that were used. Consequently, commercial culture producers started to specialize in specific cultures for specific foods. More and more starter cultures today are composed of single or multiple and defined strains. When it comes to probiotics, where regulatory requirements (especially in the EU) now demand greater documentation for efficacy, some industries invest great sums of money in the development of foods containing their own well-characterized strains of given species. This means that consumers now see claims on the labels of probiotic foods that even quote strain names (Chr. Hansen A/S, 2004; Saxelin, 2008).

In fact, emphasis by an industry on the desirability of its own particular strain of a beneficial microorganism is no moot point; another strain in the same species could have been implicated in disease. For instance, the literature abounds with reports of cases of endocarditis from which strains of *Lactobacillus plantarum* could be isolated and which may have been the causative agent (see, e.g. Zech *et al*., 1983; Davies *et al*., 1986; Bär *et al*., 1987). However, in most of the reported cases, the patient had suffered from a clinical manifestation that merited hospitalization such that the patient most probably was more susceptible to infection by *Lactobacillus* spp. than when healthy. Salminen *et al*. (2006) have catalogued 85 cases from Finland of *Lactobacillus* bacteraemia. These workers found 11 different species of the genus, several of which are the documented species contained in probiotics. Even the strain *L. rhamnosus* GG, which has been widely used in commercial probiotic foods for more than two decades, has been shown to be associated with infant mortality in mice (Wagner *et al*., 1997).

2.3 Constraints of the Law on Beneficial Microorganisms

Together, the EU and the USA are the dominant single-market systems in the world. Decisions on the safety of the food chain that are taken by their parliaments and their authorities inevitably influence policies in other countries and other regions of the world. Even the emerging economic giants in Asia, instead of being proactive and charting their own courses in food safety, are reactive to the goings on in the EU and USA. Thus, as we progress towards regulating beneficial microorganisms, whatever can be read out of the regulatory dynamics in the EU and USA might well predict what will happen to the regulation of these microorganisms in other parts of the world.

The differences between the EU and the USA in the way they regulate beneficial microorganisms reflect their different histories. When the pharmaceutical industry was developing in the first half of the 20th century, the USA was already a century and a half old. At that time, and due to events in the pharmaceutical and chemical industries, the federal (i.e. US-wide) food and drug authorities realized that the time had come to put constraints on what should be added to food (US FDA, 1999). In contrast, the EU is not yet a single country but is becoming an ever closer union, by its own concerted design (OJEC, 1992). Interestingly, in very recent years, changes in the way the EU regulates beneficial microorganisms for food and feed have very directly mirrored the EU's own development towards an ever closer union. In fact, the progression of events for beneficial microorganisms could serve as the epitome of how the EU itself progresses towards its integration to a single union and to an ever closer harmonization among the member states. This progression will become evident in the following sections.

Two forces in the EU that are quite distant from the science of microbiology have had a strong influence on the way that beneficial microorganisms for the food chain are regulated. These forces are EU law and the European Commission's reaction to consumer discontent. Because these two factors will continue to determine developments in the use of beneficial microorganisms for years to come, they will be elucidated in the following three sections.

2.3.1 EU harmonizes law on beneficial microorganisms

Currently (in 2012), the EU has 27 member states, plus four countries associated with it in the European Free Trade Association (EFTA: Norway, Iceland, Lichtenstein, and Switzerland). EFTA countries must follow relevant EU legislation to be part of the EU single market. EU efforts at coordinating the use of beneficial microorganisms in the member states are a natural consequence of the general and increasing coordination across many fields of technology and politics in Europe. Historically, European efforts at coordinating the region started officially in 1951. Forty years later, the Treaty of Maastricht established the EU, with its official political ambitions of a union (European Commission, 2007). With the Treaty of Lisbon, from 1 December 2009, 'the [European] Union' became the single name for all references to EU matters: The Union shall replace and succeed the European Community (OJEU, 2010). The consequences of this for the marketing of beneficial microorganisms should not be underestimated.

From 1991, Article 1 of the Treaty of Maastricht set the stage for very many developments in the regulatory affairs of beneficial microorganisms for food and feed; see Box 2.1. Then, in 2002, EU Regulation No 178/2002 laid down the general principles for food law for all member states and established the European Food Safety Authority (EFSA) (OJEC, 2002). In that law, two of the most basic principles are the single EU food market and an absence of competition among the EU member states; see Box 2.2. These principles are the basis for the harmonization of the way the member states must regulate beneficial microorganisms for food and feed in the areas still open to member state legislation.

As a powerful instrument to harmonization and to the 'ever closer union' (cf. Box 2.1), EU laws are now most often constructed as

Box 2.1. EU member states now to cooperate and be organized as The Union.

Treaty on European Union ('Maastricht Treaty') (European Commission, 1992)

'Title I – Common Provisions

Article 1

By this Treaty, the HIGH CONTRACTING PARTIES establish among themselves a EUROPEAN UNION, hereinafter called 'the Union'.

This Treaty marks a new stage in the process of creating an ever closer union among the peoples of Europe, in which decisions are taken as openly as possible and as closely as possible to the citizen.

The Union shall be founded on the European Communities, supplemented by the policies and forms of cooperation established by this Treaty. Its task shall be to organise, in a manner demonstrating consistency and solidarity, relations between the Member States and between their peoples.'

Box 2.2. No barriers to trade or distorted competition on the EU food market.

Regulation No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (European Parliament and Council, 2002).

Preamble (§ 30)

'A food business operator * is best placed to devise a safe system for supplying food and ensuring that the food it supplies is safe; thus, it should have primary legal responsibility for ensuring food safety. Although this principle exists in some Member States and areas of food law, in other areas this is either not explicit or else responsibility is assumed by the competent authorities of the Member State through the control activities they carry out. Such disparities are liable to create barriers to trade and distort competition between food business operators in different Member States.'

* Article 3

'3. 'food business operator' means the natural or legal persons responsible for ensuring that the requirements of food law are met within the food business under their control;'

Regulations and not as Directives. This change in construction was evident in the late 1990s and throughout the 2000s. Regulations are, so to speak, genuine EU laws, directly applicable in all member states, with no room for national laws (Article 249, Treaty Establishing the European Community) (OJEC, 1997). Directives, in contrast, must first be set forth in national law by the member states before they have any effect. Thus, passing EU laws as Regulations has had three important effects on the regulatory affairs of microorganisms. First, all EU member states must interpret the law in more or less the same way, instead of there being, e.g. 27 different ideas of what 'documented safety' is. Secondly, making EU laws as regulations means that the period needed to implement the law can be kept quite short, instead of delays of months and years needed for the implementation of a Directive. Finally, single laws for all of the EU have made it both necessary and easier for the new EU authorities to establish themselves, at the same time as much power of decision about beneficial microorganisms has been relocated from national authorities to the EU authorities. For instance, for genetically modified microorganisms (GMMs) of any kind, the role of national authorities is now only literally to dispatch applications for approval from the member state directly to the offices of EFSA (2006b).

For a detailed description of how the EU regulatory system for beneficial microorganisms functions in practice, see the next chapter (von Wright, Chapter 3, this volume).

2.3.2 Reaction to discontent among EU consumers

As well as the role of the EU in law making, consumer opinion in the EU has also been very influential in shaping the way that beneficial microorganisms for food and feed are regulated in the EU. Safe food and feed have obvious relevance for all individuals; thus, every individual is a consumer in discussions on food safety. During the 1990s, consumers in the various EU member states were disquieted by a series of food and feed contamination events. Because some of these events involved food and feed being traded between EU member states, the events seemed to reflect a lack of safety measures at the level of the EU. More specifically, they seemed to reflect a lack of accountability and responsibility on the part of the member state food safety systems (European Commission, 2000). Two examples will illustrate how consumer concerns resulted in the moving of the regulation of beneficial microorganisms from a member state level to the EU level.

In 1999, feed in Belgium was found to be contaminated with dioxins, which had spread to several categories of animals and to foods. It was believed that oil for feed had been stored in tanks previously used for industrial mineral oil (Tyler, 1999). Eggs and chickens containing the dioxins were sold in France, the Netherlands and Germany, and consumers in several countries were frightened by the event. Therefore, the EU Commission declared an EU-wide ban on the products. Eighteen months later, EU agricultural ministers adopted a regulation that put legally binding limits on dioxins in food. The EU Health and Consumer Protection Commissioner David Byrne used the passing of the regulation to show the Commission's emphatic opposition to the contamination incident: 'I am pleased to see the Ministers recognize that we need to be uncompromising and severe on contaminants in food. This new legislation, in setting legally binding limits, sets a new milestone in the EU's feed and food safety strategy' (European Commission, 2001).

In the late 1990s and 2000, the populace of the EU was affected by a number of zoonoses, or infections in farm animals (usually due to bacteria) that are transmitted to humans via the food chain. In 2002, the European Council supported a Directive and a Regulation aimed at monitoring and combating the bacteria, especially in the poultry industry. On the occasion of the support of the laws, Commissioner Byrne expressed clearly what the Commission also expected to achieve: 'These proposals demonstrate how the Commission's 'farm to fork' approach is being implemented in practice to ensure safe food for consumers' (European Commission, 2002). The European Commission had published its White Paper on Food Safety in January 2000 (European Commission, 2000). The primary objective of the White Paper was to propose a way to establish an EU-wide system for food safety and to re-establish and maintain consumer confidence in the EU's ability to ensure safe food. Box 2.3 quotes the White Paper and the European Commission's reason for establishing the EU authorities for food and chemicals, i.e., EFSA in 2002 and the European Chemicals Agency (ECHA) in 2007.

During the same period of time as the two contamination events described above, the EU institutions were compiling the new Treaty of Nice (signed in 2001), which defined a new distribution of decision-making power for the EU. The treaty gave the European Parliament much more legislative power than it had had before (European Parliament, 2001). This meant that the members of the Parliament were now in a position to respond to the wishes of their constituencies and intensify EU efforts at guaranteeing a safe food supply. Less than a year after the Treaty of Nice was signed, the EU Parliament and Council had passed the Regulation that lays down the basic principles for EU food law and established the EU authority for food safety (OJEC, 2002).

Late in 2010, EFSA published a consumer opinion survey that elucidated where the EU public saw food-related risks (EFSA, 2010). The survey was the result of 26,600 personal interviews in all 27 EU member states; a similar survey was conducted in 2005. It was apparent from the new survey that there had been no great increase in confidence that the food authorities (either in any member state or in EFSA) could ensure a safe food supply.

Box 2.3. Re-establishing consumer confidence in the EU food supply: the White Paper (European Commission, 2000).

'CHAPTER ONE: INTRODUCTION

'These facts therefore demand a comprehensive and integrated approach to food safety. This does not mean that the EU should be exclusively responsible for all aspects of food safety. However, it demands that all aspects of food safety are addressed at EU level. For example, EU legislation has to be enforceable in an efficient way in the Member States in line with the principle of subsidiarity. Responsibility for enforcement above all should remain primarily a national, regional and local responsibility. However, the Internal Market means that these are not exclusively national responsibilities: each Member State has a duty towards not only to its own citizens but to all citizens of the EU and third countries for the food produced on their territory. …

Community and Member State food safety systems have been under unprecedented pressure during recent feed and food emergencies. These emergencies have exposed weaknesses which call for action by the responsible authorities (Commission, Member States and the Parliament), to re-enforce, improve and further develop existing systems.

Food safety needs to be organised in a more co-ordinated and integrated way. This will allow existing weaknesses to be addressed, whilst at the same time creating a genuinely world-leading food safety framework, which can deliver a high level of public health and consumer protection in accordance with the requirements of the EC Treaty. However, the most comprehensive system cannot function without the full collaboration of all parties involved. The proper functioning of any system depends decisively on the commitment of the Member States and operators, as well as third countries.

The European Union needs to re-establish public confidence in its food supply, its food science, its food law and its food controls.'

Citizens also felt less confident in being able to deal personally with possible problems resulting from new technologies (<30%), such as genetically modified foods and nanotechnology in connection with foods.

2.3.3 Dilemma for regulators

Earlier in this chapter, we saw that strains of the normally benevolent species *L. plantarum* and *L. rhamnosus*, indeed, in some cases might be the cause of disease. Thus, for approval of a beneficial microorganism, it would seem justified when the authorities require documentation for both safety and efficacy on the level of strains, and not just species. By the same token, this scrutiny by the authorities would most probably be in the interest of an industry applying for approval, because with its own safety and efficacy data the industry can uphold its proprietary claim to the strain. Indeed, the importance of knowing the identity of a beneficial microorganism on the level of strain is one of the cardinal principles proposed by the FAO/WHO working group on probiotics in its guidance to evaluate the probiotics (FAO/WHO, 2002).

However, when it comes to regulating the food industry's widespread use of undefined and multi-strain starter cultures, the food authorities in most countries of the world are in a formidable dilemma (EFSA, 2005). On the one hand, the scientists at the food authorities require state-of-the-art characterization of all strains in a starter culture; these scientists know, though, that this is physically impossible with 50 or 100 different strains in the starter. On the other hand, the authorities' own scientists also know that most of the undefined cultures have empirically proven track records of both safe performance and gastronomically appealing fermentations. In other words, the authorities are probably in doubt as to what documentation they could and should require of industry to ensure the safety of consumers. An outright ban on a culture in a popular food is out of the question. In most cases, the dilemma will probably be resolved by establishing that the ultimate responsibility for safe cultures lies with the producer of the food (OJEC,

2002). Such self-scrutiny is the basis of the system of GRAS (Generally Recognized As Safe) in the USA, but is virtually not at all implemented in the EU (EFSA, 2007; US FDA, 2010b).

Since its start in 2002, the EU food authority EFSA had most probably already acknowledged the administrative burden of authorizing the myriad of microorganisms in the beneficial cultures used in food and feed in the EU. EFSA did recognize the efficiency of the US system of GRAS for evaluating the safety of cultures with a long history of safe use (EFSA, 2005). Therefore, in 2007, EFSA introduced its own, internal working system of Qualified Presumption of Safety, or QPS (EFSA, 2007). This system allows EFSA to dispense with most safety deliberations and frees resources to focus specifically on aspects of a given microorganism that deviate from the organism's QPS counterpart. This deviation might be of a genetic or metabolic nature, or in its use. The US GRAS system is touched upon immediately below, and both the EU and US systems are described in greater detail in the next chapter (von Wright, Chapter 3, this volume).

2.3.4 US law and beneficial microorganisms

In the USA, food for humans and feed for animals are both termed food and are both regulated in accordance with the Federal Food and Cosmetics Act (US FDA, 1999). Beneficial microorganisms for these products are in practice always classified (by the manufacturer) as generally recognized as safe, or GRAS (US FDA, 2010b). The burden of proof for living up to this classification is on the manufacturer of the food or feed culture as sold to the consumer. The incentive for making sure that consumers or agricultural animals are not harmed by eating the microorganisms is apparent: harmed people or animals can bring economic ruin to the manufacturer, especially through lawsuits. As far as this author knows, there have been extremely few health incidents in the USA that have been shown to be due to added microbial cultures to food or feed. The sections below on food and feed, and the next chapter (von Wright, Chapter 3, this volume), give more details on the regulatory system in the USA.

2.4 Food

2.4.1 Food cultures in the EU

Today, commercially produced beneficial microbial cultures for food and feed are basically very much like many other food and feed ingredients. They are compound products that consist both of the basic ingredient (e.g. the fungus or the lactic acid bacterium) and of a series of additional compounds, such as cryoprotectants, carriers, antioxidants and bulking agents. The microorganism itself makes up a very small proportion of the total product, volume-wise. Therefore, when food and feed authorities focus on the safety of a commercial culture, they focus not only on the microorganism but also on the identity and impurities of the other components therein.

In the EU, it is not yet decided what the regulatory status is of beneficial microorganisms for food as commercial products on the market. However, as pointed out earlier in this chapter, there is a strong tendency in the EU towards creating very specific laws to ensure food safety. As will be seen, such laws already exist for beneficial cultures for animal feed. Therefore, there can be little doubt that the near future will see new EU laws or changes to existing laws in order to regulate specific categories of beneficial microorganisms for food. In order to predict how this regulation of food cultures will work, it is useful to view both the current EU legislation and current EU practice for managing beneficial microorganisms. The following paragraphs will emphasize the current EU legislation, and the next chapter (von Wright, Chapter 3, this volume) will explain current EU practice as carried out by the EFSA and by the EU Commission.

Current EU food legislation has three categories of food components that might be relevant for beneficial microorganisms for food: food ingredients, food additives and processing aids. As of January 2010, the EU

made quite fundamental changes to its food additive law with Regulation 1333/2008 on food additives (OJEU, 2008). This could end up making microbial cultures food ingredients but probably not food additives (a legal subset of ingredients). This is because the cultures themselves are normally consumed as part of many foods, and because they are characteristic ingredients of these foods, e.g. cheese, yogurts and sauerkraut, cf. Council Directive 89/107/EEC, Art. 1(2) (OJEC, 1989), and Directive 2000/13/EC, Art. 6, §4(a) (OJEC, 2000). Then what documentation of the cultures is necessary is up to the food industry, as long as consumers are not harmed. In addition, by being categorized as ingredients, cultures for cheese, butter, and fermented milk and cream need not be listed on the food packaging label (OJEC, 2000). Conversely, if microbial culture products were categorized as food additives, then, according to the new food additive law, the safety and efficacy of the cultures must be scientifically documented and then reviewed by EFSA prior to marketing. Interestingly though, the law does not yet specify to what extent safety and efficacy must be documented (OJEU, 2008). Finally, if the beneficial microorganisms were categorized as processing aids, they would be out of scope of the food additive law (OJEU, 2008).

Because the EU food additive law from 2008 is a Regulation, and not a Directive, it has put all 27 member states in a period of transition. Denmark is a case in point. The EU law now allows the Danish authority to ease some of its previous, very stringent requirements for documentation on microorganisms for food, at the same time as tightening other requirements, as is now explained. Since the 1970s, Denmark has had a pre-market approval process for new cultures for food use that was specific for the strain of the species of organism and for the manufacturer of the strain. The result was a confidential list of approved organisms (Danish Veterinary and Food Administration, 2010). Because of the new EU food additive law, Denmark had to change its requirements as of January 2010. Now microorganisms only have to be notified to, and not approved by, the food authority before marketing, and only at the species level and for a particular group of foods. However, the notification list is publically available, and the authority interprets the Danish law to only permit notification of pure cultures. The law requires the following documentation for the purity of a notified culture: 'Studies must have been conducted that ensure that the formulation of the microorganism does not contain potentially harmful organisms and/or large numbers of contaminating organisms whose identity has not been ascertained (Danish Veterinary and Food Administration (2009), Annex 4, Part I (2); translation, SW). Here again, the dilemma is unresolved between state-of-the-art scientific characterization of all strains in a starter and the starters' track records of safe use.

The postharvest preservation of plant crops is also an area in which man has put microorganisms to a beneficial function. As part of the microbial fight for limited resources, many microorganisms have long been known to produce antagonistic substances or physico-chemical conditions that keep competitors at bay (Sivan and Chet, 1989; Tanaka, 1992; Dowling and O'Gara, 1994). These antagonistic properties have also been exploited in modern times in commercial products directed against fungi that can spoil plant material (Sundh and Melin, 2011; Wisniewski and Droby, Chapter 5, this volume). For instance, they are used for the postharvest 'biopreservation' of food and feed crops such as grain, fruits, vegetables and even decorative plants. In the EU, microorganisms used in this way as fungicides are categorized as the active substance in plant protection products, and the commercial product is thus subject to a full application for pre-market approval (OJEC, 1991). The chapter by Wisniewski and Droby (Chapter 5, this volume) gives a valuable insight into such biopreservation.

2.4.2 Food cultures in the USA

In the USA, a beneficial microbial culture sold as a commercial product to food manufacturers could either be a food additive or a GRAS

substance for food (US FDA, 1999, 2010a,b). The US definition of a food additive is very broad and encompasses virtually everything that can come into contact with food. Food additives must be approved before use, and the documentation for safety is appreciable. Among currently approved food additives, the only microbial cultures that may be marketed as live cultures and additives are dried cells of *Saccharomyces cerevisiae, S. fragilis* and *Candida utilis*.

If, however, a microbial culture in the USA is safety-categorized as a GRAS substance, that categorization can be based on one of two concepts of safety: either scientific documentation of safety or a history of safe use. According to the US Food and Drug Administration (FDA), a microbial culture for food may be used in food if it was in safe use before the GRAS system was devised by the FDA, i.e. before 1 January 1958 (US FDA, 1999). This is the so-called 'grandfather clause'. A culture that was brought into use after that date may also be GRAS if the manufacturer itself has ensured that experts in the field can confirm the safety. This means that the manufacturer ought to have all documentation in place for the safety of the product. If so requested by the FDA, the manufacturer would have to present this documentation to the FDA, and if the FDA were not convinced by the documentation, the microbial product would become a food additive and require scrutiny by FDA scientists (Wessels *et al*., 2004; US FDA, 2010b). Currently, among the substances notified to the FDA as GRAS, there are several lactic acid bacteria and bifidobacteria, but these do not seem to be meant to be bulk starter cultures (US FDA, 2010c); those latter cultures are considered to be covered by the 'grandfather clause' and are GRAS substances.

2.4.3 Food cultures in Singapore

The status given to beneficial microorganisms in the state of Singapore may allow predictions to be made about the future developments for these organisms in other South-east Asian societies. Singapore has its cultural roots primarily in the Chinese, Malay and Indian traditions. Especially because of the first mentioned, the Singaporean population acknowledges to this day the close link between food and whole-body wellness, as exemplified in the extremely widespread practice of Traditional Chinese Medicine,[1] a concept that both the food and the medicinal authorities recognize. Accordingly, these authorities administer a common and rather clear delineation among foods, food supplements and medicinal products, and use what they call the 'food–health product interface' (Health Sciences Authority, 2007). These three groups of products that contain beneficial microorganisms will be discussed in the following paragraphs.

In Singapore, there are many fermented dairy products on the food market, and almost all of them are imported. They come from Australia, New Zealand, the USA, Germany, France, Switzerland, Malaysia and Thailand. There are a few local Singaporean food establishments that produce cheese and fermented products, such as yoghurt, yoghurt drinks and cultured milk drinks. According to Singaporean law, the responsibility for safe dairy products lies either with the importer to Singapore or with the Singaporean manufacturer (Singapore Government, 2002), and no pre-market approval of cultures or the fermented foods is required. The food safety authority of Singapore is the Agri-Food and Veterinary Authority, or AVA.

The classification of microbial cultures in foods is very much like that in Western countries. If the culture is inactivated or removed from the final food product, then the culture is regarded as a processing aid because it no longer has a technological function in the final product. If the culture is viable in the final product, it is regarded as an ingredient.

The concept of time-proven safety and efficacy that underlies the practice of Traditional Chinese Medicine is reflected in the requirements that the authorities put on beneficial microorganisms. There is no single piece of legislation that governs the use of beneficial cultures in food, but there are provisions within the existing legislation that specifically allow the use of bacterial or fungal cultures in the production of certain food products. The legislation (Singapore Government, 2006; available at: http://www.

ava.gov.sg/ Legislation/ListOfLegislation/; accessed 26 April 2012) lists:

- lactic acid-producing bacteria in *Lactobacillus* milk drink or cultured milk drink;
- ripening, harmless acid-producing bacterial cultures and special filamentous fungal cultures in cheese; and
- *L. bulgaricus*, *Streptococcus thermophilus*, *L. acidophilus* and *Bacterium yoghurtii* [*sic*!] in yoghurt.

As probiotic ingredients, the AVA has administratively allowed strains of *Bifidobacterium* and *Lactobacillus* that have what the AVA terms 'a proven long history of safe use in food (such as *B. bifidum*, *L. acidophilus*, *L. delbrueckii*, *L. casei* Shirota strain)'. The AVA does not prescribe minimum or maximum concentrations of the probiotic bacteria. Rather, it is the responsibility of the manufacturer to ensure safe concentrations, and to ensure that the claimed probiotic function can be achieved by the concentration present in the food.

Concerning functional claims made for probiotic foods, the AVA does require the submission of documentation for the claim. These rules are posted on the Internet, where the permitted probiotic claims are listed, as well as a requirement for labelling (and advertising) the food with the name of the probiotic (http://www.ava.gov.sg/FoodSector/FoodLabelingAdvertisement). In practice, products on the market almost exclusively list the genus and species names and not strain names.

Food supplements in Singapore that contain beneficial microorganisms are administered primarily by the medicinal authority, the Health Sciences Authority (HSA) (Health Sciences Authority, 2007). Food supplements do not require a pre-market approval, while medicinal products do. On the authorities' document that describes the food–health product interface, the HSA lists '*Probiotics (in non-milk based products) including Lactobacillus*, *Streptococcus thermophilus*, *Bifidobacteria* [bifidobacteria]' among the 45 examples of what can be marketed as a food supplement product (Health Sciences Authority, 2007). For medicinal products, the Singaporean Medicines Act prescribes that such products are the subject of an application for approval, and that safety and efficacy must be 'taken into consideration' by the HSA when the application is evaluated (Singapore Government, 2008). Documentation of safety and efficacy of the medicinal product shall follow the guidances posted on the HSA homepage (http://www.hsa.gov.sg/; accessed 26 April 2012). These guidances are essentially the common ASEAN (Association of Southeast Asian Nations) guidances, which themselves closely follow those of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

All approved medicinal products in Singapore are searchable on the HSA web site, and there are only two products on that list that contain beneficial microorganisms. These two appear to be identical except for their dosage forms: as capsule and as powder in a sachet (Lacteol Forte Capsule and Lacteol Forte Sachet, and were approved in 1998 (Aptalis Pharma [formerly Axcan Pharma], 2011). They contain lyophilized cells of *Lactobacillus*, with no species given, at dosages of 5×10^9 and 1×10^{10} cells in the capsules and sachets, respectively. Both products may be sold as General Sales List Medicines (GSL), as opposed to Prescription Only and Pharmacy Only Medicines (Health Sciences Authority, 2011). GSL medicines can be compared with over-the-counter medicines that may be sold elsewhere than by pharmacies/chemists.

2.5 Feed

2.5.1 Feed probiotics in the EU

As mentioned above, the use of microorganisms to make beneficial products for man goes back thousands of years (Fox and McSweeney, 2004; Walker, 2004). Using the same microorganisms as feed may be almost as old as their use in food. Among the fungi, yeasts have long been exploited to ferment cereal mash, grape musts and other naturally derived substrates for the purposes of brewing, baking, winemaking and distilling. After all these processes (except baking), there has been a residue of spent fermentation mass,

consisting largely of the propagated microorganisms. It can be presumed that use of the spent biomass as animal feed very quickly became apparent and exploited when animal husbandry was close to the site of fermentation. It can also be presumed that the beneficial effect on the animals' nutrition was soon appreciated, at least for the added nutritional value. A probiotic effect of live biomass, for instance as a stabilizer for gut microbiota, is most likely no recent innovation either. As a concerted and systematic agricultural practice, the use of microbial cultures in feed was recorded at least a century ago (Stone, 1998).

In the last decade there has been great focus by the consumers in the EU on the connection between animal nutrition and human health. However, many people who work with the regulatory affairs of beneficial microbial cultures in the EU have been puzzled by the regulatory priorities of EU legislation. It is sometimes asserted: 'First came the authorities' scrutiny of microbial cultures for animal feed; then it took a decade for the authorities to find out that cultures for human food also were worth the same scrutiny'. Historically the dates and sequence of events behind this assertion are correct. In 1996, all parties in the EU that marketed microbial cultures for animal feed had to submit a full dossier for approval of those cultures to the European Commission's Standing Committee for Feedingstuffs (OJEC, 1993). For cultures for food, no EU-wide regulatory initiatives were taken until the end of 2007. Then EFSA introduced its own internal system for categorizing the safety of beneficial cultures for food (EFSA, 2007). As of 2011, cultures for food are still not subjected to an EU-approval process. However, there is a reasonable explanation for this apparent paradox in priorities between animals and humans; animals are much less particular about what they eat than humans, and the diet of livestock is virtually without variation throughout their whole adult life.

Volume-wise, the largest single field of use of beneficial microorganisms in the EU is most probably as probiotics in feed for animals. Probiotics for animals have been on the market in Europe since the mid 1980s according to the EU Association of Specialty Feed Ingredients and their Mixtures (FEFANA, 2005). Since 1993, these culture products have been classified as feed additives, specifically in the category of zootechnical additives, and the subcategory (b) gut flora stabilisers: micro-organisms …, which, when fed to animals, have a positive effect on the gut flora (OJEU, 2003). Specific requirements for the documentation of safety and efficacy and premarket approval have been part of EU law since 1993 (OJEC, 1993). As a whole, the EU now has accrued much regulatory experience with dossiers for approval of products containing these cultures. Indeed, the amount of documentation of safety and efficacy required in these applications for approval are sometimes held forth as worst-case examples for what might be required for cultures for human food.

2.5.2 Feed probiotics in the USA

In the USA, animal feed is a subset of food and thus subject to the same laws as food for humans (US FDA, 1999). Cultures for animal nutrition are termed direct-fed microbial products. As for food for humans, the authority regulating such products is the FDA (US FDA, 1995). Currently, microorganisms for feed are placed in one of four categories:

- a food (feed) additive,
- a GRAS substance,
- a veterinary drug, or
- a feed ingredient as listed in the Official Publication of the Association of American Feed Control Officials (AAFCO) (US FDA, 1995).

For information on the additives and GRAS substances, see above in Section 2.4.2 on cultures for food in the USA. For a culture used as a veterinary drug, the manufacturer of the culture might make a label claim on the product that it cures, treats or prevents disease, which would make the product a drug, and require very extensive documentation. The AAFCO category has been the one most used for feed cultures. This category has particularly contained spent microbial cultures after the industrial fermentation of other

products, such as yeast fermentations (US FDA, 1995). The cultures that are classified as belonging to the AAFCO category are defined at species and not strain level, and their safety must be applicable to animals in general.

2.5.3 Feed probiotics in Singapore

The only animals produced in Singapore as food for humans are chickens, and the country has no regulation of probiotic feed cultures.

2.5.4 Silage in the EU and USA

One particular large-scale and very traditional process for which beneficial microorganisms are responsible is ensiling. In ensiling, plant material is placed in a closed container, such as a silo, for an extended period, and the resulting succulent material is used as feed. The process is largely an anaerobic fermentation, and it results in maintaining the nutritional value of the plant material during storage and in general stabilizing the material after the container is opened (EFSA, 2006a). Traditionally, the anaerobic fermentation is carried out by the naturally occurring microbiota on the plant material. However, more recently, acids and/or cultures of lactic acid bacteria are added in order to lower the pH and help preserve the plant material.

In line with the general harmonization across the EU, the EU Regulation on feed additives from 2003 determined for the first time that silage cultures should be regulated at the EU level (OJEU, 2003). The Regulation established that silage cultures and, indeed, silage additives in general, are feed additives and that they therefore must be subjected to an authorization process. The Regulation places the silage additives in the category technological additive, subcategory (k) substances, including enzymes or microorganisms intended to be incorporated into feed to improve the production of silage. According to the Regulation, only specifically approved silage cultures may be used in the future. Dossiers for the approval of silage cultures had to be submitted to the EU Commission (who passes them on to EFSA) by 7 November 2010. The Regulation states that the approval process would be completed in August 2011.

The most important part of the dossiers for approval of any food or feed additive is documentation of its safety and efficacy. In 2006, EFSA published a 10-page guidance on how these aspects for silage additives should be documented (EFSA, 2006a). Safety is for the farm animal, for the farmer, for the consumer and for the environment, and the focus of safety studies is on the silage additive product as marketed and not just on the microbial culture. Efficacy relates directly to the marketing claims made for the silage additive. EFSA acknowledges that field trials for efficacy in silage production are very difficult to standardize and thus that it is very difficult to compare silages. This is because very local climate conditions (e.g. wind and sunshine) will influence the outcome of the ensiling. As a consequence, the EFSA guidance proposes conditions for laboratory trials which, for instance, employ the use of minisilos. At the time of writing, the EFSA web page (www.efsa.europa.eu; accessed 26 April 2012) contains several scientific opinions from the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the safety and efficacy of strains of lactic acid bacteria whose approval has been sought as silage cultures.

In the USA, cultures for silage are encompassed in the AAFCO category that is explained above for probiotic cultures for feed in the USA.

2.6 Conclusions

The EU-wide regulation of feed cultures started almost 20 years ago, while cultures for food, e.g. probiotics, are only now starting to be regulated. It is, indeed, hard to predict whether cultures for food in the EU will end up being as tightly regulated as cultures for feed. There, the responsibility for safety rests virtually entirely on the EU food and feed authority EFSA. Although EFSA recently instigated the administrative short-cut route, i.e. QPS, for the safety assessment of cultures

for food and feed, the authority still holds on to the very extensive evaluation procedure for marketing approval.

In the USA, beneficial cultures of microorganisms for food and feed have been covered by some federal legal regulations since the mid-20th century. The current federal regulations are quite lenient and place responsibility for safety on the manufacturer and not on the authorities. Countries and regions in other parts of the world look to the USA and the EU for ways of regulating beneficial cultures for food and feed. It is safe to say that these countries and regions seem to have a choice between the lenient US systems or the stringent EU systems.

Note

[1] In Traditional Chinese Medicine, a consumption over a longer period of time of certain herbs and particular parts of a few animals is considered to correct imbalances in the body, e.g. in the liver, in the lung and the blood circulation in general. An example of such an herb is ginseng; and the product Cordyceps is a fungus-infected caterpillar.

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3 Microbes for Human and Animal Consumption

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3.1 Introduction

Microorganisms have been utilized for millennia to improve the keeping quality and the sensory and nutritional properties of different types of foods. Examples include fermented milks, cheeses, cured meats, leavened bakery products, pickled vegetables, wines and beers, sauces and condiments, and even coffee and tea.

The traditional food production relied on spontaneous fermentations or back slopping, and factors such as raw materials, temperature, pH or salinity were used to determine the desired outcome of the often complicated processes. These traditional

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approaches are still applied, even in the developed world, to many artisanal products and even to some industrial-scale productions. However, as a rule today, defined starter cultures of microorganisms are generally used to ensure the proper production process and the high quality of the final products. The same applies to other types of microbial cultures (probiotics, protective cultures) which are discussed in more detail below.

The present review deals mainly with the legislative status and potential safety concerns of microorganisms intentionally added into food or feed in the European Union (EU). However, when relevant, the situation is compared with that of the USA to illustrate the different approaches in these two large single-market areas. For the sake of completeness, short descriptions of the regulatory frameworks in Canada, Asia and South America are also included.

3.2 Microorganisms Intentionally Added into Food or Feed

Starter cultures represent microorganisms that are intentionally added into food, and they represent species and strains that are generally of food origin. The same generally applies to the so-called protective cultures, which are not used in the actual production process, but are added to the final product to improve the microbial quality by preventing the growth of spoilage organisms. In contrast, probiotic cultures, or microorganisms that are intended to enhance the well-being of the human or animal host by improving the properties of the endogenous microbiota, do not necessarily have a previous history in food use, but often represent originally intestinal species or strains.

In the following sections, some of the main microbial groups that are used in food and feed applications are briefly reviewed. For detailed discussions on the technological and safety aspects associated with these processes, the reader is advised to consult specific textbooks on the subject (e.g. Adams and Nout, 2001).

3.2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating bacteria belonging to the phylum Firmicutes. LAB have a characteristic fermentative metabolism which produces lactic acid as the main fermentation product, either alone (homofermentative LAB) or together with acetic acid/ethanol and CO_2 (heterofermentative LAB) (Axelsson, 2004). Morphologically they are either rods (*Lactobacillus*, *Carnobacterium*) or cocci (*Aerococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Weisella*). Their natural habitats include plant material, the gastrointestinal tract, vaginal mucosa and other nutritionally rich niches suitable for these fastidious microorganisms.

The LAB genera used in starter cultures for food or feed include *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*. In so-called thermophilic starters, a streptococcal species, *S. thermophilus*, is also used, together with certain lactobacilli. For example, a typical yoghurt starter consists of *Lc. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, which are in symbiosis with each other while growing in milk.

The LAB that are used as probiotic cultures often represent species and strains originally isolated from the gastrointestinal tract (*Lb. rhamnosus*, *Lb. acidophilus*, *Lb. reuteri*, etc.).

3.2.2 Other bacteria used as food or feed cultures

Like LAB, most other bacteria that are intentionally used as starters or probiotics are Gram positive. Some, like staphylococci and bacilli, belong to the phylum Firmicutes, while propionic acid bacteria, brevibacteria, micrococci and bifidobacteria are included in the Actinobacteria.

Propionic acid bacteria are irregular, anaerobic rods (Ouwehand, 2004). The characteristic fermentation product is propionic acid, and *Propionibacterium freudenreichii* and its subspecies *shermanii* are used as aroma and flavour producers in Emmenthaler type cheese. These species are also applied as

silage starters and protective cultures because of the antifungal properties of propionate. Brevibacteria are aerobic pigmented bacteria that are applied as smear cultures on certain types of cheese (e.g. Limburger) to induce the proper surface ripening typical for these types of products (Weimer, 2000). Bifidobacteria (Crittenden, 2004) are anaerobic irregular rods that produce a mixture of lactic acid and acetic acid as the result of their characteristic fermentation. They are intestinal microorganisms and form the majority of the intestinal microbiota of breastfed infants. They have not traditionally been used in food production processes, but have gained popularity as probiotic cultures. Micrococci are used together with certain staphylococci (*Staphylococcus xylosus*, *Staph. carnosus*) in meat starters because of their ability to reduce nitrate to nitrite, which subsequently reacts with myoglobin and forms the additional red colour of the meat (Geisen *et al*., 1992; García-Lopez *et al*., 2000).

Bacilli are a taxonomically heterogeneous group of spore-forming aerobic or facultatively anaerobic rods. With the exception of *Bacillus subtilis* subsp. *natto* used in 'Natto', a traditional Japanese food, they are generally not used in foods, although quite a number of *Bacillus*-based animal probiotics have been introduced into the market in the EU (e.g. Williams *et al*., 2009; European Commission, 2012).

3.2.3 Yeasts and filamentous fungi

Yeasts and filamentous fungi or moulds belong to eukaryotic microorganisms, meaning that their cellular organization resembles that of higher animals and plants. Fungal taxonomy is currently undergoing profound changes (Silva *et al*., 2000). However, most of the species used as food cultures are well characterized and established. For pragmatic reasons, higher fungi that do not form mycelium but live as single cells are called yeasts, and fungi that form hyphae or mycelium are called moulds.

Baker's yeast, *Saccharomyces cerevisiae*, is by far the most well-known yeast, and its applications in brewing and baking are ancient examples of the utilization of microorganisms in food processes (Viljoen and Heard, 2000). The usefulness of *S. cerevisiae* is based on its ability to ferment hexoses anaerobically to ethanol and carbon dioxide and to produce aroma compounds, which give beer, wine and raised dough their characteristic properties. *S. cerevisiae* is also used as a feed additive (Büchi *et al*., 2010; European Commission, 2012).

Fungi of the genera *Penicillium* and *Aspergillus* are the most common filamentous fungi intentionally added to foods. For example, *P. camemberti* and *P. roqueforti* are species associated with white and blue mould cheeses, as their names already indicate. *P. nalgiovense* is used as surface cultures for certain types of sausages (Blank, 2000). *A. oryzae* is used in the production of many oriental foods like soy sauce or sake (Gomi, 2000). *Rhizopus* moulds are associated with another oriental delicacy, the soybean-based 'tempeh' (Nout, 2001).

3.3 Safety Aspects of Food and Feed-associated Microorganisms

The traditional food microorganisms usually have a long history of use – often dating back thousands of years. Thus their safety is either established or the risks associated with them have become generally accepted as a part of life. It is worth noting here that ethanol produced by *S. cerevisiae* in beer and wine and for spirits is not generally considered as a microbiological risk, although the misuse of this microbial metabolite is extremely prevalent and associated with more severe health and social consequences than the occasional food-borne infections caused by the actual food-associated pathogens.

Despite the general harmlessness of the microorganism species used in food cultures, even they are occasionally associated with bacteraemia, fungaemia, myocarditis or other types of infections, usually in patients having a severe underlying disease or immunodeficiency as predisposing factors (Gasser, 1994; Saxelin *et al*., 1996; Lherm *et al*., 2002). However, only rarely has there been an established connection between the infectious microorganism and any microbial food culture that the patient might have been exposed to via the diet. The few cases there are represent clinical isolates of probiotic strains indistinguishable from

those consumed by the patient (Mackay *et al*., 1999; Rautio *et al*., 1999).

Human probiotics are interesting cases of microorganisms that often do not have a history of being present in large numbers in traditional fermented foods but, as already noted above, often represent intestinal species and have a capacity to survive in the gut and maybe also in other niches present in the body. As they are supposed to have beneficial health effects, there is a risk that they are used as a kind of self-help by people whose health and immunocompetence might be compromised. These persons probably should be advised to use probiotics under medical surveillance.

Enterococci, especially *E. faecalis* and *E. faecium* are LAB that are increasingly associated with nosocomial infections. Antibiotic resistance is a typical feature of enterococci, and several virulence factors have been proposed (Hammerum *et al*., 2010; Sava *et al*., 2010). Generally, enterococci are harmless intestinal commensals, present in many traditional products and even occasionally intentionally used as starters (Giraffa, 2000). They might represent an alarming case of a previously harmless bacterium evolving into a pathogen.

There is a growing concern that the increasing presence of antibiotic resistances among pathogenic bacteria soon can threaten the efficacy of antibiotics in human or veterinary medicine. Because the genetic determinants for antibiotic resistance can be transferred between species and even genera of bacteria, there is a growing concern that non-pathogenic bacteria may serve as a reservoir of transmissible determinants, from which the resistances could spread to actual pathogens (see Wilcks and van Hoek, Chapter 4, this volume). As outlined in the subsequent section on EU legislation on feed additives, the absence of transmissible antibiotic resistances is one of the aspects that should be confirmed in the authorization process of a microbial additive.

A safety concern associated with filamentous fungi is their ability to produce a wide variety of poisonous secondary metabolites called mycotoxins (Richard, 2007; Reverberi *et al*., 2010). The carcinogenic and hepatotoxic aflatoxins produced by *Aspergillus flavus* in hot and humid climatic conditions are the best known of these and probably, from the point of view of public health, the most relevant example, although in temperate climates, the various toxins produced by different *Fusarium* species dominate. Even the fungal species that are used in food production may produce mycotoxins, such as roquefortine, a metabolite of *P. roquefortii* (Finoli *et al*., 2001). A complicating factor is that the production of toxic secondary metabolites very much depends on the growth conditions, and a harmless mould in cheese might produce toxin if growing on bread.

The establishment of the safety of a completely novel microorganism with no previous history of use in food and feed or even of presence in the gastrointestinal tract is a very challenging task. While there are standardized and well-recognized procedures for the safety evaluation of a novel chemical, there are no validated methods to demonstrate with a reasonable certainty the safety of a novel microorganism (see Wilcks *et al*., Chapter 15, this volume). This means that the safety evaluation of a totally novel food or feed culture is a case-by-case exercise, although in the case of feed additives in the EU, there are some mandatory studies regarding the safety for target animals, user, consumer or environment (see Section 3.7 below on feed additives).

3.4 Some Terms and Definitions Relevant to the EU Legislation on the Safety of Food and Feed Cultures

One of the fundamental purposes of the EU (and other single-market areas in the world) is to enhance the free movement of both goods and people as an essential aspect of the single-market system. To this end, a harmonization of the legal and regulatory framework is naturally a prerequisite.

The basic legal instruments of EU – Regulations and Directives and their implementation – have already been described by Wessels (Chapter 2, this volume). As will become apparent in subsequent sections, the EU legislative framework related to food and feed is based on an intricate interplay between the participating actors, which is briefly outlined below.

The European Commission (henceforth 'Commission') is the government of the EU, and has both executive and – together with the European Parliament and the Council of the European Union – also legislative powers; it is the sole body that may initiate legislation. The Commission has several Directorates General, which form the executive branch of the Commission. Regarding microorganisms in food and feed, the Directorate General for Health and Consumers (SANCO) has the central regulatory role. SANCO is assisted by Standing Committees, the most important of which is the Standing Committee on the Food Chain and Animal Health (SCFCAH). The Standing Committee members represent member states and their interests. Each member state has a number of votes that are roughly proportional to the population of the country, with smaller countries, however, somewhat favoured. The decisions are made either unanimously or by a qualified majority. A qualified majority means that a decision requires votes from a combination of countries that together make up the majority of the EU population in order to get accepted.

The Council of the European Union consists of the cabinet ministers of the member states. Which ministers participate in the meetings depends on the items on the agenda. For instance, if food and feed questions are discussed, then the ministers under whose responsibilities these matters fall will attend the meeting; for most member states, this is the minister of agriculture.

The EU has also several agencies, which have been established to perform specific tasks. The European Food Safety Authority (EFSA), established in 2002, performs risk assessments related to food and feed, while the risk management measures belong to the Commission. The risk assessment is done by scientific panels consisting of independent experts, who must not represent their countries or institutions, but only science. Before the establishment of EFSA, the corresponding task was done by different Scientific Committees assisting SANCO, for instance the Scientific Committee for Food (SCF) or Scientific Committee on Animal Nutrition (SCAN). These committees are not to be confused with the Standing Committees already mentioned.

3.5 Starter Cultures – Additives, Processing Aids or Food Ingredients?

3.5.1 Situation in the EU

In the EU, there is presently no union-wide agreement on how the starter cultures used for food production should be legally defined and under what legislation they should be properly based. The two alternatives are either additives or processing aids. By definition, an additive is a substance that is intentionally added to a food for some specific technological purpose, and although an additive could also have some nutritional value, the intended use does not have to be nutritional. In contrast, a processing aid is used to facilitate some process, and ideally it should no longer be present in the final product; there is no obligation to list these among the constituents.

According to the recommendation of SCFCAH (SANCO, 2006), starters should be regarded mainly as processing aids. Only if a specific technological effect in food is sought, such as preservation, should a microbial culture be considered as an additive. It is specifically stated that probiotic cultures should not be classified as additives. So far, these recommendations have not led to any specific EU-wide harmonization of the national regulations. In view of recent developments, food cultures might be eventually classified as food ingredients, as pointed out by Wessels (Chapter 2, this volume).

While the situation with microbial cultures intended for food use currently is obscure in the EU, the case of microbial products intended for animals is very definite. These are unequivocally considered as feed additives, and this definition also covers the starters used for silage preparation. The relevant regulations are 1331/2003 EC and 767/2009 EC, and the requirements will be discussed in more detail under the specific sections on animal feed additives below (within Section 3.7).

3.5.2 The regulatory framework in the USA

In contrast to the EU, where the regulation of microbial cultures depends on whether they are

intended for food or feed, the US legislation is simpler and more straightforward. In the USA, animal feed is a subset of food and thus subject to the same laws as food for humans. Microorganisms deliberately added either into food or feed are considered as additives. The only exception to this is if the manufacturer of the microbial product makes a claim that the product cures, treats or prevents disease, which would make it a drug, and drugs require very extensive documentation. As additives, they have to be approved by the US Food and Drug Administration (FDA) and are subject to the authorization process defined in the Federal Food, Drug and Cosmetics Act. Alternatively, a microorganism and its specific use for a certain purpose can be considered as Generally Recognized as Safe (GRAS) (Wessels *et al*., 2004).

The GRAS status allows for an applicant to bypass the provision of the data to the FDA that would be required for an additive in the normal authorization process. There are two ways to achieve this status. Either the microorganism or substance can be shown to have a history of safe use dating to before 1 January 1958, or qualified experts summoned by the applicant have evaluated that the microorganism is safe under the conditions of the expected use. The FDA does not perform the GRAS evaluation, but either agrees or disagrees with the documentation presented by an applicant to support the proposed GRAS status.

It should be noted that a company can use microbial cultures for food and feed purposes without any notification to the FDA. However, in that case, the liability in the case of a safety incident would rest solely with the company, while a GRAS status agreed by the FDA would make the legal consequences much less severe. The list of GRAS notifications since 1998 can be found on the on the FDA web site (http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing; accessed 27 April 2012).

3.6 Food Microorganisms Specifically Regulated in the EU

Although the microbial cultures used for food production are not subject to specific regulation as additives in the EU, there are three specific cases, in which microorganisms for food use are subject to EU-wide authorization procedures:

- genetically modified microorganisms (GMMs);
- microorganisms considered as novel foods; and
- health claims associated with human probiotics.

3.6.1 Genetically modified microorganisms (GMMs)

The contained use of GMMs is regulated by Directive 2009/41/EC (OJEU, 2009). The applications falling under the scope of this Directive, however, do not include the use of GMMs as such in food or feed, and thus are not considered here further. Regarding direct food or feed use, the relevant regulations are Directive 2001/18/EC (OJEC, 2001) on the deliberate release of genetically modified (GM) organisms, and, especially, Regulation 1829/2003/EC (OJEU, 2003a) on GM food and feed.

Directive 2001/18/EC requires that the application (called 'notification') for placing a GMM on the market must be submitted to the Competent Authority (CA) of the member state in which the placing on the market is first planned, and the application must be accompanied by a safety assessment for humans, animals and the environment. The notification is evaluated by the CA, who submits its assessment to other competent CAs of the EU. If there are no comments or objections, the application is approved (for 10 years). In the case of disagreements, the Commission asks the opinion of EFSA, and after obtaining that opinion, submits the matter to SCFCAH. If SCFCAH cannot make the decision, the matter is moved to the Council of the European Union. If even the Council fails, then the Commission has the final word.

Regulation 1829/2003/EC specifically deals with the authorization of GM food and feed. The Regulation requires that GM food/feed must not have adverse effects on human and animal health or the environment or differ from the food/feed which it is intended to

replace to such an extent that its normal consumption would be nutritionally disadvantageous for consumers/animals. The notification, again submitted to the CA of some member state, is in this case passed to EFSA, which makes its own risk assessment. After receiving the assessment, the Commission makes its proposal, which is then subjected to the SCFCAH. Again, if the Committee is unable to approve the proposal by a qualified majority, the matter is passed to the Council, and failing Council approval, the Commission makes the final decision.

In practice, the scope of the Directive 2001/18/EC and that of the Regulation 1829/2003/EC often overlap, and the notifier may choose whether to submit the application entirely according to 1829/2003/EC or to submit the application, totally or partially, via both regulatory pathways.

3.6.2 Novel food legislation

The Novel Food Regulation 258/97/EC (OJEC, 1997), which aims to ensure the safety of foods which do not have a history of use in the EU, also defines foods that consist of, or are isolated from, microorganisms, fungi or algae as a category of novel foods. Taken literally, this would mean that any microorganism that was not used in food in the EU before 1997 should be subject to a specific safety evaluation, particularly because the Regulation does not define the level of novelty (genus, species or strain). In practice, although some products derived from microorganisms, such as a dextran preparation produced by *Lc. mesenteroides*, have been authorized according to 258/97/EC (Commission Decision 2001/122/EC), no actual microbial cultures have been considered novel enough to fall under the scope of this Regulation.

The authorization process for novel foods defined in the Regulation very much resembles the one defined for GM organisms in Directive 2001/18/EC, with the national authorities having a central role in risk assessment. However, the Regulation is being revised, and in the future, the role of EFSA in the risk assessment will be emphasized.

3.6.3 Human probiotics and health claims

The health claims associated with foods, including foods containing probiotic microorganisms, have been regulated by Regulation 1924/2006/EC (OJEU, 2006) since 2007. As this, indeed, is a Regulation, the EU member states may no longer have their own legislation here.

The health claims are divided into three groups:

- General function claims (article 13) that are based on generally known scientific facts ('calcium is good for bones') and describe the effects of a food or food component on growth, development, and functions of the body as well as on psychological functions, behaviour, weight control, satiety, etc.
- Novel function claims (article 13, paragraph 5) that are based on new observations and studies.
- Reduction of disease risk claims and claims referring to children's development and health (article 14).

The general function claims can be generically used once the list of acceptable claims has been introduced into the EU. The claims associated with probiotics belong to the two other categories and require approval from EFSA before they can be used. Several probiotics are under evaluation by EFSA, but so far no health claims have been approved. It should be noted that EFSA does not evaluate the safety of these products (unless they are considered novel foods), only their efficacy.

3.7 EU Legislation on Microorganisms Used as Feed Additives

Beneficial microorganisms can be added to feed for animals either in silage, as probiotics to improve the digestibility of feed material, to stabilize the gut microbiota of the animal, or as preservatives. Silage cultures, probiotics and preservatives are all defined as feed additives in the EU. The authorization of feed additives is covered by Regulation 1831/2003/EC (OJEU, 2003b) on additives for use in animal nutrition,

complemented by Regulation 429/2008/EC (OJEU, 2008) on the detailed rules on the preparation and presentation of the applications.

The application for authorization should be submitted to the Commission, which forwards it to EFSA for assessment. After receiving the opinion of EFSA, the Commission presents its proposal to the SCFCAH. If no decision is reached, the process follows the familiar route to the Council of the European Union and, if the Council fails to reach a decision, it goes back to the Commission.

Regulation 1831/2003/EC requires that a feed additive shall be allocated to some of the following categories:

1. Technological additives: any substance added to feed for a technological purpose.
2. Sensory additives: any substance, the addition of which to feed improves or changes the organoleptic properties of the feed, or the visual characteristics of the food derived from animals.
3. Nutritional additives.
4. Zootechnical additives: any additive used to favourably affect the performance of animals in good health or the environment.
5. Coccidiostats and histomonostats.

Most microbial additives are classified as zootechnical additives or technological additives. Within each category, there are also certain functional groups, listed in Annex I of the regulation. Within the zootechnological additives, the relevant functional groups are either digestibility enhancers or gut microbiota stabilizers. Silage additives form one of the functional groups of the technological additives, and preservatives another.

Each additive has to be assessed both for safety and efficacy. The safety assessment includes the effects on the target animal, user, consumer and environment. The efficacy assessment is related to the category and functional group. With zootechnological additives, the effects on animal performance, or in some cases on morbidity and mortality, should be assessed, while with technological additives, the desired technological function has to be verified. Detailed guidance documents on how to perform the safety and efficacy assessments have been published by the EFSA Panel on Additives and Substances used in Animal Feed (FEEDAP).

3.7.1 Safety assessment of additives

Target animal safety

Target animal safety should be established using a tolerance test designed according to the instructions in the technical guidance on tolerance and efficacy studies in target animals (EFSA, 2008b). The aim of the test is to provide a limited evaluation of the short-term toxicity of the additive and to establish a margin of safety if the additive is consumed at higher doses than are recommended.

Three groups of animals should be used in a tolerance test: a control group, the use-level group (receiving the additive at the maximum recommended dose) and a multifold-dose-level group. If possible, at least a tenfold overdose should be applied. The animals should be observed for visual evidence of clinical effects, performance characteristics, product quality where relevant, haematology and routine blood chemistry, and also for other parameters likely to be related to the biological properties of the additive. If a 100-fold overdose is tolerated, no haematology or blood chemistry is required.

The duration of the studies depends on the target animal category and is indicated in Table 1 of the EFSA guidance document (EFSA, 2008b).

Safety for the user

For additive products, the user is the person handling the feed, such as a lorry driver or the farmer. The studies to be considered according to the technical guidance on studies concerning the safety of the additive for users/workers (EFSA, 2008d) include tests for respiratory toxicity (in case the additive contains more than 1% on a weight basis of particles with a diameter $\leq 50\,\mu m$), skin and eye irritation, and skin sensitization. All available data on systemic toxicity should also be presented. The tests should be performed using the formulated commercial product. In practice, microbial additives, as proteinaceous substances, are automatically considered as respiratory sensitizers, and the general recommendation is to treat them accordingly.

Consumer safety

The technical guidance for establishing the safety of additives to consumer (EFSA, 2008c) focuses on chemical additives and their toxicity, and aims to establish a relevant Acceptable Daily Intake (ADI) value for the additive or its relevant metabolites that could be detected in animal products entering the market. Thus, this approach cannot be directly applied for microbial products. The former SCAN had formulated guidelines in 2001 for evaluating microorganisms and enzymes for feed (European Commission, 2001). These guidelines are still applied, although they are currently being revised by EFSA.

According to the 2001 guidance, the safety concern for the consumer is not the microorganism in the additive, but the potential unknown metabolites produced during the manufacturing process that can possibly contaminate the animal products. Therefore, both genotoxicity tests (assays for point mutations and clastogenicity) and 90-day repeated dose-feeding studies on laboratory animals are formally required. The toxicity studies are not required if the product is intended for companion animals only.

Environmental safety

The technical guidance for assessing the safety of feed additives for the environment (EFSA, 2008e) deals only with chemical additives, and so cannot be applied to microbial products. In practice, microbials are evaluated on a case-by-case basis, and generally no environmental studies are required, provided that the intended use is not expected to affect the levels of the microorganism in the receiving environment. This is the case with practically all microbial additives assessed so far.

3.7.2 Specific safety concerns

Safety of the Bacillus *species used as feed additives or for enzyme production*

While human probiotics are mainly lactic acid bacteria or bifidobacteria, several *Bacillus* species are currently used as feed additives (Williams *et al*., 2009). Because some bacilli, especially the *B. cereus* group, are known producers of enterotoxins and toxic cyclic peptides (the emetic toxin of *B. cereus*, and related so-called surfactins), the bacilli intended for additives or for the production of feed enzymes should be screened for the absence of toxins. The relevant guidance is EFSA guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA, 2011). For bacilli other than *B. cereus* the tests include screening for surfactins both by phenotypic criteria (haemolysis) and by PCR for ribosomally synthetized peptides completed with a cytoxicity assay for unknown toxins. If *B. cereus* strains are intended for feed use, the guidance recommends a complete sequencing of the genome and a bioinformatic confirmation of the absence of genes for toxins.

The presence of antibiotic resistance genes

The spread of antibiotic resistance genes to the food chain is a growing concern and has prompted the need to regulate their presence in microbial feed additives. The microorganism in the additive can harbour transmissible resistance genes. Although the microorganism is not pathogenic and does not pose a microbiological risk, there is a possibility that it could eventually transfer the resistance to pathogenic bacteria of either medical or veterinary importance, thus decreasing the efficacy of antibiotic therapy. These aspects are discussed in detail by Wilcks and van Hoek (Chapter 4, this volume).

The determination of the Minimum Inhibitory Concentrations (MICs) of a selected number of antibiotics representing the most important antibiotic groups is currently required for each bacterial strain used as a feed additive. This policy was started in 2001 when SCAN proposed the first list of MIC break points. MICs exceeding these break points indicate either an innate or acquired resistance in the microorganism. The latest update of the list is presented in the FEEDAP technical guidance of 2008 (EFSA, 2012). The document lists break points for lactobacilli (both for obligate homofermentative and for obligate and facultative heterofermentative

species), bifidobacteria, enterococci, pediococci, leuconostocs, *Lb. lactis*, *Strep. thermophilus*, bacilli, propionibacteria and Gram-positive bacteria in general. Additionally, MICs are proposed for *Escherichia coli*. The list of bacteria reflects the most common genera and species notified to EFSA.

In the case where the MIC indicates a resistance, the genetic basis of the antibiotic resistance has to be elucidated. A mutational resistance, although acquired, does not usually indicate an increased risk of the resistance spreading to other bacteria. Instead, a resistance associated with elements such as plasmids, transposons or integrons implies a possibility of the resistance factors spreading, making the strain unsuitable as an additive.

3.7.3 The efficacy studies

With zootechnical additives, the applicant should generally provide three studies on target animals showing a statistically significant ($P < 0.05$) positive effect on relevant parameters related to animal performance or on morbidity/mortality. Also, in certain cases, a meta-analysis can be considered. The detailed instructions on the efficacy studies and their duration for each target animal category are given in the technical guidance on tolerance and efficacy studies in target animals (EFSA, 2008b).

Microorganisms used as silage starters are considered technological additives. In the opinion of FEEDAP, for the establishment of guidelines on the assessment of safety and efficacy of silage additives (EFSA, 2006), the efficacy studies are recommended to be performed in mini-silos with a possibility to vent gas and effluent, and with 1 kg or more of fresh, homogenous feed material. The study should be performed at ambient temperatures (15–25 °C) and it should last at least for 90 days. The parameters to be followed include dry matter and calculated dry matter losses (corrected for volatiles), pH, concentration of volatile fatty acids (VFA) and lactic acid, concentration of alcohols and ammonia nitrogen.

3.8 Towards a European Generic Risk Assessment Approach for Microorganisms: Qualified Presumption of Safety

As noted above, in the EU there are only few instances in which there is a specific legal requirement for a safety assessment of a microbial culture intended for human food. In contrast, for microbiological feed additives, there exists a detailed and well-established regulatory framework according to which the safety aspects of the additives also have to be evaluated.

Taken literally, these requirements would mean that a *Lactobacillus* culture could be added into yoghurt without even a notification, but the same species would be subject to extensive safety studies when added into animal feed. The former SCAN, which performed the safety evaluation before the establishment of EFSA and its scientific panels, acknowledged this discrepancy, and initiated a system for a generic safety assessment of microorganisms. The initiative was taken up by the newly established EFSA in 2005, when an EFSA Scientific Colloquium on the proposed system, called Qualified Presumption of Safety (QPS) took place (EFSA, 2005). The system was formally adopted by EFSA in 2007 (EFSA, 2007). The QPS concept and its present scope have been recently reviewed (Leuschner *et al.*, 2010).

QPS aims to provide similar flexibility to the GRAS system in the USA, but takes into account specific safety concerns relevant to the European risk perception. So, for example, the transmissible antibiotic resistances, which are not considered a major problem in food or feed microbial cultures in the USA, are one of the central concerns in the EU.

The basic idea of the QPS approach is that a microorganism that has an established safety record can be notified to EFSA without most of the safety studies formally required in the guidance documents cited above, provided that certain qualifications apply. For example, a LAB strain with a history of safe use can be notified as a feed additive without studies for target animal, consumer and environmental safety (it should be noted, however, that the user safety

still has to be established). The only qualification required is the demonstrated lack of transmissible antibiotic resistance determinants.

Thus, an unequivocal taxonomic characterization, a well-established safety and the fulfilment of specific qualification are all prerequisites for a microorganism to be recommended for QPS.

Currently, the QPS list includes, among others, bifidobacteria, lactobacilli, lactococci, pediococci, leuconostocs, oenococci, propionibacteria, certain bacilli and yeasts, all of which predominate in EFSA notifications. For example brevibacteria, micrococci or starter staphylococci are not in the list, because, so far, there have been no EFSA notifications regarding these microorganisms (it should be remembered that EFSA, as a rule, does not assess the safety of food microorganisms intended for humans, unless they represent novel foods or GMMs). Although enterococci are regularly notified to EFSA, they have been excluded from the list because they require safety assessment at the strain level, making their generic assessment impossible. The same applies for filamentous fungi.

Since the formal adoption of the QPS approach by EFSA in 2007, the concept has been successfully used by the FEEDAP panel to assess microbial feed additives, and is also gaining increasing importance in the risk assessments performed by other EFSA panels, such as panels for food additives and nutrient sources added to food (ANS) and for food contact materials, enzymes, flavourings and processing aids (CEF). The list of QPS organisms is annually reviewed by the panel for biological hazards (BIOHAZ).

3.9 Regulatory Situation in Canada, Asia and South America

Although the focus of this chapter has been on the EU and to some degree on the USA, one should not overlook the regulatory developments in other geographical and economic areas. Generally, it appears that health claims associated with functional foods (probiotics included), rather than safety concerns, have triggered the legislation process. Developed countries like Canada and Japan on one hand, and vigorously emerging states like China, India and Brazil, on the other, each represent different situations regarding microorganisms intentionally added in foods.

In Canada, the authority responsible for overseeing the regulations on probiotics has been, since 2004, the Natural Health Product Directorate (Sanders *et al*., 2005). The focus of the regulations is on health claims, but it is also clearly stated that probiotics should be non-pathogenic microorganisms. Microbial feed additives are under the jurisdiction of the Canadian Feed Inspection Agency.

Japan has actually been a pioneer in the legislation of probiotic products since the introduction of the legal concept 'Food for Specific Health Uses' (FOSHU) by the Ministry of Health and Welfare (now the Ministry of Health, Labour and Welfare) in 1991 (Ohama *et al*., 2006). The FOSHU legislation is a formalized procedure for the approval of functional foods. Safety assessment, either from a history of safe consumption or from specific studies, must be included in the FOSHU application.

In China, the main actors are the Ministry of Health and the State Food and Drug Administration. The former has established a list of microbial species that can be used as food cultures without falling under the Chinese Novel Food Regulation (as is the case of many probiotic strains). Any functional claims are assessed by the State Food and Drug Administration (Kun Lee *et al*., 2012).

In South America, Brazil has the most advanced legislation on functional foods (De Luces Fortes Ferreira and Bonnet, 2011), mainly focusing on the health claims. It is to be expected that the South American Regional Trade Agreement (MICROSUL), which aims to form a Southern American single market, will eventually also lead to the establishment of a common regulatory framework in this region.

3.10 Conclusions

Microorganisms intentionally added to food are part of our everyday life and often have an extensive history of apparent safe human consumption. This fact has undoubtedly

contributed to the relatively minor attention paid by the regulators to the safety aspects of starter cultures, probiotics and protective cultures. In the EU, there has also been relatively little harmonization between regulations on food and feed. This has resulted in a situation where microorganisms added to feed are extensively regulated, while a safety assessment of food microorganisms is required only in cases when they are either GM or novel foods.

In the USA, the GRAS system has proven to be an efficient and pragmatic approach to address this kind of discrepancy. However, as such, it is not directly applicable in the EU because of not only different legal traditions, but also of differences in risk perception and safety concerns. The QPS concept aims to introduce a generic safety assessment approach to microorganisms notified to EFSA that takes into account these specific European viewpoints. While its main field of application has been the safety assessment of microbiological feed additives, it also has a promise to be a useful concept in the eventual future regulatory framework for microbial food cultures.

Globally developing legislation could lead to the undesirable situation that different countries or single-market areas impose different requirements on identical microorganisms. Thus, there is an obvious need for international communication and harmonization of practices without, of course, compromising the safety and efficacy of the products.

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4 Antibiotic Resistance in Relation to Starter Cultures and Probiotics

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4.1 Introduction

In recent years, discussions have been raised as to whether bacteria used as starter cultures (fermentation starters for, e.g. the production of meat or cheeses) and probiotics (live microorganisms which when administered in adequate amounts confer a health benefit on the host; FAO/WHO, 2001) could act as reservoirs for antibiotic resistance (AR) genes. When ingesting these bacteria, there is a risk that resistance elements could be transferred to the indigenous microbiota of the gut, and subsequently to pathogenic bacteria. This chapter summarizes current knowledge on AR genes and mobile elements in the genera used as starter cultures and probiotics, and on pitfalls in determining antibiotic resistance in specific strains.

A risk-based approach has been chosen in this chapter, focusing on the most important bacterial species used as starter cultures or probiotics and with AR genes relevant for human and animal health. Bacterial species of the lactic acid bacteria (LAB), bifidobacteria and coagulase-negative staphylococci are treated. We also focus on tetracycline and erythromycin, because they are two of the most widely used antibiotics in both clinical and animal therapy, and their resistance determinants are most commonly found among food-associated bacteria (Roberts, 2005, 2008; van Hoek *et al*., 2008a).

The determination of antibiotic resistance can be a challenging exercise. A gene may be detected, but is it functional and will it be expressed? Therefore, both phenotypic and genotypic analyses are important.

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Approved standards for phenotypic and genotypic determination of the presence of antibiotic resistance in food isolates are scarce, as are standards for the specification of minimum inhibitory concentration (MIC) values (Huys *et al*., 2010; Mayrhofer *et al*., 2010).

In a recently published technical guidance report (EFSA, 2008), the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) defined MIC break points to categorize bacteria as susceptible or resistant. The report also classified antimicrobial resistance into three categories: (i) intrinsic or natural resistance inherent to a bacterial species; (ii) acquired resistance caused by the mutation of indigenous genes; and (iii) acquired resistance due to the acquisition of exogenous resistance genes. FEEDAP concluded that strains carrying the third class of resistance are unacceptable as animal feed additives (EFSA, 2008). This is also reflected in the EU Council Regulation 429/2008 (OJEU, 2008), where it is stated that microorganisms used for feed production should be free of resistance towards antibiotics relevant for humans and animals. If resistance is discovered, the genetic basis for the resistance shall be revealed, and the likelihood of transfer to the gut microbiota shall be assessed. Only if it can be shown that the antibiotic resistance is a result of a chromosomal mutation(s), and non-transferable, can the strain be used.

EFSA has taken responsibility in launching the European initiative towards a 'Qualified Presumption of Safety' (QPS) concept (see von Wright, Chapter 3, this volume) which, like the 'Generally Recognized as Safe' (GRAS) system in the USA, is aimed to allow strains belonging to species with an established history and safety status to enter the market without extensive testing requirements. The presence of transmissible antibiotic resistance markers in the evaluation of strains is an important safety criterion. EFSA considers resistance to antibiotics, especially transferable resistance, a safety concern and a decision criterion for determining a strain's QPS status (EFSA, 2007). Distinguishing between intrinsic and acquired resistance is not always a straightforward process though and may require the comparison of antimicrobial resistance patterns in many strains of the same species from different sources.

4.2 Antibiotic Resistance in Food-associated Bacteria

As mentioned previously, the risk presented by AR genes is highest when they are located on mobile elements – such as plasmids and transposons – and therefore prone to transfer to other bacteria. However, resistance towards kanamycin, streptomycin, trimethoprim and vancomycin is often intrinsic, or a result of the mutation of indigenous genes. For instance, mutations of the *rpsL* gene for the ribosomal protein S12 can be responsible for streptomycin resistance in, e.g. *Bifidobacterium* (Kiwaki and Sato, 2009; Sato and Iino, 2010). Resistance due to mutation can also occur to erythromycin and tetracycline, the two antibiotics in focus in this chapter. An example is the erythromycin resistance of the commercial strain *B. bifidum* strain Yakult YIT 400, which is evidently linked to mutation in the 23S ribosomal RNA genes located on the chromosome (Sato and Iino, 2010), and therefore the risk of transfer is minimal. Then again, acquired antibiotic resistance mechanisms are increasingly described in the literature on beneficial and/or starter culture bacteria. For example, a Belgian study found a potentially probiotic strain of *Lactobacillus plantarum* containing a *tet*(S) gene giving tetracycline resistance which was located on a plasmid of approximately 14 Kb (Huys *et al*., 2006).

Examples of acquired AR genes in *Bifidobacterium* spp., *Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus thermophilus* and coagulase-negative *Staphylococcus* spp. are presented in Table 4.1. It is evident that tetracycline and erythromycin resistance determinants are widespread in species considered as beneficial and widely present in our food. The most frequently found tetracycline determinant is *tet*(M) (Roberts, 2005). In recent years, *tet*(W) has become nearly as prominent, although it has not been detected in all genera associated with food and feed (Cauwerts *et al*., 2006a; Kastner *et al*., 2006; Aires *et al*., 2007; van Hoek *et al*., 2008a). With

the exception of *Bifidobacterium*, the most commonly described erythromycin resistance gene among the genera discussed in this chapter is *erm*(B) (Mayrhofer *et al*., 2007; Huys *et al.*, 2008; Table 4.1).

A phenomenon that is not evident from Table 4.1 is that multiple tetracycline resistance determinants can be found in a single isolate. This was, for instance, demonstrated for the Sola cheese *Lb. sakei* isolate RitS 9 which possesses two different tetracycline resistance mechanisms, i.e. the ribosomal resistance protein Tet(M) and the *tet*(L) determinant coding for an efflux pump. Additional characterization revealed that *tet*(M) was located on a transposon on the chromosome, whereas *tet*(L) was present on a plasmid, pLS55 (Ammor *et al*., 2008c). Two *tet* genes in an individual bacterium have also been described by others (Chopra and Roberts, 2001; Cauwerts *et al*., 2006a; Flórez *et al*., 2007). Moreover, three tetracycline resistance determinants have also been reported, i.e. *tet*(L), *tet*(M) and *tet*(W) have been found in *Lb. johnsonii* isolates, whereas *B. thermophilum* B0219, besides *tet*(L), also harboured two different mosaic *tet* genes, *tet*(O/W) and *tet*(W/32/O) (van Hoek *et al*., 2008a).

4.3 Phenotypic and Genotypic Methods for Assessing Antibiotic Resistance

4.3.1 Phenotypic methods

Soon after the discovery of antibiotic resistant bacteria, phenotypic susceptibility testing became an integral part of infection management. The fundamental principles underlying the susceptibility testing methods have remained largely unchanged (Jorgensen and Ferraro, 2009).

Disc diffusion is a qualitative method used to screen for the antibiotic susceptibility of isolates. The test is performed by applying an inoculum of approximately 10^8 bacteria ml^{-1} to the surface of a Mueller-Hinton agar plate. Paper discs with a fixed antibiotic concentration are placed on the inoculated agar surface. After incubation, inhibition-zone diameters are measured and used as indicator for the cut-off between susceptible, intermediate and resistant strains (Fig. 4.1).

Quantitative antibiotic resistance data can be generated by a broth (micro)dilution method or by the Etest. Phenotypic antimicrobial resistance data to a certain antibiotic is expressed as the MIC, which is defined as the lowest antibiotic concentration that results in no visible growth of a strain. Broth dilution tests were one of the earliest antimicrobial susceptibility testing methods. Twofold dilutions are prepared of test antibiotics (e.g. 1, 2, 4, 8 and 16 mg m^{-1}) in an appropriate standardized susceptibility test medium. The antibiotic containing tubes are inoculated with a standardized bacterial suspension of $1–5 \times 10^5$ bacteria ml^{-1}. After overnight incubation at the appropriate temperature, the tubes are examined for bacterial growth and the lowest concentration of the antibiotic that prevents growth represents the MIC value. The tests have been adapted to high-throughput approaches, resulting in small, disposable, plastic 'microdilution' trays that make broth microdilution tests very practical.

Etest uses an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. A strain inoculum with a density corresponding to a McFarlane standard of 1 (3×10^8 bacteria ml^{-1}) is prepared. A sterile cotton swab is dipped into this standardized inoculum and used to inoculate an agar plate of the appropriate test medium. Inoculated plates are allowed to dry before application of the Etest strip and overnight incubation. The MIC value is determined by the intersection of the strip and the lower part of the ellipse-shaped growth inhibition area.

4.3.2 Genotypic methods

Early detection of AR genes was performed using sequence analysis and Southern blotting, but since the discovery of Taq polymerase, numerous PCR tests either with or without DNA probes have been developed to detect and identify resistance determinants (Arlet and Philippon, 1991; Aminov *et al*., 2001, 2002). Unfortunately, like most methodologies,

Table 4.1. Examples of erythromycin and tetracycline resistance genes found in bacteria isolated from fermented food products and (potential) probiotic strains.

Bacterium	Tetracycline resistance gene(s)	Erythromycin resistance gene(s)	References
Bifidobacterium			
B. adolescentis	*tet*(W)		van Hoek *et al.*, 2008a
B. animalis subsp. *lactis*	*tet*(W)	*erm*(X)	Kastner *et al.*, 2006; Masco *et al.*, 2006; Aires *et al.*, 2007; Ammor *et al.*, 2008a,b; van Hoek *et al.*, 2008a,b; Gueimonde *et al.*, 2010; Hammad and Shimamoto, 2010
B. bifidum	*tet*(M), *tet*(O), *tet*(W)		Moubareck *et al.*, 2005; Masco *et al.*, 2006; Aires *et al.*, 2007; Ammor *et al.*, 2008b; van Hoek *et al.*, 2008a
B. breve	*tet*(M), *tet*(O), *tet*(W)		Aires *et al.*, 2007; van Hoek *et al.*, 2008a
B. longum	*tet*(M), *tet*(O), *tet*(W)		Moubareck *et al.*, 2005; Aires *et al.*, 2007; Ammor *et al.*, 2008b; van Hoek *et al.*, 2008a
B. pseudocatenulatum	*tet*(W)		Moubareck *et al.*, 2005; Aires *et al.*, 2007
B. pseudolongum	*tet*(W)		Mayrhofer *et al.*, 2007; van Hoek *et al.*, 2008a
B. thermophilum	*tet*(L), *tet*(O/W), *tet*(W/32/O), *tet*(W)	*erm*(X)	Mayrhofer *et al.*, 2007; van Hoek *et al.*, 2008a,b,c
Enterococcus			
E. casseliflavus	*tet*(M), *tet*(S)	*erm*(B)	Teuber *et al.*, 1999
E. durans	*tet*(L), *tet*(M)	*erm*(B)	Huys *et al.*, 2004
E. faecalis	*tet*(L), *tet*(M), *tet*(S)	*erm*(A), *erm*(B)	Teuber *et al.*, 1999; Huys *et al.*, 2004
E. faecium	*tet*(L), *tet*(M), *tet*(S)	*erm*(B), *mrs*(A/B), *mrs*(C)	Huys *et al.*, 2004; Hammad and Shimamoto, 2010; Toomey *et al.*, 2010
E. gallinarum	*tet*(M), *tet*(O), *tet*(S)		Huys *et al.*, 2004
E. hirae	*tet*(L), *tet*(M)	*erm*(B)	Teuber *et al.*, 1999; Cauwerts *et al.*, 2007
Lactobacillus			
L. acidophilus	*tet*(M)	*erm*(B)	Cataloluk and Gogebakan, 2004
L. alimentarius	*tet*(M)		Gevers *et al.*, 2003a,b
L. amylovorus	*tet*(W)	*erm*(A), *erm*(B)	Cauwerts *et al.*, 2006b; van Hoek *et al.*, 2008a; Mayrhofer *et al.*, 2010
L. brevis	*tet*(W)	*erm*(C)	Aquilanti *et al.*, 2007
L. casei	*tet*(M)	*erm*(B)	Cataloluk and Gogebakan, 2004
L. crispatus	*tet*(K), *tet*(M), *tet*(W)	*erm*(B)	Cataloluk and Gogebakan, 2004; Cauwerts *et al.*, 2006a,b; Egervärn *et al.*, 2009; Mayrhofer *et al.*, 2010
L. curvatus	*tet*(M)	*erm*(B), mrs(A/B)	Gevers *et al.*, 2003a,b; van Hoek *et al.*, 2008a; Toomey *et al.*, 2010
L. fermentum		*erm*(B), *erm*(T), *vat*(E)	Fons *et al.*, 1997; Gfeller *et al.*, 2003

L. gallinarum	*tet*(K), *tet*(L), *tet*(M), *tet*(W)	*erm*(B), *erm*(C)	Cauwerts *et al*., 2006a,b; van Hoek *et al*., 2008a; Mayrhofer *et al*., 2010
L. gasseri	*tet*(M)	*erm*(B)	Cataloluk and Gogebakan, 2004
L. johnsonii	*tet*(L), *tet*(M), *tet*(W), *tet*(O/W/32/W/O)	*erm*(B), *erm*(C)	Cataloluk and Gogebakan, 2004; Aquilanti *et al*., 2007; Ammor *et al*., 2008b; van Hoek *et al*., 2008a,b; Egervärn *et al*., 2009; Mayrhofer *et al*., 2010
L. paracasei	*tet*(M), *tet*(W)	*erm*(B), mrs(A/B)	Huys *et al*., 2008; van Hoek *et al*., 2008a; Comunian *et al*., 2010; Toomey *et al*., 2010
L. paraplantarum	*tet*(W)		Egervärn *et al*., 2009
L. plantarum	*tet*(K), *tet*(M), *tet*(S)	*erm*(B)	Gevers *et al*., 2003a,b; Cataloluk and Gogebakan, 2004; Huys *et al*., 2006; Aquilanti *et al*., 2007; van Hoek *et al*., 2008a; Toomey *et al*., 2010
L. reuteri	*tet*(K), *tet*(L), *tet*(M), *tet*(W), *tet*(Z)	*erm*(B), *erm*(T), *erm*(C), *lnu*(A),[a] mrs(A/B)	Tannock *et al*., 1994; Cauwerts *et al*., 2006a,b; Kastner *et al*., 2006; Aquilanti *et al*., 2007; van Hoek *et al*., 2008a; Rosander *et al*., 2008; Egervärn *et al*., 2009, 2010; Toomey *et al*., 2010
L. sakei	*tet*(L), *tet*(M)		Gevers *et al*., 2003a,b; Ammor *et al*., 2008c; van Hoek *et al*., 2008a
L. salivarus	*tet*(K), *tet*(L), *tet*(M), *tet*(O)	*erm*(B), *erm*(C), *mef*(A)	Cauwerts *et al*., 2006a,b; Aquilanti *et al*., 2007; Hummel *et al*., 2007
Lactococcus			
L. garvieae	*tet*(K), *tet*(M)	*erm*(B), *erm*(C)	Aquilanti *et al*., 2007
L. lactis	*tet*(L), *tet*(M), *tet*(S)	*erm*(B), *erm*(C)	Teuber *et al*., 1999; Aquilanti *et al*., 2007; Ammor *et al*., 2008b; Flórez *et al*., 2008; van Hoek *et al*., 2008a; Devirgiliis *et al*., 2010
Streptococcus			
S. thermophilus	*tet*(L), *tet*(M), *tet*(S)	*erm*(B)	Flórez *et al*., 2008; van Hoek *et al*., 2008a; Rizzotti *et al*., 2009
Staphylococcus			
S. epidermidis	*tet*(K)	*erm*(A), *erm*(C), *mph*(C), *msr*(A)	Lüthje and Schwarz, 2006; Even *et al*., 2010
S. equorum	*tet*(K)	*lnu*(A), *mph*(C)	Lüthje and Schwarz, 2006; Resch *et al*., 2008; Even *et al*., 2010
S. haemolyticus		*erm*(C), *lnu*(A), *mph*(C), *msr*(A)	Perreten *et al*., 1998; Lüthje and Schwarz, 2006
S. piscifermentans	*tet*(K)		Resch *et al*., 2008
S. saprophyticus	*tet*(K)		Even *et al*., 2010
S. succinus	*tet*(K)	*lnu*(A)	Resch *et al*., 2008
S. xylosus	*tet*(K)	*lnu*(A), *mph*(C), *msr*(A)	Kastner *et al*., 2006; Lüthje and Schwarz, 2006; Resch *et al*., 2008; Even *et al*., 2010

[a] *lnu*(A) is a lincosamide resistance gene.

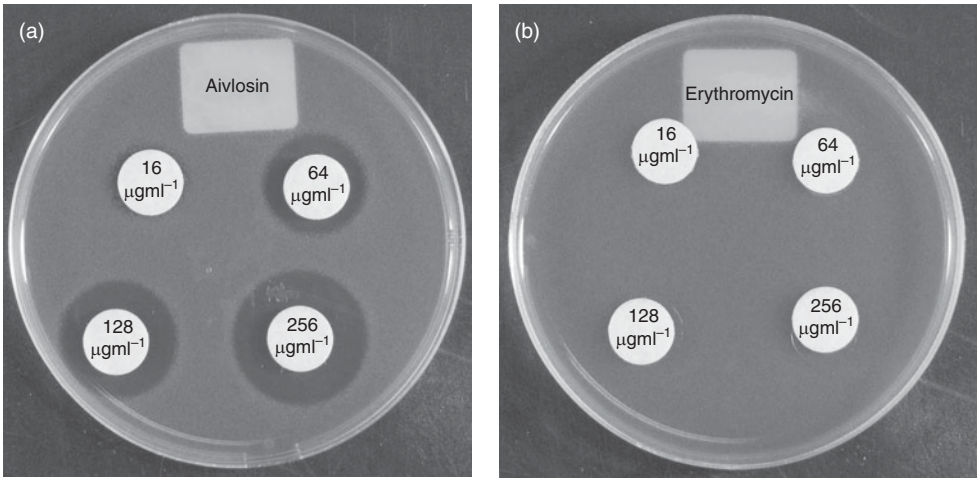

Fig. 4.1. Phenotypic disc diffusion test of the erythromycin-resistant *Lactobacillus amylovorus* LMG 18197 (harbouring *erm*(A)) with different concentrations of MLS (macrolide–lincosamide–streptogramin) antibiotics: (a) Aivlosin (3-acetyl-4 isovaleryltylosin) and (b) Erythromycin.

these techniques also have limitations, e.g. they only detect one or a few genes at a time.

With the development of the microarray technology (Schena *et al*., 1995), which consists of an arrayed series of thousands of microscopic spots of short DNA fragments, each representing a specific sequence, a high-throughput platform was introduced which is a suitable tool for detection of numerous AR genes. Although the principle behind the microarrays of DNA–DNA hybridization (i.e. Southern blotting) has been in practice in molecular biology for a long time, the key difference with this technology is the enormous number of genes that can be monitored on a single microarray. As new resistance determinants are constantly being discovered, the versatility of this method is an important feature. Initially, thematic microarrays dedicated to one antibiotic class were constructed (Lee *et al*., 2002; Call *et al*., 2003; Volokhov *et al*., 2003; Cassone *et al*., 2006), although within the last 5 years, more universal microarrays for the detection of AR genes belonging to different antibiotic classes in various microorganisms have been developed (Perreten *et al*., 2005; Frye *et al*., 2006, 2010; Patterson *et al*., 2007; Batchelor *et al*., 2008; van Hoek and Aarts, 2008).

4.3.3 Phenotypic versus genotypic assessment

Using the microarray technology, AR genes have been identified in several susceptible LAB and bifidobacteria. For instance, *B. adolescentis,* which has shown a tetracycline-susceptible phenotype carrying *tet*(W) (Mättö *et al*., 2008). However, sequence analysis revealed a gap in this gene, which resulted in a frameshift in the ORF (open reading frame) and an altered translation. Similarly, Florez *et al*. (2006) reported that a phenotypically tetracycline-susceptible bifidobacterial isolate harboured a shortened *tet*(W) gene sequence, which was consequently non-functional. Additionally, the presence of *erm*(B) and *tet*(S) was also demonstrated in two different *Strep. thermophilus* strains displaying susceptible phenotypes (van Hoek *et al*., 2008a). In the *Strep. thermophilus* DT isolate, the *tet*(S) gene had four single nucleotide insertions resulting in frameshifts and an incomplete Tet(S) protein, whereas in strain *Strep. thermophilus* S127, various PCR tests could not demonstrate a complete *erm*(B) gene preceded by a putative leader peptide.

These truncated, mutated or silent AR genes could potentially be activated again by, for example, mutation(s) or (homologous)

recombination conferring an antibiotic resistance phenotype. These potential phenomena indicate a need to use both phenotypic and genotypic assessment of antibiotic resistance for safety evaluation of new probiotic strains or starter cultures.

4.4 Horizontal Gene Transfer and Assessing Transferability

One of the cornerstones in the European QPS system is the assessment of any present AR genes and the potential for the transfer of these genes (EFSA, 2007). Moreover, a generic qualification for all QPS bacterial taxonomic units is that the strains should not harbour any acquired AR genes to clinically relevant antibiotics. As previously stated, the presence of acquired AR genes on mobile elements is of concern, because these have the greatest potential for transfer. Therefore, is it important that if resistance to antibiotics is envisaged in an industrial strain, the nature of resistance (intrinsic or acquired), and whether it is transferable or not, needs to be determined. A definite answer to this can be very difficult to find, as if no transfer is observed in one setting, this could be a false negative answer, i.e. an experiment in other settings or to another recipient could show that the resistance in question is transferable.

This section gives an overview of the present knowledge of mobile elements in the bacteria that are most commonly used as starter cultures and probiotics, and of different methods to assess the transferability of AR genes.

4.4.1 Mechanisms of transfer

Whereas vertical transfer is the clonal spread of a particular strain, resulting in identical daughter cells, horizontal gene transfer (HGT) is the exchange of genes between bacterial cells. So far, three mechanisms have been recognized whereby bacteria can transfer their DNA horizontally: (i) natural transformation by uptake and incorporation of free DNA; (ii) conjugation requiring cell-to-cell contact; and (iii) transduction, in which transfer is mediated by bacteriophages (Frost *et al*., 2005). Of these mechanisms, conjugation is considered to be the most important for spreading AR genes in microbial communities. The explanation for this is that conjugation allows DNA to move across species, genus and, in some cases, even kingdom lines, whereas transformation and transduction are usually restricted to transfer within the same species. Furthermore, many AR genes are found on mobile elements that are capable of transfer by conjugation.

The elements responsible for conjugation are conjugative plasmids, or integrative conjugative elements (ICE) such as conjugative transposons (Smilie *et al*., 2010; Wozniak and Waldor, 2010). Besides coding for their own transferability, these elements are also able to transfer smaller non-conjugative, mobilizable plasmids harbouring specific genes by a process called mobilization.

4.4.2 Mobile elements found in strains used as starter cultures and probiotics

Mobile elements have been identified in LAB and bifidobacteria, as well as in coagulase-negative staphylococci, showing the importance of testing a strain for AR genes and their nature and position in the genome before accepting that strain for use in food.

Bifidobacteria

Plasmids are rarely found in bifidobacterial species. So far, only small cryptic plasmids have been identified in a minority of tested bifidobacterial species (Ventura *et al*., 2007). Transposons and insertion sequences, however, seem to be relatively abundant in bifidobacteria. Several genomes of bifidobacterial strains are available in which the presence of transposons and insertion sequences has been identified (Garrigues *et al*., 2010; Sun *et al*., 2010; Wei *et al*., 2010), indicating that the transfer of AR genes cannot be ruled out.

As mentioned earlier, the tetracycline resistance gene *tet*(W) has been discovered in several *Bifidobacterium* species (Table 4.1), and detailed analysis of this gene has shown that a novel transposase flanked by imperfect

repeats is conserved in the vicinity of the gene of some strains, strongly suggesting the presence of a novel transposon (Kazimierczak *et al*., 2006; Ammor *et al*., 2007). Furthermore, transfer of *tet*(W) among *Bifidobacterium* species has been observed, although at low frequency, indicating that the transposons might be conjugative (Kazimierczak *et al*., 2006).

In addition, an erythromycin resistance gene, *erm*(X), found in strains of *B. thermophilum* and *B. animalis* subsp. *lactis* has been suggested to be part of transposon Tn*5432*, which is present in several opportunistic pathogens (van Hoek *et al*., 2008b). This indicates that *erm*(X) may be transferable, but further studies are needed to conclusively show whether this is the case. According to EU legislation though, a transposonal location of an AR gene will probably exclude such strains from being used as probiotics (von Wright, 2005).

Genetic analyses of the types of antibiotic resistance present in other *Bifidobacterium* strains showed that the resistances (to erythromycin, neomycin and streptomycin) were evidently acquired by mutations of the structural genes on the chromosome and not by import of mobile genetic elements such as insertion sequences, phages or plasmids (Kiwaki and Sato, 2009; Sato and Iino, 2010). So, in each case, careful analysis is required to establish whether the detected resistance is intrinsic, the result of mutation(s) of an intrinsic genetic determinants, or via an acquired AR gene.

Lactic acid bacteria (LAB)

Conjugative plasmids and transposons are common features in many LAB species (Teuber *et al*., 1999). In addition, HGT is a frequent phenomenon in LAB, which is also verified by sequence comparisons and hybridizations (Bellanger *et al*., 2009). A detailed description is given below of the known mobile elements in the different genera of LAB that are the focus of this chapter.

ENTEROCOCCUS. The genus *Enterococcus* is the best studied group among LAB regarding HGT. This is probably due to its importance in causing human infections, especially nosocomial infections, as a result of the production of various virulence factors and also because resistance to antibiotics is a growing problem with the emergence of multi-resistant strains in the two most prevalent species, *E. faecalis* and *E. faecium*.

Transferable AR genes are frequently found in *Enterococcus* species isolated from food (Teuber *et al*., 1999; Wilcks *et al*., 2005). Several mobile genetic elements, both plasmids and conjugative transposons, have been identified in strains of *Enterococcus*. Some of the best studied are the so-called pheromone plasmids, in which transfer is limited to within the species *E. faecalis*. The conjugative transfer is initiated by recipients excreting pheromones that attract donor cells, resulting in aggregation between recipient and donor, and finally transfer of the pheromone plasmid (Wardal *et al*., 2010). Several antibiotic resistance and virulence genes have been found on the approximately 20 pheromone plasmids known to date.

Mobile genetic elements, plasmids and transposons with a much broader host range are also found in *Enterococcus*. A well-characterized broad host range conjugative plasmid, pAMβ1, isolated from *E. faecalis*, carries a constitutive MLS resistance (cross resistance to macrolides, lincosamides and streptogramins), and transfer of this plasmid to several bacterial species has been observed. Furthermore, several plasmids resembling pAMβ1 have been found in other Gram-positive genera, showing its broad host range. The conjugative transposon Tn*916* that confers resistance to tetracycline was first identified in *E. faecalis*. It belongs to the Tn*916*-Tn*1545* family of conjugative transposons that are able to be transferred and maintained in a wide range of clinically important Gram-positive and Gram-negative species (Clewell *et al*., 1995).

Because of the abundance of virulence and antibiotic resistance traits in this genus, no *Enterococcus* species have been listed in the QPS system, and it is imperative that each particular strain is carefully evaluated to avoid presence of any known virulence factors, and to ensure that the strain is sensitive to clinically relevant antibiotics. In general, *E. faecium* appears to pose a lower risk than

E. faecalis for use in food, as the strains of the former generally harbour fewer recognized virulence determinants and antibiotic resistance genes, probably as a result of the presence of pheromone plasmids in the latter.

LACTOBACILLUS. The potential spread of AR genes has not been extensively studied in lactobacilli. Consequently, reports on the conjugal transfer of native *Lactobacillus* plasmids are limited. However, recent studies have demonstrated the potential of AR genes to spread from *Lactobacillus* isolated from food.

Gevers *et al*. (2003a) isolated tetracycline resistant *Lactobacillus* isolates from fermented dry sausages and studied their conjugative transfer potential. Seven out of 14 isolates were able to transfer the resistance to *E. faecalis in vitro* and the tetracycline resistance could be linked to the presence of *tet*(M) genes located on a member of the broad host range Tn*916*-Tn*1545* conjugative transposon family. Some of these isolates were also able to transfer *tet*(M) to *E. faecalis in vivo* in studies with di-associated gnotobiotic rats (Jacobsen *et al*., 2007). Furthermore, an *Lb. plantarum* strain isolated from French raw milk cheese was shown to transfer a plasmid harbouring *erm*(B), both *in vitro* and *in vivo*, in di-associated gnotobiotic rats (Feld *et al*., 2008).

LACTOCOCCUS. Several gene transfer mechanisms have been found in lactococci (Gasson, 1990), including an aggregation mediated high-frequency conjugation system. In a study of the fermenting microbiota of an Italian cheese, Devirgiliis *et al*. (2010) discovered several *Lc. lactis* strains harbouring either *tet*(M) or both *tet*(S) and *erm*(B), which in both cases could be attributed to the presence of plasmids. Furthermore, plasmids harbouring *tet*(M) were able to transfer the gene to an *E. faecalis* strain (Devirgiliis *et al*., 2010), showing the risk of HGT of AR genes among food-borne commensal bacteria. In another study, Tn*916*-like elements found in tetracycline resistant *Lc. lactis* strains isolated from Polish raw milk were transferred to *E. faecalis* both in filter mating experiments and *in vivo*, using gnotobiotic rats (Boguslawska *et al*., 2009). Furthermore, intraspecies transfer of this element was found to take place in filter mating experiments in an animal rumen model and in an alfalfa plant model (Boguslawska *et al*., 2009; Toomey *et al*., 2009).

STREPTOCOCCUS THERMOPHILUS. Information on the transfer of AR genes by *S. thermophilus* is limited, although there are recent studies showing that both plasmids and ICE are capable of transfer. A strain of this species isolated from raw milk was shown to be able to transfer a plasmid-harbouring *erm*(B) to an *E. faecalis* recipient in *in vitro* filter mating studies and in an animal rumen model (Toomey *et al*., 2009). Additionally, tetracycline resistant *S. thermophilus* strains isolated from soft cheeses were found to harbour several tetracycline resistance genes – *tet*(L), *tet*(M) and *tet*(S) – that were localized on plasmids and transposons. The gene *tet*(M) was located on a Tn*916*-Tn*1545* family transposon, which is probably responsible for the acquisition of this AR determinant by the bacterium (Rizzotti *et al*., 2009). Furthermore, *tet*(S) and *tet*(M) could be associated with the presence of plasmids. However, the authors were unable to detect transfer when using the tetracycline resistant strains as donors in filter mating, though it cannot be ruled out that the strains are capable of transferring the tetracycline resistance genes under other conditions. In addition, the high sequence similarity of the isolated genes with other tetracycline resistance genes in databases, and their position on mobile genetic elements, indicate that transfer can happen from species present in the same dairy environment as *S. thermophilus*.

Conjugative transfer of an integrative conjugative element, ICE*St3*, was recently shown to occur from *S. thermophilus* to *S. pyogenes* and *E. faecalis* (Bellanger *et al*., 2009). This ICE did not contain any AR gene, but the study shows the ability of this genus to spread its DNA, and the need for assessing the potential transfer of recognized resistance genes.

Coagulase-negative staphylococci (CNS)

Knowledge about the presence of mobile genetic elements in two of the most widely used starter cultures of coagulase-negative *Staphylococcus* (CNS) for meat fermentation, i.e. strains of *S. carnosus* and *S. xylosus*, is quite

limited. *S. carnosus* belongs to the non-pathogenic *S. simulans*-group, whereas *S. xylosus* belongs to the phylogenetic *S. saprophyticus* group, which contains *S. saprophyticus*, an important opportunistic pathogen in human urinary tract infections. Studies have shown that *S. xylosus* strains isolated from food or used as starter cultures were resistant to lincomycin, penicillin, fusidic acid, oxacillin, ampicillin, chloramphenicol, tetracycline and erythromycin (Irlinger, 2008; Resch *et al*., 2008). CNS of food origin could therefore represent a reservoir of AR genes. Conversely, strains of *S. carnosus* exhibit markedly less antibiotic resistance than strains of *S. xylosus*. It has therefore been proposed that there is a correlation between the incidence of antibiotic resistance in food-associated CNS and their relatedness to (opportunistic) pathogenic *Staphylococcus* species (Resch *et al*., 2008), and that the non-pathogenic CNS have received the AR genes from their pathogenic counterparts. This assumption is substantiated by the fact that the genetic basis of some of the antibiotic resistance encountered in *S. xylosus* could be established, and that this often resided on plasmids (Irlinger, 2008; Resch *et al*., 2008), indicating that the resistance observed is acquired, and not intrinsic to the species.

The genome of the meat starter culture bacterium *S. carnosus* TM300 has recently been sequenced, and besides one prophage and one genomic island, the genome does not contain mobile elements such as plasmids, insertion sequences or transposons (Rosenstein *et al*., 2009; Rosenstein and Götz, 2010). No information about the presence of mobile elements in *S. xylosus* is available, but its pathogenic counterpart *S. saprophyticus* only contains five mobile elements (Rosenstein *et al*., 2009), which could indicate that also *S. xylosus* has very few such elements. So more research is needed on the presence of mobile elements in food-associated species of CNS, and their role in spreading AR genes in the food chain.

4.4.3 Methods/models to study transfer

HGT of AR genes between bacterial cells can be studied in different models, starting with simple laboratory bench *in vitro* models and progressing to complex *in vivo* models, e.g. the gastrointestinal (GI) tract of humans or animals.

In vitro models are often divided into filter or plate mating and broth mating. Most conjugative plasmids are transferred only when bacteria are attached to solid surfaces, but a few plasmids transfer with a very high frequency in liquid media, e.g. the pheromone plasmids from *E. faecalis* (Wardal *et al*., 2010) and the *agr* plasmids from *Lc. lactis* (Gasson, 1990). *In vitro* studies are cheap, easy and fast compared with *in vivo* experiments and allow the conjugation to proceed under controlled conditions. It is possible to optimize the mating procedure to offer the best conditions for transfer, and the impact of specific factors on transfer frequency can be analysed. Furthermore, the transfer potential of different mobile elements and mating pairs can be compared under similar conditions.

Attempts have been made to standardize testing for HGT of AR genes (Lampkowska *et al*., 2008). However, transfer depends on the properties of the mobile element under study – the donor as well as the recipient. Therefore, it is difficult to set up standardized protocols and there are no optimal transfer conditions universal for all mating pairs. Lampkowska *et al*. (2008) also showed that, even if different laboratories used the same optimized method, a high variability in transfer rates was observed.

The results of *in vitro* tests may be difficult to transfer to the real life situations, and therefore several more realistic models have been developed. Models have been developed where transfer in food, on plant surfaces and in the GI tract of humans and animals can be studied. The GI tract is considered to be a hot spot for HGT among bacteria (Licht and Wilcks, 2006), and so this section will give a short overview of *in vivo* models that simulate the human gut.

Models based on rodents are frequently used to simulate the GI tract of humans. The starting point for the development of rodent models is often germ-free rats or mice, i.e. animals without an indigenous microbiota. These animals enable the establishment of a gnotobiotic model, i.e. a model with a known

and defined microbiota. The mono-associated or di-associated rodent model (harbouring, respectively, one or two strains) constitutes the simplest type of model, though gradually more complex models can be made by inoculation with several different bacterial species. These can be specifically chosen and carefully composed or be a more or less unspecified microbiota derived from human faecal samples for development of the human microbiota-associated (HMA) model. Other frequently used models are antibiotic-treated animals, i.e. conventional rats or mice given a high concentration of antibiotics, often streptomycin, that enables the incoming strain to be established in the GI tract.

Some studies comparing *in vitro* and *in vivo* models, using the same donor and recipient, have demonstrated difficulties in comparing results from one situation to another. In some studies, the transfer rate *in vitro* was higher than that *in vivo* (Jacobsen *et al*., 2007), whereas in other studies it was *vice versa* (Feld *et al*., 2008), all depending on the mobile element, the donor and the recipient. This shows that we are still quite far from understanding what the optimal transfer conditions are, and which factors in the GI tract may favour or inhibit a transfer event. Those conditions and factors are most probably dissimilar among the different combinations of mobile element, donor and recipient.

However, it should be emphasized that, even if extensive studies have been performed showing no transfer, transfer cannot be totally excluded. Therefore, in many cases, companies will decide not to pursue the development of a strain harbouring AR genes or, alternatively, will try to eliminate the resistance gene by curing methods.

4.4.4 Curing for antibiotic resistance

The transfer rates of AR genes that are stably located on the chromosome, for instance in the vicinity of a housekeeping gene, are considered low (Courvalin, 2006). For example, the probiotic strains *B. lactis* DSM 10140 and *B. animalis* subsp. *lactis* Bb-12 (also known as strain VTT E-012010), were found to be tetracycline resistant and to harbour *tet*(W) with chromosomal integration (Kastner *et al*., 2006; Saarela *et al*., 2007). Owing to its location, no actions have been undertaken to remove the AR gene from these probiotics.

The potential spread of AR determinants increases when they are carried on mobile genetic elements, in particular self-transferable plasmids or transposons (Masco *et al*., 2006; Rizzotti *et al*., 2009). For instance, the *tet*(W) gene, with similar characteristics to those just described, was identified in the commercially available probiotic strain *Lb. reuteri* ATCC 55730 (Rosander *et al*., 2008) as located on a plasmid. Moreover, this strain also contained another plasmid carrying a lincosamide resistance gene, *lnu*(A). Consequently, the presence of two resistance plasmids in a probiotic bacterium directed the scientists to remove the transferable elements by plasmid curing via protoplast formation. Other curing techniques are also used, including chemical curing with agents such as novobiocin, sodium dodecyl sulfate, acridine dyes and ethidium bromide, or the use of high temperatures. For instance, novobiocin was used by Huys *et al*. (2006) to cure the potential probiotic *Lb. plantarum* CCUG 43738 from a *tet*(S) determinant of approximately 14 Kb on a plasmid. Treatment of the CCUG 43738 strain eliminated this plasmid and restored the tetracycline-susceptible phenotype of the host strain.

4.5 Conclusion

Careful selection and screening processes have to be undertaken before applying an LAB, bifidobacterial and/or coagulase-negative staphylococcal strain as new starter culture or probiotic product. AR genes are common features in the genera used as starter cultures and probiotics, and therefore a comprehensive risk assessment is needed to avoid the spread of any transferable AR genes to pathogenic species.

A problem associated with safety determinations of starter strains and probiotics is that, once a resistance phenotype and an associated resistance determinant have been identified, it becomes difficult to show that this determinant is not transferable. It is impossible at this time to set up a standardized method

by which transfer of an antibiotic resistance determinant can be ruled out. In addition, laborious and cumbersome studies are sometimes required to prove that, for example, the resistance phenotype is due to a mutation in an indigenous gene and therefore most probably not prone to transfer.

We have clearly described the possible risks to animals and humans related to antibiotic resistant LAB, bifidobacterial and/or coagulase-negative staphylococcal strains, and their role in the exposure of pathogenic bacteria to AR genes. But the rise of antibiotic resistant pathogens in the last decades is also related to the massive use of antibiotics in all those fields in which LAB bifidobacterial and/or coagulase-negative staphylococcal strains are also employed, such as agriculture, and veterinary and human medicine, and therefore a more prudent use of antibiotics is suggested.

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5 Biopreservation of Food and Feed by Postharvest Biocontrol with Microorganisms

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5.1 Introduction

The primary justification for conducting postharvest biocontrol research was to replace or reduce the use of synthetic chemicals (Wilson and Wisniewski, 1989) because of concerns regarding their potential impact on human health (National Research Council, 1987), and especially on children's health (National Research Council, 1993) and the environment. The discovery of biotypes of postharvest pathogens that were resistant to the major postharvest fungicides, as well as the potential loss of registration for some of the commonly used postharvest fungicides, also added to the need for alternative strategies. There was an assumption that prospects for the success of postharvest biocontrol products were greater than those of biocontrol agents developed to manage soil and foliar diseases. Factors supporting this premise were the ability to better regulate the physical environment (temperature, humidity, etc.) during postharvest processing and storage, the ability to target high numbers of the biocontrol agent directly to the desired location of activity, and the overall value of the commodity at the postharvest stage of production. In practice, however, despite the advantages noted, the impressive progress made in postharvest biocontrol research and the development of commercial products, acceptance and widespread use of this approach as a postharvest disease management strategy is still limited in large-scale commercial settings (Wisniewski *et al*., 2001, 2007; Droby and Lechter, 2004).

In the early 1980s, one could find one or two publications per year on postharvest biocontrol, while a current literature search on this

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topic will bring up at least a 100 related publications per year, and over a 1000 articles covering the whole time period. It is now evident that postharvest biocontrol has matured into a major field of research and commercial endeavour. Wilson and Pusey (1985) noted the potential for postharvest biocontrol, and Wilson and Wisniewski (1989) enumerated many of the first principles and concepts defining postharvest biocontrol research. Several reviews have also been written over the years (Janisiewicz, 1998; Wilson and Wisniewski, 1994; Droby *et al*., 2000, 2003b; El Ghaouth *et al*., 2004). More recently, Droby *et al*. (2009) evaluated the progress on this topic of research and, importantly, tried to identify the challenges and ideas that will generate research and product development in the next two decades.

5.2 Use of Natural Antagonists for Food Preservation

The use of fungi and bacteria to either modify food or preserve it has been an integral part of human civilization. To extend this concept into a scientific approach for managing postharvest decay is perhaps very logical rather than far-fetched. For postharvest biocontrol, there has been an underlying hypothesis that there are species of microbes present on fruit and vegetable surfaces, as well as on harvested grain, that are antagonistic to decay fungi. By identifying these species, and reapplying them to the surface of harvested commodities in high numbers, one could extend the shelf life of the commodity without the use of a synthetic chemical.

In 1984, strain B-3 of *Bacillus subtilis* was found to control brown rot of peaches caused by *Monolinia fructicola* (Pusey and Wilson, 1984) and the organism was patented. Subsequently, it was determined that the main mode of action of B-3 in controlling brown rot was the production of the antibiotic iturin. It was felt, at that time, that there would be resistance to the application of an antibiotic-producing microorganism on to food, and commercialization of B-3 was not pursued even though in pilot tests it demonstrated control of brown rot comparable to that by synthetic fungicides (Pusey *et al*., 1988). Interestingly, from a scientific, commercial and registration standpoint, this viewpoint may not have been valid, as new concepts are being discovered about the actual role of antibiotics in microbial biology (Mlot, 2009), and several biocontrol products have been developed that utilize antibiotic-producing strains of *B. subtilis*.

In order to reduce the potential for selecting antagonists that produce antibiotic compounds, and also the concerns regarding consumer acceptance of placing large numbers of bacteria on a food surface, research on postharvest biocontrol has emphasized the identification and utilization of various species of yeasts (Droby *et al*., 2009), and a new *in planta* selection protocol was developed by Wilson *et al*. (1993) which has since become a standard approach for efficiently identifying potential new antagonists. Rather than *in vitro* screening of organisms in Petri plates, which favours the identification of antibiotic-producing organisms, the method involves placing washing fluids obtained from the surface of fruit into fruit wounds that are subsequently inoculated with a rot pathogen. Organisms are then isolated from wounds that do not develop infections. These are plated out, isolated in pure culture and identified. Using this procedure, pure cultures of potential antagonists are produced and screened individually in fruit wounds to assess their potential as biocontrol agents. This procedure has been used worldwide in many research programmes and has led to the identification of numerous yeast antagonists, many of which display antagonistic activity to a broad spectrum of postharvest disease fungi on a wide variety of crops. A comprehensive list of identified antagonists has been recently published in a review by Sharma *et al*. (2009).

5.3 Development and Commercialization of Biocontrol Products

At present, only a handful of postharvest biocontrol products are commercially available

Table 5.1. Postharvest biocontrol agents registered for commercial use.

Antagonist	Trade name	Country of registration
Aureobasidium pullulans	Bio-Protect	Austria
Candida oleophila	Aspire[a]	USA, Israel
Candida oleophila	Nexy	Belgium
Candida sake	Candifruit	Spain
Cryptococcus albidus	YieldPlus[a]	South Africa
Metschnikowia fructicola Kurtzman and Droby	Shemer	Israel
Pseudomonas syringae Van Hall	Biosave 110, Biosave 111	USA

[a]Not commercially available

and these have limited use, mostly in niche markets (Table 5.1).

Biosave (*Pseudomonas syringae* Van Hall) is registered in the USA and used mostly for the control of sweet potato and potato diseases (Stockwell and Stack, 2007). Shemer (*Metschnikowia fructicola* Kurtzman and Droby) is registered in Israel and used commercially for the control of sweet potato and carrot storage diseases (Kurtzman and Droby, 2001; Blachinsky *et al*., 2007; Eshel *et al*., 2009). Two early yeast (*Candida*)-based products, Aspire (Ecogen, USA) and YieldPlus (Anchor Yeast, South Africa), are no longer available. BioNext (Belgium) is developing a commercial product, Nexy, based on the same yeast used in Aspire, *Candida oleophila*, and a product based on the yeast *Candida saitoana* is being developed by Neova Technologies (Abbotsford, British Columbia, Canada). Candifruit, based on *Candida sake* CPA-1, is produced in Spain by Sipcam for managing postharvest diseases of temperate fruit crops and citrus. Bio-Protect, based on the yeast-like basidiomycete *Aureobasidium pullulans* is produced by Biofarm Co. in Austria for control of postharvest decay and fire blight on apple. How these products will fare will largely depend on their ability to control postharvest diseases in a reliable, cost-effective and easy-to-use manner.

An interesting application in the management of postharvest diseases has been the use of the yeast *Pichia anomala* to inhibit mould spoilage of grain used as animal feed during air tight storage (Druvefors *et al*., 2002, 2005). Of 57 species of yeast tested, *P. anomala* had the highest level of biocontrol activity against *Penicillium roquefortii*, the leading cause of spoilage and mycotoxin production in stored grain (Druvefors and Schnürer, 2005). Schnürer and Jonsson (2011) have recently reviewed 30 years of effort to study and commercialize this yeast.

5.4 Mode of Action

A full understanding of the tri-trophic interactions taking place at a wound site between the antagonist, host tissue and pathogen is a prerequisite for the development of successful biocontrol strategies. When studying these interactions, competition for space and nutrients, host resistance and wound response, as well as interaction with other microorganisms, have to be taken into consideration. This conceptualization, however, raises some critical questions: (i) what are the effects of antagonists on wound healing and host resistance; (ii) how important and widespread are the direct effects of antagonists on pathogens; (iii) how do incidental microorganisms or mixtures of antagonists affect pathogen–antagonist interactions; and (iv) how does the nutrient/chemical composition at the wound site affect the antagonist, other microbiota, the infection process and the wound response?

To inhibit infection, an antagonist must be present in the wound site before the arrival of the pathogen or within a short period thereafter. Several studies on the mode of action of biocontrol agents of postharvest diseases of fruits and vegetables have reported rapid colonization of surface wounds by the antagonist. The high population of antagonist cells presumably then outcompetes the pathogen for nutrients and space. Indirect evidence has been provided to demonstrate the role of competition for nutrients as part of the mode of action here: (i) there is inhibition of spore germination or growth of the pathogen during co-culturing with the antagonist; (ii) the inhibition of the pathogen is dependent on the concentration of the antagonist propagules; and (3) partial or

complete reversal of inhibition can be achieved by the addition of exogenous nutrients.

In most reports, a quantitative relationship between antagonist concentration and efficacy has been demonstrated. Thus, a delicate balance apparently exists at the wound site between the number of antagonist cells and the number of pathogen propagules which affects the outcome of the interaction and determines whether or not the wound becomes a site of infection. Manipulation of the initial concentration of the antagonist cells and/or the fungal spores clearly affects infection.

While nutrient competition appears to play a major role in the biocontrol activity of many postharvest antagonists, it is rare for only one mechanism of action to be involved in the suppression of a disease (Droby *et al*., 1989, 2000; Janisiewicz *et al.,* 2000). A successful biocontrol agent is generally equipped with several attributes, which often work in concert and may be crucial for controlling disease development. For example, colonization and nutrient competition may be related to: the ability of biocontrol agents to adhere to specific sites, including both host and pathogen tissues (Wisniewski *et al*., 1991, 2007); the exudation of specific enzymes (Castoria *et al*., 1997; Yehuda *et al*., 2003); the ability to induce resistance (Droby *et al*., 2002); the ability to regulate population density at specific sites (McGuire, 2000); the secretion of antimicrobial substances (water soluble or volatile); and, perhaps, the production of specific active metabolites induced upon interaction with fruit/plant tissues (Janisiewicz *et al*., 1991; Smilanick and Denis-Arrue, 1992; Schotsmans *et al*., 2008).

One of the more novel discoveries regarding the mode of action of yeast antagonists is the ability of some of them to adhere to and parasitize pathogen hyphae (Wisniewski *et al*., 1991). This report was the first to document the ability of yeasts to parasitize higher fungi. Other key factors that appear to play a role in the efficacy of yeast antagonists are the production of lytic enzymes by the yeast (Bar-Shimon *et al*., 2004; Friel *et al*., 2007), and their ability to tolerate high levels of salts (Wisniewski *et al*., 1995).

Castoria *et al*. (2003) demonstrated that the ability to tolerate the high levels of reactive oxygen species (ROS) produced by fruit tissue is an essential characteristic of effective yeast antagonists. This discovery has raised many new questions about the role of ROS in biocontrol activity. Reports on the induction of resistance responses in fruit by the application of antagonists within a wound or on the fruit surface have also been important in understanding the biology of postharvest biocontrol (Wilson and Wisniewski, 1994; Droby *et al*., 2002; El Ghaouth *et al*., 2003). More recently, molecular approaches have been used to examine the role of glucanases on the biocontrol activity of the yeast *C. oleophila* (Yehuda *et al*., 2003), and the ability to enhance biocontrol activity by the over-expression of antimicrobial peptides (Wisniewski *et al*., 2003; Janisiewicz *et al*., 2008).

5.5 Integration of Biocontrol Approaches

Over the past several years, researchers have sought new ways of increasing the efficacy of postharvest biocontrol agents by the use of natural antimicrobials, salts that alter the pH and manipulation of the physical environment (e.g. heat treatment) (Droby *et al*., 2003a, 2009). Attempts to increase efficacy were based on the ability of the compound or environmental manipulation concerned to enhance the competitiveness of the biocontrol agent, increase the antimicrobial activity of the biocontrol agent in an additive or synergistic manner, or induce resistance responses in the harvested commodity.

The development of integrated approaches to biocontrol has led to an expanded view of this field, one that goes beyond the 'entomology' paradigm defined as the control of one organism by another organism (Droby *et al*., 2009) to one in which one biological process (disease) is regulated with another biologically based process (e.g. microbial antagonism, the use of natural compounds, induced resistance, genetic resistance, etc.). It is a basic premise that paradigms drive scientific research and have a major impact on how we explore and interpret systems. Importantly, this evolving, expanded view represents a

distinct paradigm shift and is leading to a fundamental change in the way that we think about biological control and the development of biocontrol products and strategies.

5.6 Safety of Biocontrol Agents Used on Food

As already noted, concern has been raised about the safety of using microbial antagonists as postharvest treatments and allowing consumers to ingest them in their diet. Although this may represent an obstacle to public acceptance of the technology, the majority of postharvest biocontrol agents identified for potential development and marketing have been isolated from fruits and vegetables and so are indigenous to agricultural commodities. Humans are already exposed to them daily when consuming fresh vegetables and fruit. Even though these antagonists are introduced in large numbers to the surface of a commodity, they survive and grow only in very restricted sites on the fruit surface (e.g. surface wounds). After their introduction on intact fruit surfaces, antagonist populations usually diminish to the level of natural epiphytic microbiota within a very short period of time.

The safety of biocontrol yeasts used to preserve food was recently reviewed by Sundh and Melin (2011). They indicated that the use of hemiascomycetous yeasts in the biopreservation of food raised less concern than the use of filamentous fungi and bacteria because of their general lack of production of mycotoxins. There are reports of yeasts being isolated from immunologically compromised people and yeasts are also thought to be associated with some gastrointestinal and inflammatory problems (Jensen and Smith, 1976; Garcia-Martos *et al*., 1999). However, as indicated by Fleet and Balia (2006), the risk of yeasts causing gastrointestinal infections is very low.

There are only a few reports indicating that yeasts can be pathogenic to plants. In one study, *Pichia fermentans*, while an effective biocontrol agent against postharvest rots of apple, was pathogenic to peach (Giobbe *et al*., 2007). Another report indicated that the yeast-like fungi *A. pullulan*s and *Rhodotorula glutini* are responsible for the russeting of apple fruit (Matteson Heidenreich *et al*., 1997). Similar concerns can surround postharvest biocontrol products that are based on bacterial antagonists. One example is the Biosave product based on *P. syringae*, which is reported to be pathogenic on several plants and fruits (Spotts and Cervantes, 1994; Stefani and Stead, 2002; Menard *et al*., 2003; Kennelly *et al*., 2007). This further illustrates the potential risk involved in the use of biocontrol agents reported to be pathogenic to either plants or animals. Unfortunately, the risks associated with the use of such microorganisms have not been properly identified in relation to risk/benefit assessment. It could be argued that the bacterium whose use is proposed is a non-pathogenic strain of the species but, without a risk analysis, it is difficult to reach firm conclusions on this important issue.

5.7 Barriers to Commercialization

While hundreds of articles have been published on the potential of various antagonists for use as postharvest biocontrol agents, few of them, as noted above, have been successfully developed into a commercial products or, if so, have been commercially successful. The reasons for this are several and have recently been discussed in relation to the development of *P. anomala* (Schnürer and Jonsson, 2011; Sundh and Melin, 2011). Barriers include, but are not limited to, cost of development, patent issues, formulation, efficacy, registration requirements, marketing and support, and reluctance by end users to use new approaches.

The most critical criterion for the success of a biocontrol product is whether or not it performs effectively under commercial conditions and provides an acceptable and consistent level of control of the target disease(s). Large-scale production of a formulated biocontrol agent is required in order to conduct meaningful tests. These are costly trials to conduct and most often are done in association with a private company wishing to commercialize the biocontrol product. It is

essential that a formulated product, despite mass production of large quantities, retains the properties of the initial, laboratory-grown cultures. The formulation must retain its species purity (not be contaminated) and the microbial cells must retain their genetic stability, cell viability and attributes as colonizers on fruit surfaces, as well as other aspects of their mechanism of action. The commercial implications of complex modes of action are that performance and efficacy may be very dependent on production, formulation, packing, application, storage, etc. This highlights the additional need to develop rapid, reliable and economically viable methods of quality testing. Currently, apart from growth assays (which may not reflect biocontrol activity), the only tests that are available rely on testing the formulated product directly on fruit (the commodity). This can be a long process if conducted in a comprehensive manner and, in the end, it may not reflect performance under commercial conditions.

Industrial fermentation is accomplished under conditions quite different from those in shake culture. The process must be cost-effective and rely on industrial by-products as nutrients; fermentation must also be completed within 24–30 h (Hofstein *et al*., 1994). Downstream processing involves various steps, such as drying, and the addition of volume materials (inert ingredients), adhesives, emulsifiers and adjuvants. All these actions may adversely affect the properties of the selected biocontrol agent. The effect of commercial conditions on the physiological state of the biocontrol agent and its activity following rehydration is also critical. Various aspects of this topic were addressed in a series of articles by Abadias *et al*. (2000, 2001, 2003), and also in a recent article by Melin *et al*. (2011). Apart from these publications, few attempts have been made to address the large-scale production and formulation technology of postharvest biocontrol agents. The effects of industrial production practices, as well as the formulation technologies themselves, need to be investigated more thoroughly for their impact on the biocontrol activity of chosen antagonists. Such knowledge may not only prevent a decrease in efficacy but actually lead to enhancement and increased resistance of the biocontrol agents to environmental stress.

Registration is required by regulatory agencies (e.g. the US Environmental Protection Agency (EPA), and European agencies) before any biocontrol agent can be used commercially. Although the registration process is not as expensive or time consuming as it is for synthetic chemical fungicides, this requirement must be taken into account during the development process. The registration package must contain evidence of a clean record of safety (for both humans and the environment) for the biocontrol agent, data from basic toxicological tests on the formulated product (eye and skin irritation, ingestion) and efficacy data, including semi-commercial and commercial tests using relatively large quantities of food commodities treated under conditions that resemble commercial practices. The registration of biocontrol products for postharvest use in the USA (through EPA) has been straightforward, especially if the organism is free of any medical history that would raise human health concerns. Typically, only Tier 1 testing is required, and occasionally Tier 2 testing, to determine the effect of the bicontrol agent on animals. Environmental impact studies are not required at present; these would be very costly. In Europe, however, the situation is more complex and, until recently, registration has been difficult because data requirements are extensive (OJEC, 2001; OJEU, 2005). They include data on: identity; biological properties; analytical methods; medical information; medical surveillance; tests of sensitization, acute toxicity, genotoxicity, pathogenicity and infection potential; residues on commodities; persistence and spread in the environment; and effects on non-target organisms. The whole process is very demanding and may take 5–6 years from submission of the dossier. After approval of the active substance at the European Union level, practice has been that each formulated product has to be authorized separately in each country (Sundh and Melin, 2011).

Registration guidelines in South America are quite variable from one country to the next. The subject has been recently reviewed by Cotes (2011), and she notes that in some countries, e.g. Argentina, Brazil and Chile, there are

no specific regulations for the registration of biopesticides and they are required to undergo the same complicated procedures required for chemical pesticides. However, in July 2009 Brazil defined guidelines for the registration of biopesticides to be used in organic production (IBD Certificações, 2009). Cotes (2011) reports that only Colombia has a specific regulation for biopesticides, established by Decree 1840 of 1994 and updated in 2004 (ICA, 2004). While biologically based products in Brazil and Chile must undergo lengthy and expensive toxicological and ecotoxicological tests (similar to chemicals), current regulations in Colombia only require acute toxicology studies that are reviewed by the Ministry in Health. In 2010, in Colombia (according to ICA, the Colombian Agricultural Institute) 48 biopesticides based on 15 organisms formulated individually or in blends have been registered, while in Brazil (according to the Ministry of Agriculture), currently only four microorganisms have been registered as active ingredients of 17 biopesticides (Cotes, 2011).

5.8 Concluding Remarks

The use of microbial antagonists to control postharvest diseases of fruits, vegetables and grains has grown in the past 20 years from a novel discovery to a full-fledged science. Research groups worldwide are identifying new microbial isolates, evaluating their potential as postharvest biocontrol agents and seeking to partner with industry. While it was predicted that the success of postharvest biocontrol would be relatively easy compared with foliar and soil biocontrol agents, to date only a few commercial products are available and these are used only on a very limited basis. The reasons for the limited success are several, but are mainly due to variability in the performance of antagonists, the availability of several standard and new synthetic fungicides that perform well, and reluctance on the part of end users (packing houses) to adopt new approaches and methods that may or may not be easy to incorporate into existing practices. In addition, problems associated with the patenting, registration and large-scale fermentation of the agents has also posed difficulties.

Despite these challenges, interest in the use of biological approaches to manage postharvest diseases, as well as other agricultural needs, continues to grow. Researchers, regulatory groups and environmental advocates are coming together to define the problems that need to be dealt with and the scientific knowledge that will be needed to address the barriers limiting success. It is anticipated that great strides will be made in the next 10 years and that several new products will become available. Additionally, an enlarged definition of biological control will lead to novel approaches to postharvest disease control that are solidly based on a biological foundation.

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6 Safety and Regulation of Microbial Pest Control Agents and Microbial Plant Growth Promoters – Introduction and Overview

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6.1 Introduction

Increasingly, chemical pesticides are being taken off the market owing to newly discovered hazards towards human or environmental health. In addition, there is increased public demand for organically grown food. Microbial pesticides have a potentially important role to play in our ability to protect our agriculture and forests in a sustainable manner, and most countries have strict regulations for them, as they do for chemical pesticides. However, risk assessments of microbial pesticides present unique challenges, because microorganisms are living creatures and not chemicals.

Traditional chemical plant protection products (PPPs) are increasingly criticized for different reasons, but mainly for their potentially detrimental effects on human health or the environment. Concerns about residues in crops play an important role in the perception of chemical PPPs, and microorganisms represent a promising alternative as active substances. Due to their natural occurrence in the environment, at least some humans, and other non-target organisms, have always been exposed to many of these organisms and, consequently, have had the opportunity to adapt to them. Most of the microorganisms used are not new to consumers or to the environment and, therefore, defence mechanisms against such biocontrol organisms have evolved among non-target organisms where they were necessary. However, microorganisms differ fundamentally from chemicals in their capacity to multiply in the environment and to produce substances that may be involved in their mode of action, and these may also have detrimental effects on non-target organisms.

This chapter gives a brief overview of the regulatory systems for microorganisms used to manage pest populations and plant diseases. In the four following chapters of this volume, Jaronski (Chapter 7), Alabouvette *et al*. (Chapter 8), Evans and Seier (Chapter 9) and Howieson and Fox (Chapter 10) discuss details on data requirements and safety

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assessments of microbial biocontrol agents, as well as of microbial plant growth promoters and biofertilizers.

6.2 Data Requirements

When compared with chemical PPPs, microbial PPPs are still relatively new on the market. In many regulatory systems though, administrative procedures for registration are the same as those for PPPs containing chemical active ingredients, and data requirements have evolved from those used for chemicals. In general, the active ingredient, i.e. the technically produced microorganism, and the formulated product are evaluated separately.

Separate data requirements for microorganisms and the corresponding products exist in the regulatory systems of many countries/regions, e.g. the USA, Canada and the European Union (EU). In other countries/regions, data requirements rely on those for chemicals and are fixed for microorganisms on a case-by-case basis. These case-by-case approaches make it difficult for applicants to predict the amount of information or the number of studies needed for registration. However, the data requirements in the USA, Canada and the EU are quite similar following harmonization by the Organisation for Economic Co-operation and Development (OECD).

In all regulatory systems, data on the identity and biology of the microorganism used are required. In particular, the origin of the strain used needs to be given and that strain needs to be characterized by molecular methods. Physiological properties of the microorganism (e.g. temperature and pH ranges for growth), survival under application conditions and the mode of action also need to be described. Information on analytical methods for the detection and quantification of the microorganism and of potential contaminants or pathogens needs to be provided as well. Data from studies on acute toxicity, infectivity and pathogenicity resulting from oral, intravenous or intratracheal application are required too. In addition, the genotoxicity of microbial metabolites needs to be assessed if these are known to be produced. Furthermore, short-term toxicity, infectivity, and pathogenicity need to be examined. As with chemicals, information on potential residues and on the behaviour of the microorganism in the environment is required, although in contrast to chemicals, studies are normally not necessary and data requirements can be fulfilled by using data from the published literature or internal data. The effects of the microorganism on different non-target organisms (fish, aquatic invertebrates, algae, bees, other non-target arthropods, birds, earthworms and soil microorganisms) also need to be determined – depending on the exposure to these organisms that results from the use of the product. Studies can be conducted with the microorganism alone or with the formulated product. The formulated product itself needs to be characterized in the same way as for a chemical PPP: data are required on the physical, chemical and technical properties, depending on the formulation type. Finally, uses of the product need to be defined in detail and data demonstrating its efficacy for the intended uses need to be presented.

6.3 Regulatory Procedures

Another difficulty in obtaining registration for a microbial PPP is the administrative procedure, which in many systems is identical to or very similar to the procedure applied to chemical PPPs. This procedure may be very time-consuming and actually present a major hurdle for applicants, especially small or medium-sized enterprises, which are frequent among the producers of microbial plant protection products.

The USA has one of the few systems where 'microbial pesticides' and 'biochemical pesticides' (together termed 'biopesticides') are evaluated by a separate division from that which is responsible for chemical pesticides; however, both are within the US Environmental Protection Agency (EPA). In most other administrative systems, e.g. in Canada, Australia and the EU, microorganisms and microbial plant protection products are treated in the same administration as conventional chemical actives and their PPPs.

In the USA, Canada, Australia and many other regulatory systems, applications for the registration of active ingredients and formulated products are made to the same authority. The system in the EU is even more complicated as active ingredients are evaluated at the level of the EU (Council Directive 91/414/EEC (OJEC, 1991); followed, since June 2011, by Parliament and Council Regulation (EC) No 1107/2009 (OJEU, 2009)), whereas plant protection products are registered at national level. This two-step system is quite time-consuming, and even if the legal possibility to register a product before approval of the active substance on Annex I (of Directive 91/414/EEC) existed, this opportunity was not always applied by many member states (MSs).

The change in the EU plant protection product legislation from Directive 91/414/EEC to Regulation (EC) No 1107/2009 will only partly affect the regulatory procedure. The separate assessment at EU level for the active substance and at national (or zonal) level for the formulated product remains, in principle, unchanged. Applicants are encouraged to define the actual data for the submission in a pre-submission meeting with the authorities of the MS concerned (termed the 'rapporteur member state', RMS) to which they have chosen to submit the dossier. This dossier containing all information, studies and literature surveys on the active substance and on one representative formulation is then sent to the RMS authorities, which have 45 days to check its completeness. If the dossier found to be is complete, it needs to be distributed by the applicant to all MSs, the Commission and the European Food Safety Authority (EFSA). In the meantime, the RMS prepares the 'draft assessment report' (DAR), which presents the evaluation of the dossier. The DAR is also distributed to all MSs, the Commission and EFSA. EFSA organizes a peer review during which all MSs and the applicant can submit comments or questions regarding the DAR. Subsequently, EFSA prepares a scientific report which is the basis for the decision of the Commission to include (or not to include) the active substance. Finally the Commission's Directorate General for Health and Consumers (SANCO) prepares a draft directive which aims at inclusion (or non-inclusion) of the active ingredient into Annex I of Directive 91/414/EEC, for which the MSs vote.

The formulated products are regulated at the level of the MS. Under Directive EC 91/414/EEC, applications were submitted to the individual MSs and were evaluated independently. An attempt to facilitate this was the procedure for 'mutual recognition' (MR), under which applications could be made based on an existing registration in another MS. An inconvenience of the MR procedure under Directive 91/414/EEC was that the registration in the second MS could not be modified, i.e. no other crops or applications could be included. Moreover, in some MSs, procedures for MR were not faster than an independent registration, and the advantage of the MR procedure was restricted to its reduced fees compared with an independent registration.

Under Regulation (EC) No 1107/2009, applications for registration of products are made by zones. To this end, the EU is divided into three zones following geographic and climatic criteria:

- **Zone A North**: Denmark, Estonia, Latvia, Lithuania, Finland and Sweden
- **Zone B Central**: Belgium, Czech Republic,Germany,Ireland,Luxembourg, Hungary, The Netherlands, Austria, Poland, Romania, Slovenia, Slovakia and the UK
- **Zone C South**: Bulgaria, Spain, Greece, France, Italy, Cyprus, Malta and Portugal.

For the use of PPPs in greenhouses, in post-harvest treatment, for treatment of empty storage rooms and for seed treatment, the whole EU is considered as a single zone.

Applicants will submit the dossier for the registration of a PPP to a 'Zonal rapporteur member state' (zRMS), which will evaluate the dossier. All other MSs of the same zone receive the evaluation from the zRMS (independently of whether registration is intended in these MSs or not) and may comment on it. If registration is intended in different MSs, modifications of uses are possible. These MSs would then receive the adapted dossier in the form of 'national addenda' to the dossier.

Following the evaluation, the zRMS releases the registration of the product within a year of the application (if the dossier was complete and no additional data were requested during evaluation). All other MS where registration is applied for need to register the product within 120 days.

Apart from the zonal registration for products, restricted timelines represent the major difference between the procedures under Directive 91/414/EEC and Regulation (EC) No 1107/2009. No time limits were given in Directive 91/414/EEC and the corresponding national legislation, and this often led to very long evaluation times without access to the market. Evaluation times are restricted to 27 months for active substances and to 12 months for products under Regulation (EC) No 1107/2009. These times can be extended by defined periods if additional data are requested from the applicant.

The other major difference between Directive 91/414/EEC and Regulation (EC) No 1107/2009 concerns the requirement for efficacy data of the representative product in the EU evaluation of the active substance. Under Directive 91/414/EEC, these data were not required and efficacy was only assessed at the national level for product authorizations. In case the application concerns a product for field application, the efficacy data have to be specific for the country or at least for the climatic zone. This restriction is normally not relevant for products that are only intended for use in greenhouses, as postharvest treatments and for the treatment of empty storage rooms. The requirements for efficacy data for biological PPPs are reduced in some countries when compared with those for chemical PPPs. This reduction refers both to the number of successful studies that have to be submitted and to the extent of efficacy data obtained in the trials.

In addition to the dossier, specific forms, most frequently in the national language, have to be provided when the dossier is submitted. In some MSs, summaries in the dossier (Documents M and N) have to be submitted in the national language; in others, only parts of the dossier (e.g. Document N) have to be so submitted.

The organization of the regulatory bodies within the MSs of the EU varies greatly. In some, the regulation is mainly carried out within the ministry of agriculture, in others within the ministry of environment, the ministry of health or related agencies. Often, several regulators from several ministries or agencies are involved in the evaluation of the active substances and the plant protection products. In several MSs, both the risk assessment and risk management part of the evaluation is carried out within the regulatory agencies. However, in many others, external experts are involved or even responsible for the risk assessment. Some, but not all MSs, have pre-submission meetings with applicants. These pre-submission meetings are obligatory under Regulation (EC) No 1107/2009.

Low-risk substances and basic substances represent two new categories in PPP regulation. So far, a substance can be classified as 'low-risk' if certain hazard criteria are not met, but an exact definition is still missing. Criteria for low risk may be reviewed in the future and, if necessary, specified. The status of a low-risk substance is assigned after evaluation of the dossier. Approval for low-risk substances as active ingredients will last for 15 years instead of the 10 years for other active substances. Most of the biocontrol agents currently on the market would fulfil the criteria to obtain 'low-risk' status. 'Basic substances' are defined as substances which are predominantly used outside plant protection (essentially commodity chemicals) or fulfil the criteria for a foodstuff.

6.4 Regulation of Biocides

Biocides are regulated by Directive 98/8/EC, which has resulted in a completely different regulatory system that uses different EU authorities from those used by PPPs. However, the overall procedure, with an evaluation of the active substance at EU level and national product authorizations is similar to that for PPPs. Data requirements and dossier structure are even more similar between microbial biocides and microbial PPPs than between microbial and chemical biocides. This results

from the fact that the data requirements for microbial biocides were adapted from those for microbial plant protection products. Compared with PPPs, very few biocides contain microorganisms and experience of these among applicants and regulators is still quite limited.

6.5 Conclusion

Taken together, the registration of microbial plant protection products and biocides is derived from that of their chemical counterparts. Procedures for registration are the same for both microbial and chemical products, but data requirements have been adapted for microbials. However, improvements in data requirements are still necessary. The registration of plant protection products (both microbials and chemicals) is still time-consuming, but the new EU regulation has for the first time defined timelines for the evaluation procedure, as has been the case for many years in many non-EU countries.

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7 Microbial Control of Invertebrate Pests

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7.1 Introduction

Arthropods, particularly insects, have a wide spectrum of microbial pathogens – viruses, bacteria, fungi, protozoans, as well as entomopathogenic bacterium–nematode symbioses. All these categories of microorganisms have been commercialized to control arthropods. More than 170 years ago, Augustino Bassi proposed using microorganisms as biological control agents of insect pests. In 1873, LeConte recommended the deliberate study of insect diseases to control noxious insects. The earliest actual attempts to do so date back to the late 19th century when Metchnikoff and Krassilitchik attempted to produce an insect pathogenic fungus on beer wort and disperse it to control the sugarbeet curculio (Steinhaus, 1949). In the late 1880s, in the USA, the Kansas Department of Agriculture attempted to control the chinch bug, *Blissus leucopterus*, by dispersing insects infected with another insect pathogenic fungus (Snow, 1891). Between 1939 and 1953, *Paenibacillus* (*Bacillus*) *popilliae* was applied throughout the eastern USA as part of a government programme to control the Japanese beetle, *Popillia japonica* (Fleming, 1968); this bacterium was the first microbial to be registered in the USA in 1948 (US EPA, 2004). *Bacillus thuringiensis* (Bt), which was discovered to be pathogenic for insects in 1915, was developed in France

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in the 1930s as a control agent of various lepidopteran larvae, and was registered for that use in the USA in 1950 (US EPA, 1998a); this was followed by the use of Bt strains toxic for mosquito and blackfly larvae, and certain species of fungus gnats (US EPA, 1998b). During the past four decades, the development of microbial pest control agents (MPCAs; also called microbial biological control agents (MBCAs), a term that is used elsewhere in this book) to control arthropods has gained considerable momentum. In the 1960s and 1970s, Bt underwent commercial development in the USA for the control various larval lepidopteran pests, and subsequently for the control of Coleoptera and, as already noted, for the control of larval mosquitoes and blackflies. There are currently quite a number of commercial products, worldwide, based on this bacterium. In 2006, one industry trade group (the Biopesticide Industry Alliance; BPIA) reported an estimated the worldwide biopesticide market of approximately US$541 million (BPIA, 2010). The total global biopesticide market (including microbial pesticides, plant-incorporated protectants and others, which includes natural predators, entomopathogenic nematodes and parasitoids) was estimated to be US$1.6 billion in 2009 and predicted to increase to US$3.3 billion by 2014 (Lehr, 2010). There are several hundred microbial products presently extant worldwide (Kabaluk *et al*., 2010). In 2009, these products represented about 2.5% of the overall pesticide market, but this was expected to grow to 3.0–4.5% by 2010, when the biopesticides market was forecast to reach US$1 billion (IEN, 2009; BPIA, 2010); some 171 fungus-based insecticidal products alone had been identified at the time of a review made in 2007 (Faria and Wraight, 2007).

As with most things, there are risks in using microbes to control arthropod pests. The risks specific to these microorganisms are human pathogenicity and allergenicity, and direct or indirect effects on non-target vertebrates, invertebrates and plants. Common to all microbial arthropod control agents is the fact that they are animal pathogens, but each group of MPCAs poses slightly different potential risks, based upon its biology and ecology.

7.2 Microbial Arthropod Control Agents – A Brief Introduction

7.2.1 Use strategies of microbial pest control agents

Biological control agents, including microorganisms, can be used in several different pest control strategies (DeBach, 1974).

In classical biocontrol approaches, an exotic agent is released to control an exotic pest. The exotic agent is chosen for its ability to control the pest in the country of origin and is introduced to restore that equilibrium in the new region. An assumption is made that the agent will reproduce following introduction, and will establish itself and spread through vertical and horizontal transmission, reaching an equilibrium with its host.

Conservation biocontrol consists of taking measures to enhance the effectiveness of agents that are already present but which are in insufficient numbers to significantly affect the pest population. Such measures include providing physical refuges, introducing alternate hosts, etc.

Augmentation biocontrol involves periodic, supplementary releases of a domestic agent to maintain its effect on the target pest. The agent is already present but needs assistance to achieve the desired effect. This augmentation can be inoculative, using small numbers of an agent, in order to allow it to increase over time in order to prevent or delay a pest from increasing in numbers and crop damage. Augmentation can also be inundative, whereby the agent is released in large numbers to rapidly overwhelm the target pest, much like a chemical pesticide. This last approach is the strategy most commonly used with MPCAs. Large quantities of an appropriate stage of a microorganism are produced *in vitro* or *in vivo*, harvested, formulated and applied much like chemical pesticides.

7.2.2 Viruses

The entomopathogenic viruses important for insect pest control include four families, the *Baculoviridae* (nucleopolyhedrosis viruses,

NPVs; and granuloviruses, GVs), *Reoviridae*, specifically the genus *Cypovirus* (cytoplasmic polyhedrosis viruses, CPVs), *Iridoviridae* (iridescent viruses), and *Poxviridae* (entomopoxviruses, EPVs). Of these families, the baculoviruses have been the most extensively developed commercially, beginning in the 1950s. The first successful registration and use in the USA was against the Douglas fir tussock moth in 1976 and the gypsy moth in 1978 (US EPA, 2002). Current major uses are against a number of Lepidoptera in forests and orchards. Control of lepidopteran pests in vegetable crops has lagged behind the two former uses in North America and Europe, but over 2 million hectares of soybean are treated annually in Brazil with an NPV of velvetbean caterpillar (Moscardi *et al*., 2007). Baculoviruses are double-stranded DNA (dsDNA) viruses contained within a polyhedral protein inclusion body; they characteristically develop within cell nuclei. Hosts include Lepidoptera, Hymenoptera, Diptera and some Crustacea. In general, however, host specificity is limited to one species or a group of closely related species (Granados and Federici, 1986; Blissard and Rohrmann, 1990). Historically, these viruses have been named based on their host, e.g. *Autographa californica*. There are multiple NPVs, but species-level systematics of the *Baculoviridae* is now based on genomic DNA sequence profiles as well as host range (Theilmann *et al*., 2005; Jehle *et al*., 2006) – *Alphabaculovirus* (lepidopteran-specific NPV), *Gammabaculovirus* (hymenopteran-specific NPV) and *Deltabaculovirus* (dipteran-specific NPV), while granulosis viruses are now classified in the genus *Betabaculovirus*. This evolution of nomenclature has important implications for proper risk assessment. Considerable background information on the *Baculoviridae* may be found in a volume from the Organisation for Economic Co-operation and Development (OECD, 2006) and in McWilliam (2006).

The *Poxviridae* include important virus pathogens of vertebrates (e.g. smallpox in humans), but also include a subfamily, the *Entomopoxvirinae* (EPVs), which are restricted to arthropods, namely Coleoptera, Diptera, Lepidoptera and Orthoptera (ICTV, 2012; SIB, 2012). Perhaps the one that has been most developed is the *Melanoplus sanguinipes* (migratory grasshopper) EPV, which was field tested by the US Department of Agriculture (USDA) in the 1990s, although it was never registered (Erlandson and Streett, 1997; Streett *et al*., 1997).

Iridoviruses are dsDNA, non-occluded, icosahedral viruses that have been proposed for the biological control of insect pests. They infect mainly invertebrates, but also some fish, amphibians and reptiles. These viruses are characterized by the iridescence that they confer on heavily infected invertebrates. Their biology has been summarized by Williams (1996). None have been developed to the point of commercialization. The *Reoviridae*, dsRNA viruses contained within an icosahedral body, include several important animal and human pathogens. One genus, however, *Cypovirus*, traditionally known as a cytoplasmic polyhedrosis virus (CPV), is restricted to insects, specifically Hymenoptera, Lepidoptera, Coleoptera and Neuroptera. *Cypovirus*, which infects the gut epithelium, is readily transmitted through the frass of infected hosts, but causes only a chronic disease (Payne and Mertens, 1983; Zhou 2008). Only *Cypovirus* has been commercialized (the other genera within the *Reoviridae* being vertebrate viruses).

All these entomopathogenic viruses infect their hosts perorally, through ingestion. Within the insect gut, the protein occlusion body of the *Baculoviridae* and *Entomopoxvirinae* is dissolved, releasing virions that pass through the gut peritrophic membrane and initially infect gut epithelial cells, reproducing in the cell nuclei. The viruses proliferate from their initial infection sites to other tissues by means of nucleocapsids that bud out through the cellular membranes into the insect haemocoel (the open circulatory system). Insect hosts are typically killed by baculoviruses and CPVs within 5–7 days; the iridoviruses and EPVs more typically produce chronic disease or have a slower lethality. In the later stages of infection by baculoviruses, CPVs, and EPVs the virions become occluded within a protein matrix. These occlusion bodies are released upon host cell lysis and can result in horizontal transmission of the virus upon death and disintegration of the host insect.

Commercial virus mass production is *in vivo*, using the host insect or another susceptible species. The virus occlusion bodies are harvested by homogenization of the infected insect cadavers, and purified to variable extent depending upon the producer. Attempts have been made to mass produce these viruses within insect cell lines by Dupont and American Cyanamid in the USA, but these were abandoned (Gaugler, 1997). The viruses are used almost entirely in inundative approaches as aqueous sprays of occlusion bodies. Persistence of efficacious quantities is relatively short, as they are adversely affected by UV radiation (Granados and Federici, 1986). Genetic modification to enhance and speed up efficacy has been explored, by, for example, the incorporation of a gene expressing scorpion neurotoxin (Bonning *et al*., 1991; Chejanovsky *et al*., 1995; Jarvis *et al*., 1996).

7.2.3 Bacteria

While some bacteria are infectious and pathogenic (*P. popilliae*, *Serratia entomophila*), others, namely Bt and a related, mosquito-specific species, *Lysinibacillus* (*Bacillus*) *sphaericus*, possess toxic protein inclusions that directly affect target insects without infection and growth being necessary. Similarly, *Yersinia entomophaga* and *Chromobacter subtsugae* have toxins that affect a range of Lepidoptera, Coleoptera and Orthoptera. These bacteria have to be ingested to either infect or intoxicate their target hosts. There are two additional bacteria – *Heterorhabdus* spp. and *Photorhabdus* spp. – that have insect pathogenicity, but both are obligate symbionts with entomopathogenic nematodes, and thus have been historically exempt from the requirement for registration because their nematode partners are exempt.

Two of the infectious bacteria have a high degree of host specificity: *P. popilliae*, which infects only *P. japonica* and closely related scarab grubs, and *S. entomophila*, which infects only the New Zealand grass grub, *Costelytra zealandica*. Both *Y. entomophaga and Chr. subtsugae*, however, seem to have a range spanning several families of Lepidoptera, several families of Coleoptera, ants and some Orthoptera (Martin *et al*., 2007; Brownbridge *et al*., 2008). The toxigenic bacteria (Bt and *L. sphaericus*) have a host specificity mediated by the production of different classes of toxins: exosporal insecticidal crystal protein (ICP) inclusions (β-endotoxins, Cry proteins) and cytolytic (Cyt) proteins), heat stable or heat labile alpha-exotoxins, and vegetative insecticidal (VIP) proteins (Lüthy *et al*., 1982). Primary insecticidal activity is due to the ICPs. Orally ingested, the proteins are solubilized in the insect gut (proteins active in Lepidoptera and Diptera at alkaline pH, protoxins active in Coleoptera at a slightly acidic pH), and cleaved by proteases. The activated proteins bind to midgut epithelial cell walls, cause pore formation and result in cellular lysis. Sepsis and death ensue. There are currently 208 Cry and 11 Cyt protein holotypes (Crickmore, 2010). The US Environmental Protection Agency (EPA) recognized that the ICP genes, which generally reside on transferable genetic elements (plasmids), can be readily moved from one isolate to another, and therefore reorganized registrations on the basis of Cry or Cyt types, not Bt subspecies (US EPA, 1998a). Because the endotoxin genes are contained on extrachromosomal plasmids, it has been possible to create transgenic bacteria, e.g. a Bt incorporating the genes for Cyt1A, Cry11B and *B.* [*L.*] *sphaericus* binary toxin, to achieve a wider spectrum of targets (Park *et al*., 2003). The Bts have also supplied a number of Cry genes for incorporation into and expression in plants, especially maize and cotton. These plant-incorporated protectants will not be discussed here.

7.2.4 Protozoa

Insects have a number of protozoan pathogens. The most important of these causing acute disease and mortality are Amoebozoa and Microsporidia. Other parasitic groups, such as the apicomplexans Gregarinasina and Eucoccidiorida, and the Ciliophora, do not cause acute disease and have generally been ignored as microbial control agents of arthropods. The protozoan agents attracting

commercialization have primarily been Microsporidia. While this group of obligatory intracellular parasites was historically considered in the phylum Protozoa, and then in its own phylum, these microorganisms most recently have been reclassified as fungi (Hirt *et al*., 1999; Vossbrinck *et al*., 2005), but will be treated here within the Protozoa. Microsporidia are known from a very wide variety of hosts, including vertebrates and humans. Over 1000 species have been described to date, but only one commercial product has been registered, *Paranosema* (*Nosema*) *locustae*, for the control of grasshoppers and locusts in the USA (US EPA, 2000b). The Microsporidia are primarily infectious perorally, with a secondary vertical transmission in some species in which the organism has a transovarial route, i.e. moves into and infects the egg before oviposition (Becnel and Andreadis, 1999), and a rare, third route in which it is vectored by parasitic Hymenoptera (Brooks, 1993). The infectious stage in the peroral route is a spore, which contains a nucleus, some cytoplasmic structures and a long, coiled, tubular filament. In the insect gut, the spore explosively germinates at one end, with the filament being forcefully everted from one end of the spore into the intestinal epithelium. The nucleus, with some associated cytoplasmic material, is then injected into the cell, where the microsporidian undergoes vegetative division, and meiosis and development of more spores. During the initial phase, vegetative cells can migrate into the insect's body and attack various tissues. Different species of Microsporidia display different tissue tropisms. Most entomopathogenic species induce chronic infections with sublethal effects. Microsporidians have variable host specificity, with some species infecting only one host species, and others a group of related species. For example, *Para. locustae* infects some but not all grasshopper species (Henry and Oma, 1981), *N. apis* infects only honeybees, and the mosquito pathogens, *Amblyospora* spp., possess an obligatory intermediate host, *Daphnia magna*, a small, cladoceran crustacean (Becnel, 1994). For a more extensive description of microsporidian biology, the reader is referred to Desportes-Livage (2000).

7.2.5 Fungi

Among the fungi, the important arthropod pathogenic species fall into two general taxa, the *Entomophthorales* and the ascomycetes. Many of the latter, having only asexual reproduction, were once classified as deuteromycetes, the 'imperfect fungi,' before the advent of molecular data linked them to recognized sexual stages in the ascomycetes. The insect pathogenic *Entomophthorales* are generally host specific, with a species infecting only one or a few host species. In contrast, many of the ascomycetes are generalist pathogens, capable of infecting a wide range of arthropod hosts, and even of crossing orders. They are, however, generally restricted to arthropods. There are few reports of ascomycete infection in vertebrates, including humans; these instances seem, though, to involve stressed or immunocompromised humans or stressed vertebrates (Zimmermann 2007a, 2007b). Only the ascomycetes have undergone serious commercial development because the *Entomophthorales* have been generally refractory to any cost-effective, *in vitro* mass production.

Infection by the fungi is percutaneous, with conidia germinating on the arthropod cuticle and penetrating into the host body by means of mechanical pressure and a cocktail of enzymes. Once within the insect haemocoel, the fungi proliferate vegetatively through the arthropod, killing it within days by means of cellular disruption with extracellular enzymes, preferential consumption of energy reserves (trehalose, triglycerides) and, in some species, the production of toxic metabolites. The insect is quickly mummified upon death, and if the habitat has very high humidity and permissive temperatures, the fungi will emerge from the cadaver, frequently covering it with mycelium and then reproducing in the form of aerial conidia. More often, however, conditions for sporulation are suboptimal and the fungus is unable to reproduce. More information about the infection and pathogenesis processes can be found in Charnley (1989), and Hajek and St. Leger (1994). Many of these fungi also readily grow *in vitro*, on simple organic substrates, and so can be economically mass produced. Thus, the ascomycetes have been the focus of

commercialization. The principle genera of interest are *Beauveria*, *Metarhizium*, *Isaria*, *Nomuraea* and *Lecanicillium* (formerly *Verticillium*; Faria and Wraight, 2007). Much recent information on the biology, ecology and safety of *Beauveria*, *Metarhizium* and *Isaria* has been summarized by Zimmermann (2007a, 2007b, 2008).

7.3 Risk Assessment of Microbial Arthropod Control Agents

7.3.1 General issues

Microbial arthropod control agents infect living organisms causing disease and are, after all, animal pathogens. With microbial agents, the major risks are: (i) risks to humans during production, and during and after application; (ii) direct risks to non-target organisms, especially beneficial insects; and (iii) long-term effects on ecosystem services due to host depletion by natural enemies.

As noted by the members of the REBECA (Regulation of Biological Control Agents) project in Europe (Strauch *et al*., 2006), subsequent to their review of all the extant data, there have been no hazards reported in the use of microbial biocontrol agents in a larger context, regardless of target, but particularly with reference to arthropod targets. Nevertheless, regardless of their characteristics, microbial arthropod control agents are regulated in almost all countries. For example, in the USA, the US EPA regulates any microbial agent 'intended for preventing, destroying, repelling, or mitigating any pest, or intended for use as a plant regulator, defoliant, or desiccant' (US Government, 1947; US EPA, 2007). Canada, the European Union (EU) and its member countries, and other governments have similar regulations (Kabaluk *et al*., 2010).

The various regulatory authorities are concerned with both human safety and environmental safety, and conduct risk assessments of each microorganism on a case-by-case basis, just as with chemical pesticides. Whether a substance poses a risk to humans or other organisms depends on two factors: how toxic (infectious, pathogenic) the substance is, and the degree of exposure to the microbial. Therefore, toxicity data and exposure data are considered in deciding whether to approve a pesticide for use. Human safety encompasses the infectivity, pathogenicity, irritant and sensitization properties, and allergenicity of a microbe. Environmental safety concerns the impact of a microbe on a spectrum of vertebrate and invertebrate non-target organisms.

The origins of data requirements in most countries are in chemical pesticide data requirements. Initially, in the USA, the data requirements were directly derived from those for chemical pesticides (US EPA, 1983), but many of these testing procedures were not completely suitable for microbial agents. In 1994, the US EPA created a specific division to deal with microbial agents, the Biopesticides and Pollution Prevention Division (BPPD) and, in 1996, issued improved testing guidelines, the OPPTS 885 Series (US EPA, 1996a). There is a dedicated unit, the Microbial Pesticides Branch, within BPPD, dealing with microbial pesticides. In Canada, similar guidelines are to be found in Health Canada (2001), and are administered by the Pest Management Regulatory Agency (PMRA), while the EU unified guidelines are identified in Council Directive 2005/25/EC (OJEU, 2005). Kabaluk *et al*. (2010) recently summarized registration requirements and processes in a number of countries.

There are four components to risk assessment: (i) hazard identification; (ii) hazard characterization (determining quantitative or qualitative severity of the adverse effects and relationship of adverse effect to quantity of agent); (iii) evaluation of exposure to the hazard; (iv) quantitative estimation of risk, principally as the product of the probability and the severity of the hazard.

7.3.2 Human safety risk assessment

The first aspect of a human risk assessment of an MPCA is product characterization – what is known about the particular organism within the context of its species identification. But this criterion has been complicated

by the rapid evolution of our understanding of the systematic organization of microorganisms based on molecular data. However, even our molecular based understanding is steadily changing as techniques improve. Witness the progression in the 'state-of-the-art' methods (isoenzymes; bacterial fatty acid methyl ester (FAME) profiles; random amplification of polymorphic DNA (RAPD); amplified fragment length polymorphism (AFLP); microsatellites (simple sequence repeats – SSRs) and minisatellites (which have longer sequence repeats, called variable number of tandem repeats – VNTRs); internal transcribed spacer and intergenic spacer ribosomal DNA sequences (ITS and IGS, respectively); and specific gene sequences), wherein new techniques supplant older ones, and often reorganize our comprehension of the taxonomic standing of a microbe. The fungal genus *Metarhizium* is a case in point. These fungi were traditionally organized into 13 morphospecies and two varieties. Tulloch (1976) drastically revised the genus, discarding a number of species and reducing the genus to *M. anisopliae* and *M. flavoviride*, a concept easy for regulators to understand, although there were difficulties in the confident assignment of an isolate to a given species based on colony and conidial morphology. Driver *et al*. (2000), using RAPD patterns and ITS sequence data, reorganized *Metarhizium* into one species of nine varieties. Using more refined techniques, namely specific functional gene sequences, Bischoff *et al*. (2009) reorganized *Metarhizium* into ten species, with the reassignment of some of the Driver *et al*. (2000) varieties to different species. Now, *M. anisopliae* F52, commercialized in the USA and the EU, has become *M. brunneum*; another strain, ARSEF2575, which has been used in many published studies, is no longer *M. anisopliae* but *M. robertsii*. Similarly, the fungus *Verticillium lecanii*, which comprises a number of commercial insect and mite control products, but is also known as a mycoparasite and is in the same genus as a number of plant pathogenic species, has become *Lecanicillium lecanii*, *L. muscarium* and *L. longisporum* (Zare and Gams, 2001), and these taxa are restricted to insect pathogens. Other examples are the US-registered grasshopper agent, *N. locustae*, which is now *Para. locustae* based on molecular data, and is thus separated from the large genus of *Nosema*, which includes two important honeybee pathogens and a number of lepidopteran pathogens. Yet another example is the reassignment of *B. popilliae* to the genus *Paenibacillus* (Pettersson *et al*., 1999). The genus *Paenibacillus* contains *P. alvei* and *P. larvae*, which cause diseases in honeybees. The nomenclatural changes have in some cases clarified risk assessments, but in other cases have perhaps increased confusion about the potential risk of an agent. But how do various traits concerning potential risk relate to the molecular data, and to the resulting taxonomic constructs? This is not at all clear. In some cases, such as the *Baculoviridae*, the reassignment of specific viruses to four new genera has clarified our understanding of their host spectra (Jehle *et al*., 2006). The clear taxonomic differentiation of Bt from other sporeforming *Bacillus* spp. (*B. cereus*, *B. anthracis*) also lends support to its safety. With the entomopathogenic ascomycetes, however, the relationships are not really clear because of the novelty of our taxonomic understanding of many strains, and they await directed research in proteomics, for example to determine the presence/absence of specific, active mycotoxin genes in the new *Metarhizium* species. The regulators, therefore, have to continue to rely on a strain-based evaluation of candidate MPCAs. Despite an ever-changing taxonomy, proper identification and comparison with related organisms will continuously be an important tool in assessments.

The second component of product characterization is a detailed description of the production process and discussion of unintentional ingredients (metabolic by-products, mutant strains, microbial contaminants – especially mammalian pathogens or antagonistic microbes, and chemical contaminants). The major regulatory agencies demand that every microbial manufacturing process description should address the detection and control of unwanted microorganisms and of contamination by suspected toxic components, and that the registrant pays particular attention to measures to minimize the potential growth of contaminating organisms. In addition, multiple production lots (five in the

USA, Canada and the EU) must be evaluated in terms of the numbers of contaminating microorganisms per unit weight or volume of active ingredient (microbial agent preparation). While some level of contamination is allowed, the tolerable levels of key human pathogens – *Shigella*, *Salmonella*, *Vibrio,* other *Enterobacteriaceae* – are very low, of the order of <1 microorganism g^{-1} of active ingredient. In the EU, total mesophiles cannot exceed 10^8 colony forming units (cfu), *B. cereus* 10^7 cfu, while the limits for *Escherichia coli* and *Staphylococcus aureus* are none per gram or millilitre, and those for *Salmonella* spp. are none in 25 g or 25 ml. In the USA, for Bt, which may produce β-exotoxin, a demonstrated heat-stable, mammalian toxin (Vankova, 1978), or *Bacillus* enterotoxins, the requirement is that each fermentation lot be tested 'by subcutaneous injection of at least 1 million spores into each of five laboratory test mice. The test results must show 'no evidence of infection or injury in the test animals when observed for 7 days following injection', to verify that no *B. anthracis* (closely related to Bt) or other human pathogens are present or are eliminated in the final product (US EPA, 2009). In addition, for an exemption of a Bt product from an assessment of residue tolerance (critical for a microbial agent to be competitive with chemicals), a fly larva toxicity test must be conducted with master spore preparations brought into production to demonstrate absence of β-exotoxin, or a registrant must periodically determine that β-exotoxin synthesized during spore production is eliminated by the subsequent spore-harvesting procedure. In its Bt re-registration eligibility document, the US EPA (1998a) also requires that a *Daphnia* toxicity test be conducted for each specific new manufacturing process, as a screen to eliminate excessive exotoxin synthesis.

The entomopathogenic fungi can produce a number of metabolites, some of which have been shown to have toxic properties (summarized by Molnar *et al*., 2010). These metabolites are thought to aid pathogenicity or to act as antibiotics in suppressing saprophytic microorganisms in the dying and dead host (Vey *et al*., 2001). Published studies with a number of isolates of *Metarhizium* (summarized by Zimmerman, 2007b) have failed to reveal any human toxicity or pathogenicity. But there was one exception: using US EPA test protocols, Mycotech Corporation in the USA observed rapid toxicosis and mortality of mice associated with conidia of two *Metarhizium* isolates from Madagascar administered intranasally in a pulmonary toxicity test (but not intraperitoneally); heat-treated conidia were not toxic (Goettel and Jaronski, 1997; Jaronski, unpublished data). Thus, given the wide range of phenotypic characters among isolates of *Metarhizium*, there are grounds for caution. The position of the US EPA is that any metabolites of concern must be below detectable limits in the technical-grade active ingredient (conidial powder or other preparation that is incorporated into a formulation), or standard toxicological tests must be conducted. For the *Beauveria* metabolites beauvericin and bassianolide, this acceptable level of detection was 5 ppm in the US EPA registration of strain GHA. In a study that also included other *Beauveria* metabolites, Strasser *et al*. (1998) reported a detection limit of 5 ppm beauvericin, 4 ppm oosporein, and 45–46 ppm bassianin and tenellin. So these levels would theoretically be the US EPA limits.

In contrast, in the EU, under Directive 91/414/EEC (OJEC, 2000), there is considerable concern that metabolites of concern may be introduced and accumulate in the environment. What exactly is the relevance of production of a 'metabolite of toxicological concern' *in vitro* to the *in vivo* situation, or to overall human and environmental safety? In some cases, such as the β-exotoxin of Bt, the toxicity of the material does raise serious questions. In other cases, such as with a number of the fungal metabolites, there is greater uncertainty. The dihydroxybenzoquinone oosporein is produced by many strains of *Beauveria bassiana* and *Beauveria brongniartii* and is toxic *per os* with day-old poultry and also intraperitoneally in mice and rats (Manning and Wyatt, 1984; Vey *et al*., 2001). The maximum amount of oosporein produced by a *B. brongniartii* strain in liquid culture was 270 mg l^{-1}, while the metabolite was present at a level of 2.0–3.2 mg kg^{-1} in the barley solid substrate on which the fungus

was grown. In a mycosed larva, there were only 200 μg and, more importantly, only 0.02 mg m^{-2} in soil enriched with the commercial product or 6.4 mg m^{-2} in soil enriched by mycosed larvae (Strasser *et al*., 2000). These authors consequently pointed out that 'The concentration of oosporein detected in the soil is usually 2.5 million times lower than that of the pesticides methyl bromide and dazomet' and should therefore not be of regulatory concern.

The various regulatory systems focus on toxicology studies needed for human health risk assessment to provide a reasonable certainty of no harm from the aggregate exposure to pesticide residues. Human safety is assessed with mouse or rat test systems – acute toxicity/pathogenicity following pulmonary (intratracheal), oral and intravenous/intraperitoneal administration. Dermal and ocular irritation, as well as sensitization potential, is evaluated in rabbits, per requirement 40 CFR Part 158 in the USA (US EPA, 2007) and Directive 2000/54/EC in the EU (OJEC, 2000).

Most governmental agencies follow a tiered approach (e.g. US EPA,1996a). The lowest tier (Tier I in the USA) acute tests are intended to provide initial information on the toxicity, infectivity and pathogenicity of an MPCA using a single, high-dose exposure, typically 10^8 organisms, during a post-exposure observation period, typically of 21 days (although, at least in the USA, this period is flexible). In addition to death or clinical signs of disease, the protocols are intended to observe gross pathology at necropsy, 3 days after dosing, and again at weekly intervals. Clearance of the microbial is also an important data end point and is monitored in the faeces with orally administered microbes, and in multiple tissues and organs with the other routes of administration (with the exception of intraperitoneal administration), as well as from any observed lesions.

There is a tiered system in US, Canadian and EU data generation processes. If persistent or significant signs of pathology of the microbial are observed in the test animals in Tier I, acute oral toxicity/pathology tests may be required in non-rodent animal species. If toxin production by the MPCA is suspected, or if toxin production is indicated by significant or persistent signs of toxicity in the test animals in the absence of signs of infectivity or pathology, the toxin(s) is/are to be identified and isolated, and an acute toxicity study conducted with the toxic component(s). If significant infectivity or unusual persistence of the MPCA is observed in the absence of signs of toxicity or pathogenicity, a subchronic (90 day) study (Tier II) would be required. A Tier III scheme contains tests that may resolve issues of known or suspected human pathogenicity and tests for particular adverse effects of intracellular parasites of mammalian cells. In the face of adverse Tier I data, however, the registrant may desire to simply terminate testing and registering of the agent at that point, as continuing would incur considerable additional cost and lose the safety cachet for that microbial.

The necessity for mammalian testing of MPCAs cannot be argued, except perhaps for the *Baculoviridae* (Strauch *et al*., 2006). Baculoviruses are not pathogenic for non-lepidopteran invertebrates, much less vertebrates, because of their extreme host specificity, nor are they known to produce any toxins (see OECD, 2006, and McWilliam, 2006, for discussions of baculovirus safety). The other viruses, bacteria, protozoa and fungi used for arthropod control are, after all, (non-human) animal pathogens. There is disagreement, though, about suitable end points and levels of microorganism clearance from the test animals. In the EU, where there has been more controversy about the registration data requirements for microbial agents, the EU REBECA (Regulation of Biological Control Agents) Project has recommended changes because the 'current system for the assessment of risks for human and animal health caused by microbial BCAs [MBCAs] needs a revision [in the EU].' (Strauch *et al*., 2007a,b). In terms of human safety, it is recommended, for 'Group 1' organisms (per Directive 2000/54/EC; OJEC, 2000), i.e. those microbes for which no human pathogenicity has been previously observed, such as all the currently registered microbials, that: (i) that waivers of the requirements to investigate allergenic effects be granted (because all living organisms can cause such allergies and people who

are allergic can protect themselves); (ii) culture supernatants, crude extracts of the microbial culture, or the formulated product be tested for toxicity rather than single purified metabolites; (iii) the significance of minor adverse effects in intratracheal administration in pulmonary pathogenicity tests be reduced and greater reliance be placed on intraperitoneal injection studies in rats; (iv) the microorganism clearance aspects in vertebrate testing be de-emphasized because they are much less informative than clinical signs of pathogenicity; (v) key indicators for human (mammalian) safety should include the criteria of no growth at temperatures of >35°C, and absence of clinical reports and indications in relevant scientific literature or databases; and (vi) data on susceptibility of the microbial to chemotherapeutics be included in a risk assessment. Further, REBECA argued that since microorganisms are part of the natural environment, the evaluation of risks associated with the application of microorganisms always needs to be related to the natural exposure of non-targets, including humans. For infections in immunosuppressed humans, the REBECA group recommended that such potential should not hamper the registration of a microorganism because contact between immunosuppressed individuals and all microbes, much less MPCAa, needs to be avoided in any case.

7.3.3 Environmental safety risk assessment

An ecological risk assessment begins with defining the use context of the microbial (patterns of use, frequency, arena of use, etc.) and selecting appropriate non-target species for testing. The results of the host specificity testing, in terms of direct (acute mortality) and indirect (fecundity, fitness) effects, are then coupled with what is known about the dispersal ability of the microbial, and its potential for recycling after use (establishment), to develop an assessment of risk. Ecological safety of MPCAs by regulatory agencies is oriented towards risk assessment using representative birds, fish, aquatic/marine crustaceans, and beneficial arthropods. The latter group always includes honeybees. Testing also includes additional arthropods, three in the USA and Canada, and 'several appropriate' species in the EU (OJEC, 2001). All three systems emphasize that test species be related to the potential use of the microbial and should include organisms used for biological control and organisms important for integrated pest management in the crops involved. EU guidelines, but not US guidelines, also require testing of arthropod pathogens with earthworms and soil microorganisms.

As with mammalian testing, there is a tiered approach with non-target arthropods, beginning with maximum dose laboratory assays to identify a Lowest Observable Effect Level (LOEL) and/or the LD_{50}/LC_{50} for the microbe (Tier I in USA). In such laboratory assays, the doses are in increments to 100× the LD_{50} /LC_{50} of the pathogen in its natural host, or 10–100× the recommended field dosage. If adverse effects are seen in Tier I assays, potential exposure of the non-target arthropod to the MPCA is estimated by means of a Tier II testing for population dynamics of the microbial (fate and expression) in the environment. Data normally generated separately on the persistence of the microbe in the face of UV radiation, temperature and other environmental factors become relevant in designing the Tier II tests. If Tier II tests show that there may be significant exposure to the microbe (i.e. if the minimum infective dose is less than the exposure or if there are other considerations that would decrease the observed effects in the environment), Tier III tests may be required to determine a dose–response effect or to examine certain chronic effects. Tier 4 tests would be conducted under simulated or actual environmental conditions to evaluate any specific problem that could not be resolved by lower tier testing.

There is variability in the specific test guidelines among regulatory bodies. In the USA, duration of the Tier I laboratory tests can be as short as 8–10 days (for fungi) or as long as 30 days (for bacteria and viruses) (US EPA, 1996b). In the EU, however, guidelines call for studies involving multiple applications of an MPCA and the weathering of its residues on a crop before bioassay with the

non-target arthropod. For example, in the testing of *B. bassiana* strain GHA against predaceous *Typhlodromus pyri* mites, the protocol called for each formulation to be applied six times at 1 week intervals to potted grapevines placed in a tunnel greenhouse fitted with UV-transparent plastic, at which point leaves were removed and infested in the laboratory with protonymphs, which were then followed to adulthood. Adult mites were then monitored and the number of eggs produced per mite after a 1 week oviposition period was determined (Jaronski, unpublished).

The use pattern can determine which tests will be mandated and which waived (US EPA, 2000a). For example, in the registration process for *B. bassiana* strain HF23 in the USA, the registrant of this strain successfully obtained waivers of data requirements for testing with freshwater aquatic invertebrates, non-target plants, non-target insects, honey bee toxicity, estuarine/marine animals and wild mammals (US EPA, 2006). These waivers were based on the use pattern of the fungus: control of house flies in chicken manure and poultry production/livestock houses. Based on the fact that no direct outdoor uses were proposed, the fungus had very limited survival in poultry litter and manure, could not grow at 37 °C and is already ubiquitous in the environment, the US EPA concluded that exposure of non-target organisms would be minimal and warranted waivers of the specific tests. In contrast, an MPCA intended for use in a natural habitat having a variety of non-target, especially beneficial, species would undergo a greater degree of scrutiny. Although information on plant pathogenicity is listed among the data requirements of most regulatory bodies, the biology of the arthropod pathogens minimizes this risk and, in many cases, e.g. the US EPA, testing with the pure microbial is waived, although there is possible danger from the formulation and, in many cases, phytotoxicity testing of the formulated agent may still be required.

There has been some discussion about the applicability of such laboratory-based, acute-effects testing (especially the Tier I levels) for many insect pathogens, especially in the EU, where the strictness of the regulations has been accused of being a major disincentive in the commercialization of microbial agents (Strauch *et al*., 2006). For baculoviruses, the long history of use without incidents (OECD, 2006) has spurred recommendations from the European scientific community to simplify registration requirements and conduct evaluation at a species, not a strain, level (Strauch *et al*., 2006). At the same time, there have been reports of virus latency (Cory, 1997), by which a virus could be maintained in an insect population at low but significant levels and without causing overt morbidity until another stressor (another pathogen) appeared. Many of the Microsporidia show high host specificity, but do not have a great degree of use history so there is little precedent on which to rely. Solter *et al*. (2005) reported that host spectra for two *Nosema* species based on laboratory bioassays was greater than seen in the field; they concluded that, in this case at least, both horizontal and vertical transmission provided physiological barriers to host switching. Solter and Maddox (1998) observed that the microsporidians *Nosema* and *Vairimorpha* spp. isolated from various forest Lepidoptera produced infections in gypsy moth (*Lymantria dispar*) larvae that were often atypical compared with infections produced in the natural hosts.

The high host specificity of infectious bacteria (*P. popilliae*, *S. entomophila*) implies very little risk for non-target organisms. In addition, they have very fastidious growth requirements, and *P. popilliae*, at least, will remain dormant in the soil until ingested by its specific host. In the absence of the proper host, bacterial levels dwindle in the soil to ineffective background levels. The Bt ICPs also show considerable specificity (in terms of protein solubilization and activation, toxin binding and insertion into a membrane), which tends to support their environmental safety. The commercial *B. t. kurstaki* HD1 products containing Cry1Aa, 1Ab and 1Ac are highly toxic for *Trichoplusia ni* (cabbage looper), *Helicoverpa zea* (corn earworm), *Heliothis virescens* (cotton bollworm) and *Ostrinia nubilalis* (European corn borer), but not for *Spodoptera* (army worm) species nor for *Plutella xylostella* (diamondback moth), whereas other Bt products containing Cry1Ca are moderately toxic to this latter genus but

not to *T. ni*. Neither of the two Bt taxa has observed toxicity for non-lepidopterans. Correspondingly, Bt products containing the Cry3 ICP family are toxic only to certain Coleoptera. Nevertheless, all pesticide regulatory agencies require non-target testing. This testing is necessary because unexpected effects may occur. For example, in other less popularized studies of the effects of Bt proteins on non-target invertebrates, it was shown that immature lacewings (*Chrysoperla carnea*) fed on prey that had eaten Bt maize (expressing the Cry1Ab protein) suffered greater mortality than control lacewings fed on prey that had eaten non-Bt maize (Hilbeck *et al*., 1998a). Only 37% of the lacewings survived when fed larvae of the cotton leafroller (*Spodoptera littoralis*) or the European corn borer that had eaten Bt maize. In comparison, 62% of the control group survived when fed on caterpillars that had eaten non-Bt maize. In a subsequent study using an artificial liquid diet it was determined that immature *C. carnea* were sensitive to the Cry1Ab toxin at a level of 100 μg ml^{-1} of diet (Hilbeck *et al*., 1998b). More recent laboratory studies have confirmed that *C. carnea* fed *S. littoralis* larvae fed on Bt maize suffered higher mortality and longer developmental times than when fed with control larvae (Dutton *et al*., 2002).

Another risk is that a highly efficient pathogen would so drastically affect the target population that other natural enemies (predators, parasitoids, other pathogens) would be deprived of sufficient resources and be driven towards local extinction. Parasitoids and oligophagous pathogens would be most susceptible, while most predaceous insects have enough polyphagy to switch to other prey. So far, there is little historical evidence for such an outcome. None of the commercial MPCAs have shown absolute or near absolute efficacy. In fact, many microbial products are criticized for insufficient efficacy in a chemical-paradigm use, to the extent that even genetic manipulation to overcome this perceived defect has been pursued, e.g. entomopathogenic fungi overexpressing proteases or expressing scorpion venom, or baculoviruses expressing venom (Hoover *et al*., 1995; St. Leger and ChengShu, 2010). Perhaps the best arena for examining such potential indirect adverse effects is with Bt maize and Bt cotton, which were adopted across large areas in the USA in 2010 (22.4 million ha maize, 3.2 million ha cotton) and which have a high degree of efficacy in controlling the target pests (NASS, 2010). Wolfenbarger *et al*. (2008), in reviewing the effects of Bt maize and Bt cotton in the USA, concluded that there were no consistent effects on non-target insects, but rather a general absence of large-scale adverse effects, especially when compared with crops treated with chemical insecticides. In Bt maize, while fewer specialist parasitoids of the target Lepidoptera occurred, the numbers of generalist parasitoids, specialist parasitoids of other insects, and predators, were higher than in pesticide-sprayed crops. In Bt cotton, there were slightly fewer predators than in unsprayed non-Bt cotton, due in large part to moderate reductions of the predaceous Nabidae and Coccinellidae. Thus, in a case where a significant effect on the target pest existed, even indirect adverse non-target effects were minimal. Similarly, *B. t. israelensis* has been intensively applied to the Volta River watershed of Niger for more than 10 years to combat the blackfly vector of onchocerciasis. During that time, adverse effects to non-target aquatic insects were minimal and transient, especially when compared with chemical treatments (Federici, 2003).

Persistence of a microbial is an important aspect of its potential environmental impact. Although many of the viruses disperse quickly after application (most likely through host mobility before death), no inundative application of *Baculoviridae* has produced epizootics in less susceptible species. Cory (2003) ventured the opinion that it is unlikely that NPVs can produce sustainable epizootics in species that do not regularly succumb to epizootics. Infectious bacteria may have long persistence. *S. entomophila* titres persisted at least 8 months following application to the target soil arena when hosts were present, but declined rapidly in the absence of the insect (Jackson, 2003). These bacteria recycle and can provide long-term suppression of their host. *P. popilliae* was observed 25 years after its introduction into turf to control the Japanese beetle (Ladd and McCabe, 1967), but subsequently scarab beetle populations increased despite its presence;

bacterial mutation and selection for less virulent genotypes, as well as changes in the susceptibility of the host were hypothesized. The establishment of *P. popilliae* in the field appeared to be dependent on the achievement of sufficient larval densities. *L. sphaericus* persisted at least 5 months in the bottom mud of pools where it had been applied and significantly controlled target mosquito populations for 5–6 weeks (Nicolas *et al*., 1987). As obligate, frequently chronic, pathogens, the Microsporidia persist in their host populations to a variable extent. For instance, the North American *P. locustae*, which has a very broad host range within the Acrididae, atypical for this group, has been observed to persist 11 years post introduction into grasshopper populations in Argentina, to spread to susceptible but non-target acridids, and to cause heavy infections, albeit at a prevalence below 50%, most frequently 4–20% (Lange and Azzaro, 2008; Lange and Cigliano, 2010). *P. locustae* did not persist to any great extent after inundative applications in the USA and Canada (Johnson, 1997). The chronic nature of acridid infections by this microsporidian can have important implications, not only for long-term persistence and spread, but also in long-term effects on host insect reproductive fitness and population levels. The ascomycete fungi targeting foliar arthropod pests have relatively poor persistence, a matter of a few days, and also no record of biologically significant establishment, which are reasons for their commercial attractiveness. Reproduction is limited by the requirement for near saturated humidity for at least 48 h, otherwise the fungus dies with its host. Except in glasshouses, very moist habitats (coffee) and in very protected situations (within maize leaf sheaths), this requirement is rarely met. In soil systems, persistence can be longer. In a meta-analysis of extant data, Scheepmaker and Butt (2010) observed that *B. bassiana*, *B. brongniartii* and *M. anisopliae* demonstrated a decline in populations over time, the rate of which could vary considerably depending on a host of factors. Persistence at biologically meaningful levels could be as long as several years. The many ecological factors affecting these fungi in inundative use have been summarized by Jaronski (2007, 2010).

Microbial insecticides, other than Bt, have not enjoyed as wide adoption as in the Bt crops, so there has been little opportunity to explore ecosystem-wide effects. Stiling and Cornelissen (2005) attempted to summarize the efficacy of biological control agents via a meta-analysis of 145 published studies to that date. While microbial agents were not a significant focus of the analysis, they were included. Bacteria and fungi each reduced pest abundance 2.7-fold, with a wide variance about each mean value (Figs 4A and 4B in their study). In contrast, predaceous mites were far more effective in reducing pest abundance (a 7.4-fold proportional change).

The biggest challenge in environmental risk assessments of microbial arthropod control agents comes with the entomopathogenic ascomycetes that are generalist pathogens. In laboratory bioassays, where simple physiological susceptibility of a non-target organism is determined, with the test insect often inadvertently stressed to some degree, there is a greater probability of adverse effects. Jaronski *et al*. (2004) proposed that adverse effects in these Tier I laboratory assays do not really predict the impact of a microbial under natural conditions in the field. While *B. bassiana* GHA was readily pathogenic for honeybees in a traditional laboratory bioassay using isolated worker bees, outdoor tests involving intact, healthy bee colonies demonstrated little to no impact. Bees treated three times at 5 day intervals with 5×10^{13} conidia ha^{-1} (the label rate for control of whiteflies in vegetable crops) suffered no accelerated mortality compared with controls, and infection was observed in only 1.2% of the treated bees during a 30 day period. At five times the label rate, 2.5×10^{14} conidia ha^{-1}, treated colonies had a 3–8% infection rate among the treated bees; and mortality rates of workers among replicate colonies were not consistently correlated with treatment. Subsequently, Al-mazra'awi *et al*. (2006) used bees to successfully vector *B. bassiana* GHA into canola to manage *Lygus* populations. The key to minimal adverse effect seems to be associated with a healthy, active honeybee colony, wherein numerous factors operate to minimize effects, while the traditional bee-testing protocol caused considerable stress among

the test bees. Several other cases, in which the adverse effects of a fungus indicated in laboratory testing were not borne out in the field, were outlined by Peveling *et al*. (1994), Vestergaard *et al*. (2003) and Jaronski *et al*. (2004). Jaronski *et al*. (2004) recommended that minimal non-target testing be required for registration of indigenous fungi, especially species that have considerable host specificity. For non-indigenous species of potential concern (wide host spectrum, as with many of the ascomycetes), testing protocols should incorporate pertinent exposure to the pathogen and realistic environmental conditions. In addition, public research efforts to evaluate model pathogens in the field should be funded to increase our overall understanding of pathogen–target–non-target interactions.

Risk assessment is not a clear-cut process, and the regulatory agencies have to make decisions about whether the risks and benefits are in enough balance to make a decision: 'When the available data are inadequate or non-conclusive, a prudent and cautious approach to environmental protection, health or safety could be to opt for the worst-case hypothesis. When such hypotheses are accumulated, this will lead to an exaggeration of the real risk but gives a certain assurance that it will not be underestimated' (Ehlers, 2007).

At times, the regulatory process has not been transparent. Strauch *et al*. (2007a) noted: 'It becomes very clear in an early stage of this action that the development of such proposals is hindered so far by: significant lack of validated risk assessment methods for microbials, knowledge gaps on the natural distribution of the biocontrol micro-organisms, knowledge gaps on natural exposition of humans and other non-target organisms, missing definitions allowing the identification of low risk products. This is hampering an adequate risk assessment of microbial plant protection products.'

In addition, the increasing tendency toward the Precautionary Principle in the EU and its member states has further clouded the process and potentially made it more difficult to provide sufficient information for regulatory approval (Myhr, 2010). This Principle states: 'Where the levels of uncertainty are high, where potential impacts are very large, and/or where those impacts may be irreversible, there are grounds for adopting a precautionary approach until such time as we have sufficient scientific knowledge to make a risk-based decision' (CEC, 2000).

The question remains about what to do with the risk assessment data. How does a regulatory body quantify the risk of a microbial? This question seems to be more important in the EU than in the USA, and has generated considerable discussion. For the US EPA, the pesticide risk quantifiers of human safety effects that lead to acceptable residues for a chemical have not really extended to microbials, because if a microbial agent does not qualify for an exemption from an assessment of residue tolerance (based on the acute vertebrate toxicity/pathogenicity tests), then registration efforts are abandoned for commercial reasons. There are no registered microbial agents in the USA that have an assessment of residue tolerance. In the EU, an acceptable MPCA registration results in the statement 'there are clear indications that it may be expected that [agent] does not have any harmful effects on human or animal health or on groundwater or any unacceptable influence on the environment, … subject to compliance with the particular requirements of Directive 91/414/EEC, for each [agent]-containing plant protection product for which Member States will grant or review the authorization. … Extension of the use pattern beyond those described above will require an evaluation at Member State level' (OJEC, 2001).

In assessing the ecological risk of a microbial agent, the testing data have to be coupled with the ecological context of use, the dispersal and persistence of the agent, the potential for permanent establishment, and the indirect as well as direct effects on non-targets. Several 'competing' risk quantifiers currently exist. An environmental impact quotient (EIQ) (Kovach *et al*., 1992) incorporates risk to consumers, producers and the environment, based on toxicity to mammals, vertebrates and non-target invertebrates, and its application has extended to MPCAs. The EIQ is continually updated and is currently maintained at Cornell University (2012), but

has not been applied to entomopathogenic microorganisms. Laengle and Strasser (2010) have levied a number of criticisms of the EIQ system as this scheme pertains to microbials. The Norwegian Agricultural Inspection Service has developed a quantified risk index for pesticides that incorporates toxicity and exposure (Norwegian Agricultural Inspection Service, 2002). Components of the index include terrestrial and aquatic adverse effects, potential for leaching (movement for microbials), persistence and bioaccumulation. Quantitative scores for each category are then used to calculate an index as the squared sum of all components. Including exposure into the risk increases the utility of the index, and the risk index is based on data supplied to registration agencies. With this Norwegian index, all the microbials have a minimal score, which is a bias, and also prevents quantitative comparisons with chemicals.

Biocontrol with macroorganisms provides a better framework for evaluating biocontrol microorganisms. Lynch *et al*. (2001) created a semi-quantitative scheme incorporating not only non-target mortality, but also population-level effects, resulting in a severity index of 0–9. A direct mortality level of 40% was proposed as a minimum level at which significant population level effects were possible. This index, however, relies solely on reports of field effects and cannot readily use typical registration data. An improved framework is provided by van Lenteran *et al*. (2003). There are two components to this framework: (i) the *likelihood* for establishment, dispersal, non-target impact (breadth of host range, direct and indirect impacts on non-targets); and (ii) the *magnitude* of the above factors. Numerical values are assigned to likelihood (from 1 for very unlikely to 5 for very likely) and magnitude (from 1 for minimal to 5 for massive effect). These values are then multiplied for each aspect (establishment, dispersal, host range, direct effects, indirect effects) and the products summed to yield a risk index (5 for the most benign agent to 125 for an agent with the greatest adverse effect potential). The authors proposed that classical biocontrol organisms with a risk index of less than 35 should result in little to no objection for release, while agents with an index of >70 points would result in a decision to not release the agent. This risk index must also be considered in the context of size of the potentially affected region, permanence of the habitat and use practices, rather than just be an abstract number. It should be remembered that these index thresholds are for classical biocontrol agents that have the need and capacity to reproduce and spread subsequent to introduction, unlike the bulk of MPCAs which are used inundatively and do not reproduce or establish themselves. van Lenteren *et al*. (2003) went on to assess a number of agents used in the EU. The only microorganisms included were three entomopathogenic fungi, *B. bassiana*, *M. anisopliae* and *M. acridum*, all used inundatively as biopesticides. The authors concluded that 'Entomopathogens (*Beauveria*, *Metarhizium* and *Steinernema* (an entomopathogenic nematode)) all score intermediate because of their broad host range, but their very limited dispersal capacities strongly reduces risk'.

Laengle and Strasser (2010) modified the van Lenteren *et al*. (2003) assessment matrices for microbial pest control agents with more detailed components and a more complicated algorithm for calculating a risk index. Host specificity is divided into: (i) number of affected species, and (ii) taxonomic level of host range (genus, family, order, class, phylum). Dispersal includes distance and quantity factors. A scale for vertebrate effects is added. The other components are better quantified as to severity than in the van Lenteren *et al*. (2003) system. With the Laengle and Strasser (2010) system, the microbes can be compared with chemical pesticides. Several current microbial agents were then evaluated. The results are given in Table 7.1. Despite acknowledged limitations (some degree of subjectivity, weakness in predicting environmental impacts), the Strasser and Laengle system has considerable advantages.

Additional basic aspects of risk assessment are cost–benefit and relative risk analyses. These aspects are perhaps more strongly practised in the USA and Canada, where the risk of a microbial agent is weighed against the impact of the current chemical pesticides in that crop. In those countries, risk is considered relative to benefits if some adverse effects

Table 7.1. Relative risk index of selected microbial arthropod control agents in comparison with three chemical insecticides (adapted from Laengle and Strasser, 2010). A Risk Index score <100 implies low risk; 100–500, 'reduced risk'; >500 'high risk'.

Organism	Risk index
Bacillus thuringiensis kurstaki (foliar spray)	280
Beauveria bassiana (foliar spray)	260
B. bassiana (soil application)	96
B. brongniartii (soil application)	16
Metarhizium anisopliae (foliar spray)	96
M. anisopliae (soil application)	240
Chlorpyrifos (foliar spray)	2610
Phorate (granular, soil application)	2016
Pyrethrin (foliar spray)	216

data (e.g. some infectivity of an agent for honeybees) do exist. For example, Canada has a 'Value Considerations' section in its microbial pest control agent evaluation reports (e.g. Health Canada, 2009, 2010). In the proposed and final registration decisions, Health Canada examines the contribution of a microbial agent to manage pest problems, leading to economic, health and environmental benefits, and proposes risk-reduction measures to protect human and environmental health on the use label for a product (Government of Canada, 2002). The Canadian law defines value as 'In respect of a pest control product, value means the product's actual or potential contribution to pest management, taking into account its conditions or proposed conditions of registration, and includes the product's efficacy; effect on host organisms in connection with which it is intended to be used; and health, safety and environmental benefits and social and economic impact' (Government of Canada, 2002).

The last precaution for continuing risk assessment of a registered microbial in the USA is a requirement for prompt reporting by the registrant of any adverse incidents, including events of hypersensitivity to an agent during production or use, and adverse environmental effects. These '6(a)2 notifications' (US Government, 1947, 2011) have stringent timeliness requirements and penalties for non-compliance by registrants. In this manner, the US EPA exerts some future control over the products, both chemical and biological, that it registers for use against pests.

7.4 Additional Risk Assessment Considerations

7.4.1 Non-indigenous versus indigenous microorganisms

A non-indigenous or exotic pathogen is defined as one that is not present in a certain geographical area (by the USA) or ecozone (by Canada). In contrast, an indigenous pathogen is one that is endemic, or has been previously introduced and has become established, within an area. In the USA, the importation of microorganisms that are not indigenous, and which are not registered by the US EPA as a microbial pesticide, is regulated by the USDA, specifically the Animal and Plant Health Inspection Service (APHIS). Currently in the USA, even non-indigenous strains of an indigenous microbial species are regulated. Special permits must be obtained to work with such organisms under some level of laboratory or greenhouse containment. For release into the environment (e.g. a field trial), APHIS must conduct a risk assessment of potential environmental effects on the assumption that the absence of evidence of negative environmental impacts is not evidence of an absence of those impacts. An environmental assessment (EA) process is used in evaluating non-indigenous microbial agents. An EA on the agent is drawn up by APHIS and published for public commentary, with subsequent deliberation and decision by the agency. In the EA, the main objective is to identify potential hazards associated with a decision to allow or not to allow release of an agent, with a determination that risks associated with a particular action have been sufficiently evaluated for reasonable decision. Within the last few years, the US EPA has required an APHIS permit for any non-indigenous microbial for which registration is being sought, thus providing for APHIS assessment, as outlined above.

In Canada, unlike the USA, as the ecozone is the determinant of indigenous status, so testing a microbial outside its ecozone of origin requires a federal research permit and, to this end, a microorganism (from continental North America), requires: the documented geographical range of the microbial pest control agent; the documented geographical range of target and known affected non-target organisms; a comprehensive literature review on taxonomically closely related microorganisms with respect to effects on target and non-target organisms; and data on persistence and dispersal in the environment (PMRA, 2001). For microbes originating outside North America, much of the data for regular registration is required. The PMRA is, therefore, able to assess the risk of non-indigenous microbes and act appropriately.

In the EU, a microorganism is considered indigenous or non-indigenous 'at the species level, to the intended area of application' (OECD, 2008). However, the European Federation of Marine and Technology Societies defines non-indigenous species as 'species, subspecies or lower taxa introduced outside of their natural range (past or present) and outside of their natural dispersal potential' (Olenin *et al*., 2010). This difference in definitions has yet to be resolved (CEC, 2008).

The OECD Environment Directorate has acknowledged the difficulty in determining indigenous status of a microorganism: 'definition of a species or at least uniform tools for distinction of species and strains are needed to distinguish the micro-organism from the other microorganisms already present in the environment; this is not the case for many micro-organisms' (OECD, 2003). In other countries, the degree of regulation of non-indigenous MPCAs is very variable – from lax (some African and Middle Eastern countries) to extremely restrictive (New Zealand, Australia).

The advent of molecular tools to better define taxonomic relationships and population genetics has provided a new perspective on the indigenousness of many of the microbial pathogens. For example, detailed studies of *B. bassiana* population genetics (Meyling and Eilenberg, 2006; Rehner *et al*., 2006) have revealed that the species can be grouped into clades, and that these clades cross continents. Thus, in North America, isolates of the fungus can be more closely related to European isolates than to North American siblings, and vice versa in Europe. The implication is that strains of *B. bassiana*, and probably at least some of the other entomopathogenic ascomycetes (*M. anisopliae senso lato*, *I. fumosorosea*), have a cosmopolitan distribution and so the concept of indigenousness does not apply.

While most non-indigenous macrobial biocontrol agents are used in classical biocontrol and through small-scale inoculations, microbial insect pathogens are more likely to be used in inundative control. The potential risk of a non-indigenous microbial insect pathogen is that it would become established and spread to additional areas, affecting non-target insects. This risk is actually self-defeating for commercialization because return on investment requires either repeated sales, or a very high price for a single use. The historical record of microbes that have been commercialized is that the potential for significant establishment is rarely, if at all, realized.

7.4.2 Genetically altered versus naturally occurring organisms

There has been increased interest in the genetic modification of entomopathogens to improve their efficacy. In addition to the fungus and virus examples mentioned earlier, Bt has been experimentally engineered to express several, diverse insect toxic genes, even from other species (e.g. *L. sphaericus*) to greatly extend host range (Park *et al*., 2003). These transformations are based on the toxic genes being located on mobile plasmids. Genetically engineered MPCAs can have a number of potential risks: (i) direct effects on non-target organisms through expanded lethal host range; (ii) enhanced evolution of host resistance; (iii) adverse ecosystem services effects; (iv) unintentional development as a pest organism; (v) biological pollution/cumulative effects; and (vi) possible transfer of the genetic traits to other microorganisms. Because the new organism would be released

into an environment for the first time, if adverse effects occur, it would be likely to be impossible to eradicate if the microbe becomes established. The difference from the situation with non-indigenous microbes is that the genetically modified organism will be introduced into the environment for the first time in its modified form, whereas the naturally occurring organism has occurred in nature elsewhere (Goettel and Jaronski, 1997).

7.4.3 Use of microbial symbionts to control arthropods

New developments in the exploitation of arthropod symbionts, primarily the bacterium *Wolbachia*, have created new potential avenues for arthropod control. Mosquitoes deliberately infected with a particular strain of *Wolbachia* failed to transmit dengue fever virus (Walker *et al*., 2011). The bacterium was readily passed on to subsequent mosquito generations, reaching 80–100% prevalence among wild mosquito populations subsequent to an environmental release (Hoffmann *et al*., 2011). These observations, and others, for example, the transfection of the olive fruit fly (*Bactrocera oleae*) by *Wolbachia* from the cherry fruit fly (*Rhagoletis cerasi*), resulting in embryonic mortality from cytoplasmic incompatibility (Apostolaki *et al*., 2011), have engendered discussion about pest population control strategies using arthropod symbionts. In addition to using unmodified *Wolbachia*, recent data point towards genetically modifying the bacteria either to prevent vectoring of human, animal or plant pathogens, or to reduce the reproductive potential of a target insect. For more information about this topic refer to Floate *et al*. (2006) and Aguilera *et al*. (2011). Ostensibly, such uses of these bacteria will require regulatory oversight. These strategies, being different in their approach from the traditional methods discussed earlier, will challenge current risk assessment criteria and require new perspectives.

7.5 Conclusion

The regulation of MPCAs and the attendant risk assessments have seen considerable evolution as bio-based pest control measures have gained ever more popularity and importance. In some countries (the USA, Canada) there is a concerted effort to register more microbials in an effort to reduce chemical pesticide usage. Regulatory agencies worldwide are reviewing more data packages each year and therefore becoming more familiar with the risks from MPCAs. With this increasing familiarity on the part of the regulators, and generation of non-target effects analyses by the scientific community, there is reason to hope that risk assessments will be simplified and better science based in the future.

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8 Microbial Control of Plant Diseases

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8.1 Introduction

At this beginning of the 21st century, humanity is facing several challenges regarding food security in relation to climatic change and energy crisis, leading to a need for increasing agricultural production for both food and energy purposes. At the same time, there is an increased public demand for 'safe' foods, which does not necessarily means organic food, but food without pesticide residues. Thus crops have to be protected from pests and diseases with less use of chemical pesticides. In this context, there is a renewed interest in biological control using different types of antagonistic microorganisms.

In this chapter we consider the use of microbial biological control agents (MBCAs) to control plant diseases. But it seems important to first stress that the term 'biopesticide' or, alternatively, 'microbial pesticide', is not necessarily appropriate to describe an MBCA. The modes of action of MBCAs are complex, often multiple and generally totally different from those of chemical pesticides. Most of them do not have 'cidal' effects in that they directly kill the target organisms; instead, they inhibit their growth, detoxify the toxins produced by the pathogens and induce plant defence reactions.

In many countries, the placing on the market of MBCAs is regulated the same way as that of chemical pesticides. Because MBCAs are living organisms, however, the hazards they might present are not of the same nature as those of chemical pesticides, and the risks have to be assessed differently.

In the European Union (EU), Regulation (EC) No 1107/2009 (OJEU, 2009) concerning the placing of plant protection products on the market, implemented on 14 June 2011, contains the text of reference which regulates the use of plant protection products, including chemicals as well as MBCAs. The Regulation follows the approach established in Council

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Directive 91/414/EEC (OJEC, 1991), which was amended by Commission Directive 2001/36/EC (OJEC, 2001) regarding the data requirements for the inclusion in Annex I of microorganisms as active substances, and the national authorization of products (Annexes IIB and IIIB). Even if the latter Directive was written with the objective of adapting Directive 91/414/EEC to the case of living microorganisms, one must say that this corpus of regulation does not permit a rapid and satisfactory evaluation of risk linked to the application of biological control products. In fact, Directive 91/414/EEC was created in response to human health problems that appeared after many years of intensive use of chemical pesticides. It is not, therefore, adapted to the evaluation of the safety of MBCAs and there are no provisions for a timetable or a possible revision of data requirements for microbial active substances and plant protection products containing them.

In contrast, several countries outside the EU, especially the USA, do not regulate MBCAs in the same way as chemical pesticides. The main differences between the EU regulations and those of other countries have recently been reviewed by Hauschild *et al*. (2011). With the objective of harmonization of the requirements for MBCA registration, the Organisation for Economic Co-operation and Development (OECD) is working on guidelines to identify the hazards and evaluate the risks linked to the use of MBCAs (see Meeussen, Chapter 21, this volume).

In this chapter, we outline the specific challenges posed in the safety assessment and registration of microorganisms being commercialized for the control of plant diseases, and critically discuss these in reference to the Regulation (EC) No 1107/2009, related legislative texts and OECD proposals.

Because MBCAs (fungi, bacteria or viruses) might be closely related to species that are known to be pathogenic, the first challenge is obviously to ascertain that the MBCA is not, and will never become, infectious or pathogenic for humans, animals or plants. One must also ensure that the secondary metabolites, and in particular toxins, if they are produced, are not harmful for man, or deleterious for non-target organisms or the environment under the intended uses. In contrast to chemicals, MBCAs can survive and even multiply on plants and in the environment. So there is great concern about the behaviour and fate of the MBCAs after release. In relation to this capacity to survive and multiply, the identification of the hazards posed by possible residues is complex, as the residues consist not only of the living MBCAs themselves, but also of the secondary metabolites that the MBCAs can potentially produce on the plant parts that are used as food or feed. Finally, these basic questions apply not only to the active substance, which is the microorganism itself, but also to possible microbial contaminants and metabolites of concern that might be present in the commercial product, which could be released into the environment.

Thus, the identification of hazards and assessment of risks appears to be more complex when dealing with microbial biological control agents than with chemical control products.

8.2 Identity of the Microbiological Control Agent

Obviously, the first concern is whether the MBCA is pathogenic or infectious to humans and animals. Commission Regulation (EU) No 545/2011 (OJEU, 2011) of 10 June 2011 implementing Regulation (EC) No 1107/2009 concerning data requirements for active substances clearly states, like the previous Directive 2001/36/EC, that 'the identification together with the characterization of the micro-organism provides the most important information and is a key point for decision making'. All experts agree with this statement: a correct identification of the microorganism is the first step to ensure the safety of the plant protection product containing it. Although there has been a debate about the level of identification of the microorganism and methods to be used, scientists agree that identification must be at the strain level, using the most accurate methodology available. Indeed, the biocontrol capacities are specific to the strain and not necessarily common to all strains belonging to the same (sub)species. This does not exclude the possibility

that, for the general parts of the dossier, data for different strains of the same species might be used when a great similarity within a species has been demonstrated. Nevertheless, as illustrated below, many species of microorganisms include both pathogenic and beneficial strains. It is necessary, then, to develop methods that enable the identification of the biocontrol strain from other strains belonging to the same species. Such tools are also needed for regulatory reasons to ensure that the product contains only the strain that has been approved. Identification of an MBCA at the species and strain level cannot rely only on phenotypic or physiological characters; today, several molecular methods are available that can be routinely used to identify MBCAs (Louws *et al*., 1999).

Beside the need to distinguish an MBCA from pathogenic strains, the ability to specifically recognize a strain of an MBCA provides some protection for the owner of the strain. Because in Europe a microbial strain isolated from the environment cannot be patented, registration of a well-identified strain is a way to protect the plant protection product. Knowing that the data generated to establish the registration dossier are the property of the petitioner and typically protected for a period of 10 years, a competitor willing to market a product using the registered strain will have to negotiate access to the protected part of the dossier or conduct all the expensive studies needed to produce all the data specific to the dossier. Therefore, although not impossible, registration of a strain in practice prevents its use by another company for several years.

8.2.1 Bacteria

The use of several strains of bacteria as biological control agents has been the cause of debate as to whether products containing these strains could be responsible for human infection. A well-documented example is that of *Pantoea agglomerans*; several strains of this species are among the most promising biocontrol agents for a variety of bacterial and fungal plant diseases. Strain C9-1 of *P. agglomerans* is registered in the USA and in Canada to control fire blight of apple and pear, but commercial registration is not possible in Europe because *P. agglomerans* is listed as a biosafety level 2 organism owing to clinical reports that it is an opportunistic human pathogen. Rezzonico *et al*. (2009) conducted a comparison of many strains of this species, of both plant and clinical origin, with the aim of identifying genetic/phenotypic markers using multi-locus phylogenetic analysis and fluorescent amplified length polymorphism (fAFLP) fingerprinting. Sequence analysis revealed that a majority of the clinical isolates were improperly designated as *P. agglomerans*. In the *P. agglomerans sensu stricto* group, there was no discrete clustering of clinical/biocontrol strains and no marker was identified that was uniquely associated with clinical strains. Conversely, a putative biocontrol specific marker was identified which is only present in biocontrol strains. This marker could be used in the future for identification of biocontrol strains that can be expected to pose no risk to humans.

Another example concerns *Stenotrophomonas maltophila*. Some environmental strains could be developed as MBCAs, while others are known to be responsible for nosocomial infections. Minkwitz and Berg (2001) characterized 50 strains for phenotypic and molecular characteristics. Using 16S ribosomal DNA sequencing, the isolates could be separated into three clusters, two of which consisted of isolates originating from the rhizosphere; the third cluster consisted of both clinical and environmental strains. *In vitro* characterization of the antagonism of the strains towards several pathogenic fungi and of their production of secondary metabolites demonstrated that all the strains, whatever their origin, produced a large range of antifungal metabolites, which included antibiotics, siderophores and enzymes. However, as for other bacterial species, such as *Pseudomonas* spp., the antifungal mode of action was strain specific.

These two examples justify the need for a precise characterization of MBCAs at the infra-specific level in order to clearly distinguish between bacterial strains potentially harmful to man (or mammals), and strains present in the environment that can be safely developed as biological control agents.

8.2.2 Fungi

Fungal strains belonging to the genus *Trichoderma/Hypocrea* have been known for years as beneficial organisms with antagonistic activities against many plant pathogenic fungi. The genus *Trichoderma* is ubiquitous in soil, on organic debris, and on roots and other plant parts. It is mainly present in its anamorphic stage, which produces many unicellular microconidia. The different species of *Trichoderma* are very difficult to identify based on morphological characters, as described by Rifai (1969). Only recently has the use of molecular techniques targeting the sequence of the ribosomal DNA enabled a clear identification at the genus and species levels. Indeed, these tools enable placement of the strains in a phylogenetic tree, thus revealing the relationship among species belonging to the same genus. This allows prediction of the potential hazards based on the proximity of a strain to known pathogenic species or strains. Cordier *et al*. (2007) compared the 18S rDNA sequences of *Trichoderma* strains to identify candidates that showed some abilities for biological control. One potential MBCA strain examined was found to occur in the same cluster as several strains belonging to *T. longibrachiatum*, a species known to include strains pathogenic to man. Consequently, based on this information, it was decided to terminate the development of this strain as an MBCA as it could potentially be pathogenic to humans.

Several preparations already on the market were supposed to contain strains of *T. harzianum*. But, based only on morphological characters, these strains were misidentified. They have been recently re-identified as *T. atroviride* through sequencing of the ITS (internal transcribed spacer) region of the ribosomal DNA and of genes encoding different functions, such as the elongation factor 1α. In order to distinguish these different strains belonging to the same species, it is absolutely necessary to develop a specific marker for each strain. This has been done for strain I 1237 of *T. atroviride*, which has recently been developed as a biocontrol agent (Cordier *et al*., 2007).

If the MBCA belongs to a species known to include strains pathogenic to plants, non-target animals or man, the design of a SCAR (sequence characterized amplified region) marker is an absolute necessity as it will be the only way to accurately distinguish it from other strains that are potentially pathogenic. One of the best examples comes from *Fusarium oxysporum*, which shows a great diversity of strains. *F. oxysporum* can be found in many different environments, including soil, where it is very common and occurs in high numbers. Most of the strains found, if not all, are good saprophytes, surviving for many years in the soil and in the rhizosphere of many plant species. *F. oxysporum* is well known to include very aggressive plant pathogens and also opportunistic human pathogens. The pathogenic soil-borne strains penetrate the roots of different plants and can provoke either rots or wilts. Strains responsible for wilts show narrow host specificity, usually attacking a single plant species, which has led to the distinction of pathovars or *formae speciales*. Interestingly, some strains are not only non-pathogenic but also provide disease protection for certain plant species (Alabouvette *et al*., 2009). Several non-pathogenic strains have been proposed as MBCAs to control *Fusarium* diseases. In such a case, it is obviously necessary to develop a tool enabling precise identification of the strain in question. Recently, a SCAR marker has been designed to identify the well-known protective strain Fo47, and a method developed not only to detect but also to quantify this MBCA in the root of the plant (Edel-Hermann *et al*., 2011).

To conclude with this first aspect regarding the safety of MBCAs in relation to human, animal or plant infectivity or pathogenicity, one must stress that presently there is no universal tool to address the precise identification of an MBCA at the species and strain levels. However, currently available technology provides several tools enabling characterization of a strain at the infraspecific level. Depending on the species, it might be necessary to use several techniques or to sequence several DNA regions to reach a level of characterization sufficient to ensure the development of innocuous strains.

8.3 Contaminants

Products placed on the market contain not only the MBCA itself, and often residues of the cultivation medium, but also different additives (e.g. co-formulants) and, possibly, microbial contaminants. Therefore, it is important to ensure that the contaminants are not hazardous to man or the environment. According to Commission Regulation (EU) No 545/2011 implementing Regulation (EC) No 1107/2009 as regards the data requirements for plant protection products: 'the content of other components (such as by-products, condensates, culture medium, etc.) and contaminating micro-organisms, derived from production process' must be reported. This is a difficult challenge, because the level and the identity of contaminants might change from one production process or geographic site to another, and even from one batch to another. Thus, the regulations require that the applicant must subject both the production process and the product to continuous quality control.

From the microbiologist's point of view, a plant protection product should contain the MBCA strain without any contaminants. Produced in pure culture, in fermentation reactors, the active substance should be clean. The presence of contaminants often means that the production process has not been well optimized. Contamination could potentially occur during the different steps of the production, harvesting and formulation processes, which are usually conducted under clean, but not necessarily aseptic, conditions. The technical active substance and the preparation could be contaminated by ubiquitous microbiota from the surroundings. In such cases, the content of contaminants will be low compared with that of the MBCA and of no concern for human health. In some cases, for example in the case of viral MBCAs, which have to be produced in living insects, cells or bacterial cultures, it is difficult to avoid contaminants. In addition, some preparations are formulated with components of natural origin, such as clays, which might contain a complex microbiota. So these preparations might contain high levels of various types of contaminants. The regulations require that a petitioner reports the maximum content of contaminants and their identification to ensure that the products do not contain plant or animal pathogens (Commission Regulation (EU) No 545/2011).

The level and nature of acceptable contaminants pose problems in relation to risk assessment for humans and non-target organisms. Most countries recommend methods for determination of the presence of contaminants of human concern and have limit levels for genera such as *Listeria*, *Salmonella*, *Enterococcus*, etc. At present though, there is no harmonized or agreed European list. The OECD BioPesticide Steering Group has prepared an issue paper on this subject (OECD, 2011b). This issue paper focuses on microbial contaminants of human and animal concern, primary human pathogens. For reference and guidance to regulatory authorities as well as applicants, the issue paper includes a compilation of the methods available for screening microbial pest control products for the presence of pathogens and other contaminating microorganisms. Table 8.1 presents a list of contaminants, with the tolerated content limits proposed by OECD and proposals by industry representatives (Ravensberg, 2010). Very low limits should not be necessary for microbial contaminants that are normally present in soil and are introduced in relatively high densities with different agronomic inputs, such as manure.

8.4 Secondary Metabolites

According to Regulation (EU) No 544/2011: 'relevant metabolites (i.e. if expected to be of concern to human health and/or the environment) known to be formed by the micro-organism shall be identified and characterised at different states or growth stages of the micro-organism'. This point is one of the most controversial, as bacteria and fungi produce, usually at low concentrations, a large variety of secondary metabolites. These can include antibiotics, toxins, enzymes, hormone-like substances, surfactants and other types of compounds (Vining, 1990).

Consumer concerns regarding mycotoxins entering the food chain have prompted closer scrutiny of the secondary metabolites

Table 8.1. List of contaminant indicator species and limits proposed by OECD and the industry.

Indicator	Proposed OECD microbial contamination of product (OECD, 2011b)	Industry proposal July 2009 (Ravensberg, 2010)
Salmonella	Absence in 25 g or 25 ml	Absence in 25 g
Listeria monocytogenes	Absence in 25 g or 25 ml	Not relevant; omit
Vibrio	Absence in 25 g or 25 ml	Not relevant in EU; omit
Shigella	Absence in 25 g or 25 ml	Not relevant in EU; omit
Aerobic plate count	$<1 \times 10^5$ cfu g^{-1} or ml^{-1}	<0.1% if active ingredient level, with a maximum of 10^7 cfu g^{-1}
Anaerobic spore-formers	$<10^5$ cfu g^{-1}	Not relevant, cannot develop; omit
Yeast and mould count	<1000 cfu g^{-1} or ml^{-1}	Not relevant; omit
Escherichia coli or thermophilic (faecal) coliforms	Absence in 1 g or ml/, <10 cfu g^{-1} or ml^{-1}	Coliforms: <1000 cfu g^{-1}
Staphylococci	Absence in 1 g or ml	*Staphylococcus aureus* <1000 cfu g^{-1}
Pseudomonas aeruginosa	Monitoring[a]	Not relevant
Mouse IP/SC assay	No evidence of infection or injury in test animals	Only in some cases when need has been proven

[a]Evaluation will be based on levels that occur.

of all fungal biocontrol agents. The secondary metabolites produced during the fermentation process might be present in the technical product. This problem can often be avoided by choosing a harvesting process that enables efficient separation of the viable propagules from the substrate used for the fermentation – in which secondary metabolites may have accumulated. However, metabolites might be also produced during growth after release of the MBCA into the environment. The production of secondary metabolites depends on many factors, such as the age of the culture, the growth medium, the plant species and even the plant organ to which the biological control agent is applied (Magan *et al*., 2002; Woo and Lorito, 2007).

To better address possible hazards posed by secondary metabolites, it is important to know the mode of action of the MBCA. Furthermore, the potential toxicity of secondary metabolites will get more attention if the metabolite is part of the mode of action of the MBCA. It is necessary to remember that there may be several modes of action by which a MBCA controls plant diseases. For instance, many different modes of action have been documented in *Trichoderma* spp. These include direct antagonism through competition for nutrients, competition for space, hyperparasitism, antibiosis and, more generally speaking, the production of secondary metabolites and enzymes that have a direct effect on the target organism (Woo and Lorito, 2007). Indirect antagonism occurs primarily through the stimulation of plant defence reactions. It is obvious that there is no risk linked to hyperparasitism or competition for space or nutrients, but secondary metabolites involved in the mode of action of *Trichoderma* spp. might be toxic and of concern for human health and the environment. Indeed, among many other molecules, *Trichoderma* strains can produce the mycotoxins known as trichotecenes and peptaibols (Kubicek *et al*., 2007). It is possible, but time-consuming and expensive, to detect and quantify these molecules in the culture filtrate (Stoppacher *et al*., 2007). It is even more difficult and expensive to determine whether these molecules are produced *in situ* after application of the biological control strain to soil or to the plant organ to be protected. In fact, it has been demonstrated that the same *Trichoderma* strain does not produce the same secondary metabolites, including mycotoxins, in the environment as metabolite production depends on the plant to which the strain is applied and

the target pathogen that it confronts (Marra *et al*., 2006). It is important to stress, as well, that the secondary metabolites are only produced locally and in very limited quantities, and that strains naturally present in the environment also produce those same compounds. Thus, the relevance of the characterization and quantification of all toxic metabolites must be questioned, and it might not be necessary to spend much effort on this. Nevertheless, when the main mode of action is based on the production of antibiotics, there might be some concern over secondary metabolites with regard to human health and the environment. One must be certain that the use of an MBCA producing antibiotics will not contribute to the induction of multiple resistance to antibiotics used in human or veterinary medicine.

In conclusion, one must be realistic and admit that it is impossible to characterize all of the metabolites produced by an MBCA at the different stages of its life cycle, on all different substrates or plants. So only those present at a quantifiable level in the technical product, and known to be of concern, might be subject to risk assessment. The production of secondary metabolites will also be addressed below as it is one of the most important questions in relation to acceptable residue levels.

8.5 Residues

According to the data requirements for active substances consisting of microorganisms in Regulation (EU) No 545/2011 (Annex, Part B), residues are divided into viable and non-viable. The MBCA itself constitutes the major part of the viable residues, which also include microbial contaminants. In contrast to chemical pesticides, the MBCAs can establish and sometimes proliferate on or in food and feed. The metabolites are non-viable residues which can be produced by the MBCA on or in food and feed.

For the microorganism itself, if the toxicity testing (see below) has led to the conclusion that the MBCA is not of concern for human health, the residual microorganisms should be of no concern either. The only question is whether the microorganism might have multiplied after release and reached a density far above the dose used in the toxicity testing. When a SCAR marker has been developed, it is quite easy to assess the population level of the MBCA in food or feed, but, in the absence of a specific marker, this is almost impossible, because the plant organs will often be colonized by natural populations of organisms belonging to the same species as the MBCA.

More difficult to address is the question of the secondary metabolites that may have been produced and still be present in food or feed. According to EU legislation, full experimental residue data are required if relevant quantities of the microorganism or of produced metabolites, especially toxins, have been found to be persistent. As stated above, it is not realistic to try to characterize all the molecules that are present at the surface of, for example, a fruit. Thus, only the secondary metabolites known to be of concern for human health should be considered, and their level determined if an analytical method can be developed.

A recent example of the complexity of this question concerns the production of 2,3-deepoxy-2,3-didehydrorhizoxin (DDR) by *Ps. chlororaphis*. A strain of *Ps. chlororaphis* has been selected for its biocontrol capacities of several fungal root diseases of wheat. During the assessment for approval, it was established that it produces the toxic compound DDR. This secondary metabolite was isolated, characterized and its mutagenic potential demonstrated. As a consequence, questions arose as to whether the strain should be commercialized. A method of analysis was developed by the applicant and validated by the regulators. In the regulations, the quantification limit for DDR in the fermentation medium is 2 mg l^{-1} and the seed detection limit is 1 µg kg^{-1} (Commission Directive 2004/71/EC; OJEU, 2004). The highest amounts of DDR were detected at the end of the fermentation process, but because DDR decomposes rapidly, no detectable quantities of the metabolite could be found on treated seeds (Hökeberg, 2006). Hence, the risk might have been significant

for producers, but was not for farmers. However, producers would have to consume several litres of the fresh bacterial culture to be harmed. Finally, the decision was taken to allow this strain to be placed on the market, but with the restriction that it has to be used in a closed seed-dressing apparatus (Directive 2004/71/EC).

To conclude with these aspects of risk assessment in relation to residues and presence of secondary metabolites, we must recall that the phytopathogenic microorganisms that we are aiming to control also produce secondary metabolites and toxins, which are tolerated at low levels in feed and food. Considering that an MBCA efficiently counterbalances the target pathogens, we should not be more restrictive on a biological control agent than we are for the pathogenic microorganism it controls. For example, Commission Regulation (EC) No 856/2005 (OJEU, 2005) has set the threshold limits for the *Fusarium* toxin deoxynivalenol (DON) at 1750 μg kg^{-1} of unprocessed durum wheat and 1250 μg kg^{-1} for other unprocessed cereals. Hence, it would not be realistic to define thresholds for a fungal MBCA producing DON as a secondary metabolite that would lead to lower levels of DON than those prescribed for wheat or other cereals.

8.6 Effects on Human Health

As stated in the introduction, it is obvious that an MBCA must not have negative effects on human health. This point is very important in regard to risk assessment and has been discussed among regulators, scientists and industrial partners. There seems to be mutual agreement that regulations must protect human health, but the question is whether all studies required by regulators are needed, and whether the methods, which were initially designed to study the toxicity of chemicals, are adequate for microbials.

First of all it is recognized that microorganisms are very diverse and therefore need to be assessed on a case-by-case basis. However, there are some exceptions for homogeneous groups, for example the baculoviruses, for which OECD has proposed a common approach leading to the listing of the family *Baculoviridae*, or at least of a given species of *Baculovirus*, in Annex I. But as we gain more experience with certain groups of microorganisms, we may expect that a more common approach would be possible. For example, the European Food Safety Authority (EFSA) has produced a statement regarding the QPS (Qualified Presumption of Safety) status for *Bacillus amyloquifasciens* as a food additive and added that this QPS status should apply to *B. amyloquifasciens* strains used in agriculture as a seed treatment.

The EU legislation clearly states that evaluation should be carried out in a tier-wise manner. Tier I includes the collection of all available relevant 'basic information' and 'basic studies' which have to be performed for all microorganisms. Tier II studies are required if tests under Tier I have shown adverse effects.

For Tier I, the debate is whether all the studies that are required by EU directives and performed according to the OECD guidelines have to be performed, or whether waivers (i.e. justifications for non-submission of data) can be accepted based on common characteristics and already acquired knowledge on strains belonging to the same species as the biological control agent under evaluation.

As regards the 'basic information' required in an EU application, it must be said that review of the literature and of medical data banks provides a lot of useful information which is not always correctly used during the evaluation process. It must be clearly stated that humans are regularly exposed to a wide range of naturally occurring microorganisms and that the probability that man has not been exposed to the natural population isolated from the environment and used for biocontrol is very low. Because the medical data banks report all cases of human infections, including those caused by opportunistic microorganisms in immunocompromised patients, the survey of these data banks will give useful information on the infectivity and pathogenicity of a given species. If there is no indication of symptoms linked to this microbial species, there is a high probability that a microorganism belonging to that species is neither infectious nor pathogenic to humans.

The 'basic studies' requirement is the most discussed aspect of the EU regulation. It concerns: (i) sensitization, (ii) acute toxicity/pathogenicity, (iii) infectiveness, and (iv) genotoxicity. Indeed, some regulators will ask for experimental data to satisfy all data requirements recommended by the regulation, while others will accept waivers for some studies. In fact, one of the most critical problems is that the recommended methods which have been set up to study the toxicity of chemicals have not been adapted for the study of microbials (see Wilcks *et al*., Chapter 15, this volume). Why should time- and money-consuming studies be conducted when we know that the results will not be relevant to identifying the hazards and assessing the risks? This is perfectly illustrated by the required sensitization studies. The regulation itself recognized that 'as a consequence of the absence of proper test methods, microorganisms will be labelled as potential sensitizers, unless the applicant wants to demonstrate the non-sensitising potential by submitting data'. Thus, biological control products are given a bad image of sensitization, and might even be excluded from some uses, just because there is no method available to check their sensitization potential; and when an applicant provides experimental results, these will be appreciated differently by different experts because the method is usually not validated for microorganisms. There is an urgent need to develop adapted methodology to assess the sensitizing capacity of biological preparations (see Loprieno, Chapter 19, this volume).

Regarding 'acute toxicity, pathogenicity and infectiveness' Regulation (EU) No 545/2011 lists a series of required studies: acute oral toxicity, acute inhalation toxicity and intraperitoneal single dose, or alternatively subcutaneous route instead of intraperitoneal for microorganisms which only grow or multiply at temperatures lower than 37°C. Moreover, as microorganisms are able to grow and multiply in many different environments, an estimation of the clearance of the microorganisms and/or 'active toxin' at the administration site, in organs and in blood, has to be provided, leading evaluators to ask for difficult and costly studies. The question is whether all these studies are required for risk assessment.

The toxicity tests should take into account the main route of exposure. Considering that inhalation is the most probable route of contamination, an intratracheal acute toxicity, pathogenicity and infectiveness study seems appropriate. There is also a consensus that the oral toxicity test is needed, because ingestion of the active substance enables testing of both the direct pathogenicity/infectivity and the indirect toxicity linked to the presence of potentially toxic secondary metabolites. In our opinion, however, the need to perform the intraperitoneal/subcutaneous test as representing the 'worst case' is questionable because it is not realistic according to the use patterns of the biological control product.

As for sensitization, genotoxicity testing poses methodological problems (see Typas and Kouvelis, Chapter 18, this volume). The first level of test (Ames test) is based on the detection of mutations induced by the pesticide when growing strains of *Salmonella enterica* serotype Typhimurium on a nutrient medium enriched with the active substance. This test cannot be used when the active substance is a living microorganism. The Ames test might be required when secondary metabolites are produced. In that case, the culture filtrate, containing the excreted metabolites, can be used to perform the test. Indeed, Regulation (EU) No 545/2011 clearly states that 'relevant metabolites' must be purified and their toxicity studied as for a chemical. When secondary metabolites are essential for the mode of action of the biocontrol agent, they must be studied per se. This has been the case for the DDR produced by *Ps. chlororaphis*.

An OECD Working Document (OECD, 2008) describes the approach of different regulators and international expert groups to the issue of the genetic toxicity assessment of microbial pesticides. It concludes that existing testing guidelines for chemical pesticides may not be directly applicable to testing microbial products, and specific guidelines for each test system and type of microbial pesticide to be evaluated are needed, modified as necessary to avoid interference by constituents in the test samples. The document

recommends the development of international harmonized guidelines for genotoxicity tests for microbial pesticides and asks for appropriate OECD guidelines.

To summarize the concerns for human health, the actual requirements of EU legislation are not adapted to the evaluation of microbials. The requirements are based on the principle of precaution and on the 'worst case' approach, neglecting the fact that the human species has already been in contact with these naturally occurring microorganisms during evolution. Moreover, the available methods designed for chemicals are not adapted for production of the required data. There is an urgent need to promote research aimed at setting up methods better adapted to the study of microbials, and better in addressing important questions in relation to human health.

8.7 Fate and Behaviour in the Environment

Study of the fate and behaviour of a plant protection product in the environment poses quite different questions depending on whether the product is a chemical or a living microorganism. A chemical product can accumulate with repeated application and its degradation might take time, in particular if it is a synthetic molecule that has not been present in the natural environment. Moreover, metabolites resulting from the degradation might be more toxic than the plant protection product itself. In contrast, microbiological control is based on releasing a naturally occurring microorganism, the aim being to temporarily increase the population density of a microbial strain belonging to a species that usually already exists in the environment. Most microbial species are ubiquitous; therefore the use of the concept of indigenous versus exotic organisms is of limited value in the case of microorganisms (OECD, 2008). The strain selected for developing a microbial pest control product might be better adapted to specific edaphic or climatic conditions than other strains, but in the absence of any specific selection pressure there is no risk of proliferation of the introduced strain, as it is submitted to competition exerted by the native microflora. Nevertheless, there is a fear that an applied microorganism can multiply in the environment and become a pest or plant pathogen. This fear appears to be unjustified by facts. Plant pathogenic bacteria and fungi, having expanded over the world, are pathogenic microorganisms that were favoured by the presence and spread of the crop on which they develop. They had a competitive advantage compared with other microorganisms. In the case of MBCAs, there are no examples of a microorganism having become dominant in the environment where it has been introduced without its host plant. A bacterium or a fungus, originating from a natural environment, for example soil, will not become dominant when reintroduced into the same environment. However, as it is not possible to rule out any hazard, it is necessary to examine the fate and behaviour of MBCAs in the environment.

As a first step, the MBCA should be correctly identified and its biology needs to be studied in laboratory experiments. Beside studying its behaviour under natural environmental conditions, information is required on the background levels of the population in the environment. Finally, a tool enabling tracing of the introduced microorganism needs to be developed in order to distinguish it among other strains belonging to the same species and naturally occurring in the environment.

To trace an introduced strain in a laboratory test, the easiest approach is to use a mutant that is resistant to an antibiotic or a fungicide. For example, Edel-Herman *et al*. (2009) used a UV-irradiated mutant of strain Fo47 of *F. oxysporum* resistant to benomyl to study its population dynamics in two soils of different physicochemical properties over a year. In the disinfected soils, this strain grew and established itself at a high population density regardless of the inoculation dose and soil type. In contrast, in the non-disinfected soil, i.e. in the presence of a native community of microorganisms, the biological control strain was not able to proliferate. It did not disappear, but it became established at a population density lower than that at which it was introduced, i.e. the biological control

organism became part of the native microbial communities. But this approach using antibiotic or fungicide resistant mutants can only be used in a confined environment because it is often not safe to release such mutants into the environment. Moreover, the mutation might have modified the dispersal or survival behaviour of the microorganism.

To address the fate of an introduced microorganism in the environment, the most elegant approach consists of designing a SCAR marker that will enable tracing of the natural organism among other strains of the same species. This approach was used to study the fate of *T. atroviride* strain I-1237 introduced into two soils of different physicochemical properties. Results were analogous to those obtained for *F. oxysporum* Fo47. Indeed, strain I-1237 neither disappeared nor proliferated in the non-disinfected soils. Based on these results and on many others from the literature, we can conclude that a soil-borne microorganism reintroduced into a soil will survive but will not proliferate; it can be expected to become part of the native populations of the same species. It is, therefore, necessary to determine the background level of the native population of a given species before introducing an MBCA of that species into the soil in order to be able to follow the dynamics of the MBCA after introduction and determine how long it takes to return to the initial (background) population level. In most cases, this will take a few months.

In this context, the general EU criterion for excluding active substances showing a half-life in soil of more than 60 days from the low risk status (Regulation (EC) No 1107/2009, Annex II, 5; OJEU, 2009) is absolutely not justified for MBCAs. To be effective, the MBCA must establish in the soil for periods longer than 60 days. Moreover, the methods available to assess the population density of a microorganism in soil are not accurate enough to detect a variation of 50% of the population density. This rule, which was made for chemicals, simply cannot be applied for microbials.

Following a seminar on 'the fate in the environment of microbial control agents and their effects on non-target organisms' (OECD, 2011a), a guidance document for the environmental safety evaluation of microbial biocontrol agents has been developed by J.W.A. Scheepmaker, B. Karaoglan and S. Bär. It deals with environmental fate and behaviour as well as with environmental toxicity, and proposes decision trees. The publication of this document by OECD is planned for the near future.

8.8 Effects on Non-target Organisms

As stated above, since MBCAs are naturally occurring microorganisms, and most of the non-target organisms (NTOs) that they might encounter have already been exposed to them, so hazards towards NTOs are usually very limited. In a paper reviewing studies dealing with non-target effects of fungal BCAs (biological control agents) aimed at controlling root pathogens, Winding *et al*. (2004) stated that when non-target effects have been observed, they were generally small in scale, limited to one growth season and not proven to affect soil health. Moreover, many animals (including mammals, birds, fish and crustaceans) are kept under intensive animal husbandry conditions and their pathogens are carefully surveyed and well established. Thus, many of the possible non-target effects can be identified by a literature survey and the absence of reported hazards – despite the already existing regular exposure of the non-target organisms – would indicate a negligible risk. However, non-target effects must be addressed and specific methods need to be developed. Current OECD test guidelines have been designed to study chemical molecules and are frequently not adapted to study the possible effects of MBCAs on NTOs. The US Environmental Protection Agency (EPA) has chosen a tiered approach to evaluate NTO effects. Dose-response tests are only required if any adverse effects are observed in the Tier I hazard characterization data set.

In the draft for the above-mentioned OECD guidance document on the fate and behaviour and NTO effects, Scheepmaker and co-authors insist on a perfect identification of the MBCA, its host range and its modes of action. This basic knowledge is required to

make an informed choice of which studies must be conducted. The risk assessment should also take into account the period and the mode of application to determine which non-target populations will be exposed to the MBCA. If metabolites/toxins are known to be responsible for the mode of action, and if there is relevant exposure of NTOs, then toxicity data should be available for those substances and a risk assessment should be performed. Of course, the need for studies should also depend on the chemical nature and characteristics of the substances released. For microbials used to control plant diseases, the effect on arthropods is not a great concern, although based on recent problems caused by chemical insecticides to bees, there is some general concern about the possible impacts of MBCAs on bees. If the MBCA is applied indoors, or if it is used outdoors but directly incorporated into the soil, there is no need to worry about toxicity for bees, as there will be no exposure. In contrast, it is obviously important to study toxicity for bees if the MBCA is applied outdoors on aerial parts of crops and other plants, especially at flowering time. In practice though, microorganisms appear to be less toxic for bees than do chemicals, and there is no reference today in the literature showing an adverse effect of microbiological control agents on bees. It is remarkable that bees can even be used as vectors to deliver BCAs such as *Trichoderma* that are used to control *Botrytis cinerea* (Fravel, 2005). The bees deliver conidia of the biocontrol agents to the flowers, thereby conferring protection of the fruit against grey mould (Shafir *et al.*, 2007). Depending on the species of the MBCA, limited-scale toxicity tests such as topical application tests could be needed.

Similarly, data requirements for adverse effects on earthworms seem unnecessary because there is no pathogen of earthworms described in the literature and because earthworms have probably already been exposed to most naturally occurring BCAs. A US guideline (OCSPP 885.4340; US EPA, 1996) outlines a methodology for non-target insect tests in Tier I, although it does not include tests for soil insects. Furthermore, there is a requirement that the potential for negative effects on any beneficial arthropods used in Integrated Pest Management (IPM) shall be considered.

In the EU, tests to determine possible phytotoxicity or pathogenicity to plants of microbial substances are not required. Only observations of possible adverse effects on plants in efficacy trials in the field have to be made; if symptoms are observed, phytotoxicity trials with the plant protection product would be triggered. In the USA, effects on plants have to be assessed if the active microorganism is closely related to known plant pathogens.

For effects on soil microorganisms, the US EPA does not support testing of MBCAs, as it considers that the soil microflora is very resilient and that the relative risk from adding microorganisms to soil microbial communities is minimal. These soil microbial communities play very important roles in the ecosystem, but the soil microbiota is characterized by a high level of redundancy of the functions. Thus, the functional characteristics of component species are as important as the number of species for maintenance of essential processes, such as nitrogen or carbon cycling. In the EU, the regulation asks for consideration of the effect of MBCAs on carbon and nitrogen mineralization. Modern technology helps us to study the non-target effects on soil microorganisms. The use of molecular tools now enables tracing of the presence of genes encoding for important functions, and these have shown that release of a small quantity – in relation to the total microbial biomass – of a BCA did not modify soil functions (Sessitsch *et al.*, 2002).

Several methods based on direct extraction of DNA from the soil (denaturing gradient gel electrophoresis, DGGE; temperature gradient gel electrophoresis, TGGE; etc.) enable global assessment of the impact of the introduction of a biocontrol agent on the structure of microbial communities. For example, the T-RFLP (terminal restriction fragment length polymorphism) technique based on variations in the 16S and 18S rRNA genes was used to assess the impact of the strain I-1237 of *T. atroviride* on the structure of bacterial and fungal communities (Cordier and Alabouvette, 2009). Introduction of strain I-1237 in soil resulted in a significant modification of the

fungal community structure 3 days post inoculation. This difference remained significant up to 3 months after infestation, although at the end of the experiment (9 months), the fungal community structures were not significantly different in the inoculated and the control soils. Regarding the structure of the bacterial communities, the introduction of the MBCA induced a shift 3 days after inoculation, though after 7 days and later, the structure of the bacterial communities was similar in the inoculated and control soils.

The same approach was followed by Edel-Herman *et al*. (2009) to assess the non-target effect of the introduction of the biocontrol strain *F. oxysporum* Fo47 in soil. Again, results showed that even when an impact was detected shortly after the introduction of the BCA, the structure of the microbial communities tended to rebound quickly to their initial stage; after a few weeks, there were no differences between the infested soil and the non-infested control. These two examples are interesting because they show that the introduction of the MBCA into the soil is followed by an immediate impact, which is expected to provide the desired biocontrol effect. However, after a few weeks, this impact is no longer significant, indicating that there is no permanent effect of the MBCA on the resident communities of soil microorganisms. Moreover, studies using the same types of methods have shown that traditional agricultural practices have much more impact on soil microbiota and soil functions than the release of a BCA. This is especially prominent in the case of a manure or compost amendment that releases millions of microorganisms (Pérez-Piquerez *et al*., 2006).

8.9 Conclusion

This review aimed to list the key hazards presented by microorganisms used as BCAs of plant diseases and to describe the methods used to characterize these hazards and assess the corresponding risks. The most important risk connected with the release of an MBCA is the potential pathogenicity to humans, and everybody agrees that the risk has to be eliminated by accurate studies. The first step in developing an MBCA is therefore to correctly identify the strain at the species level and to compare it with microorganisms that are pathogenic for humans, animals or plants. Searches in databases will indicate whether other strains belonging to the same species have already been involved in human, animal or plant infections. If the species is not among those of concern, we can reasonably expect to be on the safe side, and only limited studies should be needed to satisfy the regulatory requirements. If the strain belongs to a species that includes infectious or pathogenic strains, it is necessary to rigorously demonstrate that the selected strain belongs to a clade that does not include pathogenic strains, perhaps by including determination of the presence or absence of specific virulence genes (see Wassenaar and Alter, Chapter 13, this volume). Thus, from a microbiologist's point of view, the scene is quite clear; the problem is that for the registration of an MBCA, the petitioner must fulfil requirements that have been set up for chemical pesticides. So even when the strain belongs to a non-infectious and non-pathogenic species, all toxicity studies are required according to the OECD test guidelines. In the case of environmental safety, we face the same situation; studies on fate in the environment and non-target effects are required, even when the MBCA is a strain that naturally occurs in the environment into which it will be reintroduced. For a chemical pesticide, the metabolism of the molecule and the toxicity of the degradation products must be determined. The approach to studying the fate of an MBCA in the environment needs a totally different approach, and presently there are no methods well adapted for this purpose. Currently available technology proposes some techniques, but the real problem is sampling: how many samples should be taken, and where should they be taken to adequately follow the dissemination of an MBCA in the environment? We are facing the same type of methodological problems when studying the impact of MBCAs on NTOs. There is a need to develop methods to study the fate and non-target effects of BCAs in the environment. But, as already mentioned,

because the MBCAs are naturally occurring microorganisms, one should not overestimate the risks for the environment. We can hope that as more MBCAs are registered, the regulatory authorities will gain a better understanding of the specificity of BCAs and will lessen the requirements. One approach will be to compare the new strains with strains already registered. If a newly selected strain is recognized as being closely related to strains already on the market without any concern, it would be acceptable to take into account knowledge generated for registration of the previous strain for evaluation of the new strain. For beneficial microorganisms used in the food and feed chain, EFSA has created a list of species that have been granted the status of QPS. We can expect such a list to be created for MBCAs when more strains have been evaluated. The question remains: which criteria would be acceptable for bridging between strains? This is still an open question, but demonstrating phylogenetic proximity between strains and similarity of the modes of action appears to be a reasonable basis as a start.

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9 Safety and Regulation of Microbial Control of Weeds

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9.1 Introduction

Microbial control of weeds is based almost exclusively on the use of fungi as biological control agents. Plant viruses are invariably difficult to manipulate, as most rely on other (unmanageable) biotic factors – mainly arthropod vectors – for successful transmission, while plant pathogenic bacteria are similarly problematic in terms of their delivery and infection mechanisms. Hence, there are few examples of control involving bacterial and viral agents.

There are two distinct approaches to the microbial control of weeds – classical and inundative – each with very different safety and regulatory issues to consider. Classical biological control (CBC) is directed at introduced alien plant species (neophytes) and involves the selection and movement of highly specialized or coevolved natural enemies from the centre of origin or native range of the target species into the exotic range where it has become weedy and problematic. The agent – invariably, a biotrophic fungal pathogen – is released, following approval based on a pest risk assessment, into the exotic ecosystem and it disperses naturally from predetermined inoculum sites, to establish and build up over time to epidemic (epiphytotic) proportions. The pathogen, once introduced in this way, is

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literally, therefore, a free agent and becomes a permanent part of the ecosystem. In principle, it cannot be eradicated or even controlled, if the need should ever arise. Thus, this strategy is entirely in the public domain – because all parts of society, as well as ecosystems, can be affected – particularly because public funds are most often involved. In sharp contrast, inundative biological control (IBC) typically exploits indigenous plant pathogens targeted at locally important native or naturalized weeds. These pathogens are readily cultured (necrotrophic) fungi, mass produced and formulated as bioherbicides under a patent, and funded from the private rather than the public sector. In this chapter, we compare the safety and regulatory issues between these two contrasting approaches to the microbial control of weeds.

9.2 Classical Biological Control (CBC)

9.2.1 Why do we need CBC?

Humankind has been moving plants and animals around the world since the dawn of agriculture, some 10,000 years ago. However, it is only in recent times – as trade and globalization have exploded – that the wholesale exchange and movement of exotic species has taken off and has now moved into overdrive: we are now entering the Homogocene era, loosely defined as the human-mediated or anthropogenic replacement of native biotas by non-native or alien species (Rosenzweig, 2001; Olden *et al*., 2004). Some of these species – introduced either deliberately or accidentally – have become problematic pests and a threat to both agriculture and biodiversity (Mooney and Hobbs, 2000). In fact, after habitat destruction by humans, invasive alien species (IAS), and exotic plant species, in particular, have been considered to pose the greatest threat to the world's natural ecosystems (Mack *et al*., 2000; Mack and Lonsdale, 2002; Carey, 2002), particularly in small-island systems (Trueman *et al*., 2010). Because these weedy plants can cover vast tracts of land, control by conventional means is logistically challenging, often prohibitively expensive and unsustainable. Moreover, in many cases, especially with aquatic and riparian weeds, environmental constraints usually preclude the use of chemical herbicides.

Frequently, the only choice considered for invasive alien weeds, once eradication has failed, has been that of containment, to try to reduce their impact on agricultural production, on the indigenous flora and fauna, and on society in general. Obviously, most countries, and certainly those in the developing world, do not have the foresight or political will, nor the infrastructure and resources to even attempt containment, especially of environmental weeds with no obvious economic impact; they prefer to ignore the problem of IAS or hope that it will go away. If this philosophy were followed to its logical conclusion, we would be well on the way to the predicted homogenization or McDonaldization of the world's flora (Lövei, 1997; see Ritzer, 1998 for the general concepts of the latter terminology).

Nevertheless, there is the alternative management strategy of CBC, which presents a possibility to avert, or at least to temper, domination by invasive alien plant species. Potentially, CBC offers a cost-effective, environmentally benign and sustainable solution to the problem of invasive alien weeds; either functioning in isolation – as a one-off, 'silver-bullet' remedy – or integrated into a multi-component management plan. Some programmes have been spectacular successes and others have not, or have even been considered to have had negative impacts. Fortunately, the latter examples have been in the minority and all were entirely predictable (Evans, 2000). None of the purportedly failed CBC programmes have been analysed scientifically though, and until recently, the obvious question was never asked: what would the situation have been without any biological control? This was addressed by Hoffmann and Moran (2008), and their study revealed the 'subtle but very real benefits that accrue from otherwise seemingly ineffective agents'.

9.2.2 How does it work?

It has long been recognized, but only relatively recently crystallized into a hypothesis – the 'enemy release hypothesis' (Keane and

Crawley, 2002) – that plants, once released from their natural enemies, show a dramatic increase in vigour and fecundity. In plants with certain traits, such as the ability to colonize disturbed habitats, this increased fitness gives them a competitive edge over the native flora, allowing them to become weedy and invasive. The concept of CBC is simple: to address this imbalance by reducing the fitness through the release of natural enemies from the native range or centre of origin of the alien weed target. Evidence for this hypothesis was soon forthcoming from a study of invasive neophytes in the USA which showed that they had significantly fewer biotrophic (coevolved) plant pathogens than in their native ranges (Mitchell and Power, 2003).

Obviously, ecosystem functioning is never so simplistic and, clearly, interlinking multi-trophic factors are involved in plant invasiveness, such as resource availability (Blumenthal, 2006). Another, recently recognized component can also be factored into the invasiveness equation – microbial endophytes. In the 'endophyte-enemy release hypothesis' (Evans, 2008), it is postulated that alien plants become invasive not only because they leave their natural enemies behind, but also that they arrive without, or with only a part of, their coevolved endophytes. Some of these endophytes are known to protect the plant host from both abiotic and biotic pressures, including natural enemies, but resources need to be allocated for this protection. So these neophytes have a double gain in fitness: no natural-enemy pressure; no 'bodyguards' to pay off. However, if a coevolved natural enemy were to be introduced into the system, the plant would be highly vulnerable to attack – not only because it lacked protective endophytes but also because it occurred in vigorous monocultures – the perfect setting for a natural-enemy population explosion. This could explain the phenomenon of the 'silver bullet', in which the introduction of a single natural enemy into the system can bring about the sudden and terminal decline of weed invasions. There are many such examples in the entomological literature of spectacular control of seemingly intractable invasive alien weeds following the release of insect natural enemies (Dodd, 1940; McFadyen, 1998; Syrett *et al*., 2000; van Wilgen *et al*., 2004; Page and Lacey, 2006), as well as of the control of exotic arthropod pests using parasitoids (Herren and Neuenschwander, 1991; Bokonou-Ganta *et al*., 2002), because the historical records for this type of control go back much further than the use of microbial agents. In recent times, though, similar successes have also been achieved with coevolved fungal pathogens of invasive weeds (Morin *et al*., 1996; Evans, 2002a; Barton *et al*., 2007).

Finally, in answering the question – 'How does it work?' – we only need to look at the historical records of plant-disease epiphytotics when coevolved pathogens eventually catch up with their crop hosts growing in exotic monocultures, with catastrophic socio-economic results: potato blight, coffee rust, wheat rust, rubber blight, cacao diseases (Large, 1940; Quimby, 1982; Evans, 2002b; Agrios, 2005).

9.2.3 How safe is it?

In other words: how risky is biological control? This question was posed by Simberloff and Stiling (1996a) specifically in relation to the general principles and practice of CBC, which they regarded as inherently risky, as well as the agents of CBC, which they assumed to be 'guilty until proven innocent'. Thus, we live in an ever-increasingly safety-conscious or risk-averse world, and there are sound arguments both for and against this escalating trend. Yet in the latter case, we could reach a situation where nobody will take a decision if there are any perceived risks, however small, that could rebound politically or financially on the decision makers or on their organizations. Unfortunately, with CBC, there will always be risks because it is a natural system and nature is never 100% predictable (Evans, 1998). If the precautionary principle were to be followed to the letter – as set out in the 1992 Rio Declaration on Environment and Development (Sheppard *et al*., 2003) – CBC would be deemed to be unacceptably risky. Nevertheless, a comparative risk analysis shows that the probabilities of CBC agents

becoming pests themselves is negligible compared with the number of other exotic pests (Lonsdale *et al*., 2001). In order to address this potential impasse and to provide a scientific platform for decision makers, existing standardized procedures – originally devised for quarantine pests to assess pathways of their entry into a country and the associated risks posed – have more recently been applied to CBC agents. We will return to this theme later, as such a pest risk assessment (PRA) is usually only carried out once the CBC programme has been approved, funded and is ready for implementation. Notwithstanding, there are many other, predominantly non-scientific, obstacles to surmount – especially with microbial as opposed to arthropod agents – before a weed CBC programme can even be considered for funding. Often, a climate of fear prevails in the minds of the public in general, and of administrators in particular, when the word pathogen is used; this unease is well encapsulated in the term 'pathophobia', as applied to weed biocontrol (Freeman and Charudattan, 1985). CBC with microbials is, therefore, often viewed as biological or germ warfare, with all the risks and melodrama that this terminology invokes, or it is even equated with biblical plagues (Goettel *et al*., 2001).

Similarly, public perception of biological control has been strongly affected by reports in the mass media, especially the so-called 'investigative' documentaries; here the philosophy is that the only good news is bad news. Hence, the focus has been on horror stories depicting 'biocontrol' disasters, such as the cane toad, with no attempt to balance the picture by showing safe and successful, scientifically underpinned introductions. Invariably, the first question posed after giving a presentation on CBC of weeds, to both scientific and non-specialist audiences alike, is: 'What will the agent attack/feed on once it has controlled/eliminated its target?'. The standard answer follows the theme that the cane-toad story (one could substitute here rat snake, Indian mongoose, predatory snail, myxoma virus) has little or no scientific basis and that all these so-called 'biocontrol agents' are polyphagous opportunists that may well have huge and permanent impacts on vulnerable ecosystems. In the case of the myxoma virus, in what has been termed neoclassical biological control (Lockwood, 1993; Simberloff and Stiling, 1996b), or the new encounter hypothesis (Hokkanen and Pimentel, 1984), it is even more bizarre because the pathogen that was targeted at a European rabbit in Australia came from a South American fox! Sadly, the long-term benefits from such new-encounter introductions are negligible, or even negative, because after the initial epidemics, resistant hosts will bounce back, with the non-specialist pathogen being unable to respond.

Thus, in order to allay any ongoing public doubts concerning the microbial CBC of weeds – typically driven by fungal biotrophs such as rust fungi – it is necessary to focus on the highly specialized nature of such plant–pathogen associations and to show, for example, that wheat rust has been around since the beginning of agriculture and has moved with its host around the world, but has never 'shifted' on to other plant species, even close relatives. This centres on the public ignorance of microbial genetics that envisages spontaneous mutations that will allow the agent to attack non-targets (Barton, 2004). Unfortunately, this ignorance also extends to the scientific press (*New Scientist*, 1999; also see Evans, 2000, for further examples of biocontrol 'bloomers' from this journal). Moreover, unlike the myxoma virus, the rust retains the ability to overcome whatever resistance the host develops (naturally or through breeding) because of this intimate (coevolved) association.

In conclusion, the central philosophy and keyword of CBC *sensu stricto*, as opposed to the so-called biological control examples cited above, is coevolution and host specialization: the selected agent forms part of the suite of the natural enemies from the centre of origin of the target IAS, has coevolved with that target host and is restricted to it, or to its near relatives. Consequently, as we will show, the critical, and by far the most costly part of any CBC programme, is host-specificity screening as a basis for the PRA. This is rigidly adhered to with microbials for CBC of weeds, but contrasts with the more laissez-faire approach for microbial CBC of arthropod pests and plant

diseases because, up until recently (Hajek and Delalibera, 2010; Kohl *et al*., 2011), there were no risk assessments and these agents were considered to be inherently safer when used for CBC, often unknowingly, as they were freely exchanged between collaborating scientists in different countries and continents without consideration of quarantine issues (Waage, 1997).

9.2.4 How is risk reduced?

As emphasized previously, because weed biocontrol practitioners have adopted a strictly scientific approach founded on host-specificity screening, the track record for CBC of weeds using microbials has been free of controversy so far; although only a relatively small number of agents (26 species of fungi) have been released since the first introductions were made in the 1970s (Barton, 2004). In contrast, the release of invertebrate agents has sometimes been problematic. Perhaps this should not be surprising because it is of much longer standing, going back to the late 19th century; over that period – depending on the interpretation – around 400 (McEvoy and Coombs, 1999; Barton, 2004), to 600 (Marohasy, 1996), to 900 (Julien and Griffiths, 1998) introductions have been made. There has been a gradual refining of host-specificity screening and, although the methodologies are very different between testing arthropod herbivores and microbial pathogens, weed pathologists have adapted many of the protocols developed by entomologists over the past century. This has since morphed into a Code of Conduct for the Import and Release of Exotic Biological Control Agents (FAO, 1996, 1997). It has also served as a model for a series of subsequent follow-up publications on the safe use of biological control (EPPO 1999, 2000; IPPC, 2005; OEPP/EPPO, 2010); see also Sheppard *et al*. (2006) for a more Eurocentric view. The Code aims to facilitate the safe export, import and release of CBC agents by detailing the procedures involved and, of course, it is especially targeted at countries where legislation to regulate them is lacking or considered to be inadequate. In effect, it is meant to minimize health and environmental impacts and ensure that any benefits derived from such introductions are not offset by 'significant adverse effects'. This latter statement is interesting because it implies that some degree of non-target impact or collateral damage is acceptable. Ironically, this has yet to be tested in Europe for CBC microbial agents of weeds (Vurro and Evans, 2008).

As CBC of weeds using microbial agents is of recent origin, host-specificity screening is still somewhat on a learning curve. However, thanks to the centrifugal phylogenetic testing protocol developed primarily for arthropod agents (Wapshere, 1974a,b) – which is based on genetic relatedness rather than the earlier approaches that focused on the threat to economic plants – most of the potential microbial agents (all biotrophic or hemibiotrophic fungi) have undergone extensive screening. Nevertheless, the pioneering and still among the most successful weed CBC programmes involving fungal pathogens – the skeleton weed rust (*Puccinia chondrillinae*) in Australia (Cullen *et al*., 1973; Burdon *et al*., 1981), and white smut of mistflower (*Entyloma ageratinae*) in Hawaii (Trujillo, 1985; Davis *et al*., 1992) – were undertaken before this protocol was developed and would probably not have been approved today; certainly not in the case of the latter introduction, because relatively few plant species were screened and the taxonomy of the agent was erroneous. Despite these shortcomings, there were no non-target impacts and the success of this fungal agent has since been repeated in both South Africa and New Zealand, once the true identity of the fungus had been established (Barreto and Evans, 1988), and after more rigorous host-range screening (Morris, 1991; Barton *et al*., 2007). The centrifugal phylogenetic protocol was later claimed to be too rigorous by its original author (Wapshere, 1989), who argued that it could lead to the rejection of potentially beneficial agents.

There are many examples of artificially extended host ranges during specificity screening in the laboratory, controlled environment room or greenhouse: for instance, insects feeding on non-hosts in no-choice tests in which, literally, the insects face a life or death situation. Another example is fungi

sporulating on 'new' hosts, where the fungal pathogens are given every opportunity to overcome plant resistance because of the use of unrealistically high inoculum loads, optimum infection conditions (including the use of sophisticated dew-simulation chambers) and 'vulnerable' greenhouse-grown plants. The question is one of interpreting these results – typically, with pathogens, for example, sporulating structures may be effete or sporulation density can often be significantly lower than on the control target weed – and, also, of refining or modifying the experimental procedures to satisfy the stakeholders. None the less, some still consider that it will always be a high-stakes game to successfully implement a CBC programme without collateral damage to non-target plants or more cryptic environmental impacts (McClay and Balciunas, 2005).

9.2.5 How is it promulgated and regulated?

As is obvious from the previous sections, the history of CBC of weeds has been remarkably successful and virtually trouble free as a result almost entirely of self-regulation by the biocontrol practitioners themselves (Marohasy, 1996; McFadyen, 1998; Barton, 2004). Thanks to regular international meetings, weed biocontrol entomologists and pathologists can exchange ideas and refine the science, leading to even greater degrees of safety. Because these scientists are invariably involved in non-profit or non-commercial public-funded programmes, there is a deep commitment to the ethos and science of biological control and to doing it right. Any mistakes, of course, will not only reflect on the individual scientists involved, and their organizations, but also on the whole theory and practice of biological control. There are also many critics on the sidelines waiting to feed on any crumbs of controversy that fall from the biological control table. Thus, it is a safety-first approach, as well as a pragmatic one – both financially and ecologically. For example, Berner and Bruckart (2005) outline the processes in a 'decision tree' for deciding whether to continue or abandon a CBC weed programme in the USA, based on an impact assessment of the potential pathogens. In another example, Barratt and Moeed (2005) describe the policy and practice of handling CBC programmes in New Zealand, where any introduction is regulated under the Hazardous Substances and New Organisms Act 1996 and is peer reviewed by the Environmental Risk Management Authority (ERMA). The enabling legislation focuses on health and safety and the environment, and 'provides a framework for assessment and approval to import, develop, field test, conditionally release or release' a range of products and organisms, including microbes (Barratt and Moeed, 2005).

Typically, CBC weed projects are funded in stages and, if no suitable natural enemies are unearthed in the literature and during the field-survey stage, then the relevant data and conclusions are transmitted to the sponsor and the programme is shelved – saving both time and money. Some potential CBC projects involving alien weeds, however, never even make it to this stage, as for example, the highly invasive *Rhododendron ponticum* when it was targeted for biological control in the British Isles to supplement more conventional management methods (Fig. 9.1). A precursory examination of the botanical records revealed that over 500 species of the genus have been introduced for horticultural purposes into the UK alone, many of which have been hybridized with *R. ponticum* (Mabberley, 1997). This led to the obvious (self-regulated) conclusion that CBC was dead in the water before even being launched, on logistical but primarily on safety grounds, much to the relief of the assembled horticulturalists when this decision was presented at a conference (Evans, 2003). Biological control through the IBC approach does though still remain an option for this weed (see Section 9.3.5, Europe).

Nevertheless, many potential conflicts of interest are not resolved so easily and can permanently or temporarily halt a weed CBC programme. Amazingly, for even the most invasive and environmentally damaging of invasive alien weeds, there are always supporters with economic, ecological or even emotional concerns about the risks and perceived impacts of a CBC programme, not only on the environment, but also on the target weed itself. More often than not, these are

Fig. 9.1. The rhododendron weed, *Rhododendron ponticum:* (A) the plant in part of its native range in the Algarve mountain range of Portugal (H.C. Evans); (B) the plant in its invasive range in the UK, showing the heavily infested eastern slopes of Lundy Island (Bristol Channel), where the species has formed dense, impenetrable thickets (note arrow on figure) and is threatening native flora and fauna (S. Evans); (C) applying mycelial suspensions of the potential IBC agent *Chondrostereum purpureum* (see Sections 9.2.5 and 9.3.5 (Europe)) to newly cut stumps to prevent resprouting in an area in Cornwall (SW England) where this weed is an important inoculum source of newly emerging *Phytophthora* diseases threatening native trees (S. Thomas); (D) fruiting bodies of the basidiomycete *Chondrostereum purpureum* colonizing an inoculated birch stump in Finland (M.K. Seier). This is part of a programme to control birch in selected woodland sites, such as under power lines. In the UK, the Department for Environment, Food and Rural Affairs (Defra) is funding a programme to investigate the potential of local strains of *C. purpureum* for the management of rhododendron, especially where it poses a threat to the indigenous flora in its role as an alternative host of newly emerging diseases caused by *Phytophthora ramorum* and *Phytophthora kernoviae* in native trees. Risk assessments are based on pre-existing model systems in the Netherlands (see Section 9.3.5 (Europe)).

based on spurious arguments, because no CBC agent will eliminate its host – this goes against the theory of coevolution – and, therefore, minor economic investments can still 'reap the benefits' of an invasive alien plant; this is a point identified clearly by an NGO (non-governmental organisation) in East Africa working with local cooperatives to produce cane furniture from water hyacinth. This laudable cottage industry was merely scratching the surface of the immense weed infestations threatening the biological integrity of the aquatic ecosystems of the region, and it was concluded that biological control was the only way forward. This was founded on a sound socio-economic and scientific assessment, rather than an emotional reaction to CBC centred on hearsay. The same plant in India is claimed to be an answer to the energy crisis, because harvesting and processing it could provide a source of biogas. Certainly, water hyacinth is plentiful – blocking many waterways – but, can a sustainable industry be built on it? The plant would need to be 'farmed' and the environmental damage would only be compounded.

More bizarre examples can be quoted: web groups freely exchanging seeds of their favourite flowering plant (= invasive alien weed) despite legislation specifying that the species should not be cultivated. But, perhaps the most disturbing one is that of *Chromolaena*

odorata, most commonly known as Siam weed – among the many, often evocative, alternative names, such as triffid weed – even though it is a species native to the neotropics, where promising fungal natural enemies have been identified (Evans, 1987, 1995; Barreto and Evans, 1994). This plant was trialled in West Africa as a potential green manure in the 1960s to 1970s, based on conflicting Asian experiences (Holm *et al*., 1977), but it escaped to infest huge areas of deforested land: its allelopathic properties enable it to dominate and eliminate any indigenous plant regrowth. An attempt to initiate a UN-funded CBC programme against *C. odorata* was thwarted by claims of the plant's multiple local benefits, from having a key role in slash–burn agriculture to the embalming of bodies (Moore, 2001). That the process of slash–burn should officially still be encouraged in West Africa, epitomizes the disturbing aspects of what is an ecological disaster story of an invasive alien weed that has helped to suppress and eliminate natural ecosystems, and which is there to stay, and of a plant species that will be accepted by future generations as an integral part of the flora. As one committed and outspoken biocontrol practitioner said, with deep sarcasm, when learning of the innumerable 'benefits' of the plant at an international meeting in West Africa: 'When I return to Australia, I will convey this good news to the farmers'. Australia has funded a long-standing CBC programme in New Guinea to reduce the chances of the weed arriving (McFadyen, 1989), and then embarked on an ambitious containment campaign after its subsequent discovery in Queensland (Waterhouse, 1994), which is still ongoing (Mack and Lonsdale, 2002).

The first and, until recently, the most infamous and costly conflict of interest in weed CBC was in Australia and led directly to the passing through parliament of the Australian Biological Control Act in 1984 (Cullen and Delfosse, 1985). According to Sheppard *et al*. (2003), this still remains the only country with biological control legislation. Before this Act was passed, CBC introductions could be prevented by minority groups because of the rule of law principle. In this case, the opposition came from beekeepers who viewed the target weed (*Echium plantagineum*) as a saviour (salvation Jane), in sharp contrast with the farmer's opinion (Paterson's curse). An economic risk assessment revealed that there was no contest, as the negative impact of the weed on rangeland agriculture far outweighed the value of honey production. However, the legal fees, out-of-court settlements and delays were a serious drain on the resources of the programme. The net benefits of this successful CBC programme using arthropod agents have recently been put at AU$1200 million (McFadyen, 2008): a substantial amount of honey in any beekeeper's books! An even costlier and longer legal wrangle has been going on more recently in the USA, involving CBC of salt cedars (*Tamarisk* spp.), a complex of highly invasive riparian weeds originating in Eurasia. From the beginning, the CBC programme, which has now been running for decades, came up against ornithologists – a powerful lobby group in the USA (Hunter *et al*., 1988); they argued the case that the introduction of CBC agents might threaten the habitat of birds, especially the endangered willow flycatcher, whose populations had increased because the dense weed stands favoured their nesting behaviour. The long-standing soap opera seemed to have come to an equable end for the CBC scientists with the proposed release of new and highly promising insect biocontrol agents from Kazakhstan (Mityaer *et al*., 2008). But there may be a sting in the tail – and the final nail in the CBC coffin – as, apparently, the release programme is now subject to a court injunction (Chew, 2009). Unfortunately, this has been at great cost to the American taxpayer, as well as a waste of untold scientist years.

Such conflicts of interest have not occurred in Europe, simply because no microbial CBC agents have been released or even considered for introduction as part of a weed CBC programme. Indeed, permission to release the first insect CBC agent – the psyllid, *Aphalara itadori*, against *Fallopia japonica* (Japanese knotweed) – has only just been granted in the UK, following both parliamentary and public lobbying, which attracted worldwide media interest (Shaw *et al*., 2009). There is an equally, if not more promising

fungal agent waiting in the wings (Djeddour *et al*., 2008; Kurose *et al*., 2009), but the philosophy is 'one cautious step at a time' before springing on the regulatory authorities and the public, in general, the prospects of releasing an alien pathogen into the UK (Fig. 9.2). Sheppard *et al*. (2006) in their review of European 'opportunities, regulations and other barriers to adoption' of CBC, noted that for plant pathogens, their use has been hindered because they come under the same EU (European Union) Directive as chemicals: a totally inappropriate situation (see also Seier, 2005). This regulation, Council Directive 91/414/EEC (OJEC, 1991), treats microbial CBC agents as 'plant protection products' laying out a well-defined procedure for their registration which is both lengthy and costly

Fig. 9.2. Japanese knotweed, *Fallopia japonica*: (A) survey for natural enemies on volcanic laval flows in Kyushu Island, Japan (H.C. Evans); (B) knotweed shoots pushing through and destroying road infrastructure in South Wales (UK), an adaptive trait from its native range (H.C. Evans); (C) damage to and defoliation of knotweed near Nagasaki, Japan, caused by the ascomycete fungus *Mycosphaerella polygoni-cuspidati* (D. Djeddour), with a close-up (D) of leaf symptoms, showing the herbicidal-like symptoms (H.C. Evans). This pathogen is significantly more common and highly damaging in Japan than the psyllid agent (see Sections 9.2.5 and 9.4), and shows great potential as a CBC agent, both as a conventional inoculative introduction for infested natural ecosystems, and as an inundative application for urban situations. The mycelium is readily cultured and highly infective. The UK Government (Defra, Department for Environment, Food and Rural Affairs) has released additional funding towards completing the risk assessment studies. It is anticipated, however, that there will be considerable regulatory hurdles to overcome because of the uniqueness of the pathogen (an ascomycete rather than the 'traditional' CBC choice of an obligate rust species) and the potential of exploiting it as an introduced bioherbicide.

and based on data sets that have no relevance to CBC. Discussions are currently underway at the EU level on how this *status quo* could be simplified, aided by the outcomes of the policy support action REBECA (Regulation for Biological Control Agents, 2006–2007) (Ehlers, 2008, 2011). However, how exactly the regulatory process for microbial CBC agents will be handled by the EU remains uncertain until a test case is made.

9.2.6 CBC in action: case studies of problematic safety and regulatory issues

The examples selected in this section of in-house case studies show how the PRA is constructed and the problems encountered during weed CBC programmes involving potential microbial agents, and how these are resolved: either successfully – in which case the programme concludes with the approval of the PRA by the receiving country and the introduction of the agent, or, unsuccessfully – in which case the programme is terminated or suspended pending further data.

Rubber-vine weed

The rubber-vine weed, *Cryptostegia grandiflora* (*Asclepiadaceae*), is an endemic Madagascan woody vine that has been described as the biggest threat to natural ecosystems in tropical Australia (McFadyen and Harvey, 1990). It also became a major problem for graziers in northern Queensland where it competed with indigenous grasses, blocked rivers and denied cattle access to water by forming impenetrable barriers growing up to 30 m over the upper storey eucalypts. During surveys in Madagascar, the damaging leaf rust – *Maravalia cryptostegiae*, closely related to coffee rust – was found over the disparate western range of the plant (Evans, 1993). A strain of the rust from the north-west region was screened in negative-pressure quarantine facilities in the UK against a range of plant species (>70) selected by the Queensland authorities to reflect not only Australian and Madagascan asclepiads – in accordance with the centrifugal phylogenetic protocol (Wapshere, 1974a,b) – but also regional tropical crops and anything related to coffee. In addition to standard visual and stereomicroscope observations of symptoms, it was also decided to include additional scanning electron microsope (SEM and light microscope examinations to determine the behaviour of the rust, both on and within the leaf. The latter involved a clear-staining technique (Bruzzese and Hasan, 1983), which was not a normal part of the requested PRA but was included to obtain a fuller picture of plant–pathogen interactions in hosts and non-hosts: in other words, a value-added approach of scientific inquiry and rigorousness that tends to separate CBC of weeds from most other forms of biological control. Moreover, CBC screening offers an outstanding opportunity to follow plant–pathogen interactions in a highly disparate range of species: an area that normally receives scant attention in plant pathology (Heath, 1974, 2001). Interestingly, five different resistance mechanisms – including two novel ones – were identified occurring on and within the non-host leaves (Evans and Tomley, 1994).

The selected rust strain defoliated the target weed and also sporulated on a closely related Madagascan genus, which did not elicit undue concern. However, towards the end of the 4 year screening period, a newly described asclepiad species in a closely related genus was received from Queensland and proved to be susceptible to the rust strain, albeit with limited rather than full sporulation, and with restricted internal mycelium development. All these host-range results, together with data on infection parameters and impacts (Evans and Fleureau, 1993), as well as taxonomic and hypothetical life cycle details (Evans, 1993), were included in the final report, which included an official PRA sent to the Australian Quarantine Inspection Service (AQIS) for permission to import the agent. AQIS is also tasked with sending the PRA to all Australian states for further scientific evaluation and amendments. Fearing the worst, a unique experiment was set up using a pollution-monitoring wind tunnel to better simulate natural infection in the field, especially inoculum loads (see Section 9.2.4 on these artificial parameters). Rusted plants were placed at one end of the tunnel in front

of generated wind currents and selected 'trap' species were placed at the other. Fortunately, neither the Australian nor the Madagascan asclepiad 'hosts' showed infection, while sporulation occurred on the target weed: although at a lower intensity than in the previous tests (Evans and Tomley, 1996). These results seemed to justify long-standing concerns that some agents could be rejected as unsafe because of the artificial pressure of the testing protocol and the overzealous interpretation of the results (Bruckart *et al.*, 1985; Wapshere, 1989; Weidemann, 1991).

In fact, before these extra data were generated, the Australian authorities had already approved the introduction of the rust on the grounds that: rubber-vine weed was on a rapidly advancing invasive front (covering 40,000 km^2) in Queensland, and was posing a threat to the biodiversity of Northern Territory and its prestigious national parks; therefore, the danger of doing nothing far outweighed the risk of rust attack on a rare plant in a fragile ecosystem, which was geographically and climatically isolated from the rubber-vine infested areas. The rust – certified to be pure and free of contaminants (hyperparasites) – was despatched (as a 'beneficial pest') to Australia and passed through several generations in quarantine before being released into experimental plots containing selected Madagascan and Australian asclepiads, including the new endemic species, as well as *Cryptostegia* spp. The wind-tunnel experiment was vindicated because none of the other asclepiad genera were infected, although the rust sporulated only intermittently on the target weed, but consistently and heavily on *C. madagascariensis*, a species that, together with *C. grandiflora*, was found to form part of a rubber-vine complex in Madagascar (Marohasy and Forster, 1991). At the time of collection of rust strains, it was assumed that there was only one variable species of the host genus in Madagascar. Unfortunately, the selected strain from northern Madagascar was from *C. madagascariensis*, whereas the Australian invasive species, *C. grandiflora*, came from the south-west region. New collections were made in this region of Madagascar, briefly rescreened in the UK – mainly to filter out natural enemies – and sent to Queensland. The rust was released into the field, initially on bait plants. However, because of the urgency of the situation and the size of the infestation, inoculum was mass produced in greenhouses and sprayed from the ground in crude formulations, as well as from the air along the advancing front, due to its inaccessibility (Tomley and Evans, 2004). This is the first use of aircraft as a release strategy in weed CBC – after a PRA for aerial application of the fungus was approved – and the first example of applying weed pathogens inundatively for CBC. Aircraft have previously been used in CBC by applying parasitoids against an invasive alien mealy bug in Africa (Herren and Neuenschwander, 1991), as well as in IBC, for crop spraying of one of the pioneering bioherbicides in the USA (TeBeest *et al*., 1992).

Independent monitoring by ecologists showed that rubber-vine populations were quickly reduced (up to 40%), following severe defoliation and the use of controlled burns to kill off otherwise fire-resistant plants, with virtually no seedling recruitment (Tomley and Evans, 2004). The weed has literally been stopped in its tracks, with no non-target effects. The benefits to Australian ecosystems cannot be measured financially, but the cost–benefit to agriculture in 2005 – a decade after release – has been put at over AU$230 million (Page and Lacey, 2006; McFadyen, 2008; Palmer *et al*., 2010) and, of course, this increases annually.

Bathurst burr

Bathurst burr, *Xanthium spinosum*, is a major problem to sheep farmers and the woollen industry in Australia (Parsons and Cuthbertson, 2001). During surveys in its Argentinian native range, the powdery mildew, *Erysiphe cichoracearum sensu lato*, was found to be widespread, highly damaging and, apparently, host specific because it was not found on other *Xanthium* spp. occurring in the same localities. As in the *Cryptostegia* case study above, a strain of the mildew was host-range tested in UK quarantine. The first screening against selected cultivars of sunflower – a major crop in Australia – as well as species of *Xanthium*, showed exceptional promise with symptoms only on the target

weed. In more comprehensive testing, including some of the newer Australian sunflower cultivars, several of these became infected; showing symptoms almost as severe as on the weed control tests. Although the screening protocol has been shown to be robust, susceptibility in this case does not strictly follow the phylogenetic model. This could be linked to genetic changes in the sunflower cultivars during the intensive breeding programme. Nevertheless, it throws up concerns about the stability of powdery mildew–plant associations, such that it is unlikely that these biotrophic pathogens will ever be considered for CBC.

Giant hogweed

Giant hogweed, *Heracleum mantegazzianum*, is becoming an increasingly important environmental and noxious weed in Europe (Pysek *et al*., 2007). A hemibiotrophic fungus, *Phloeospora heraclei*, showed promise during surveys for coevolved natural enemies in the Caucasus mountains of Russia (Seier and Evans, 2007), especially because it attacked and killed the seedling stage (Fig. 9.3). During comprehensive, high-level quarantine screening in the UK, the pathogen induced symptoms on commercial cultivars of related crop species, principally on parsnip and coriander. However, there is a conundrum to be solved here: this fungus has never been recorded on cultivated parsnip and coriander in the UK, or, apparently, in Europe – although it occurs on wild parsnip; also, it is not found on giant hogweed in its invasive range but occurs on native *Heracleum* spp., such as *H. sphondylium*. Owing to the high CBC potential of this pathogen, attempts are

Fig. 9.3. Giant hogweed, *Heracleum mantegazzianum*: (A) surveying for natural enemies in the plant's native range in the Russian Caucasus region, where it is a typical colonist of upland meadows (R. Wittenburg); (B) blistering caused by contact with the photosensitizing hairs and exposure to sunlight (D. Djeddour); (C–D) symptoms caused by the hemibiotrophic, ascomycete fungus *Phloeospora heraclei* on seedlings, which can lead to severe necrosis and plant death. In greenhouse tests, similar symptoms have been elicited on commercial parsnip and coriander varieties causing the programme to be stalled, awaiting further studies on the risks posed to these plants in the UK. Field data suggest that this is an artificial extension of the host range based on circumstantial evidence that both native wild parsnip and hogweed are attacked by this pathogen but these strains (pathotypes) have never been recorded on the invasive giant hogweed (M.K. Seier).

being made to solve the puzzle through a series of cross-inoculation studies, but until this is resolved satisfactorily – based on scientific evidence that non-target plants are not at risk – the programme is temporarily, at least, at an impasse.

9.2.7 Bureaucrats, botanists, beekeepers and other bottlenecks

This title was used to illustrate the safety and legislative problems that weed biocontrol practitioners come up against before, during and after the implementation of CBC programmes (Harris, 1985). It reflects the frustrations of a discipline that seems to oscillate between highs and lows. This 1980s low period had been preceded by the highs of the 1970s, when the use of microbials for the management of weeds had become more mainstream with the pioneering and hugely successful CBC programmes in Australia and the USA (Cullen *et al*., 1973; Trujillo, 1985). The pattern has been repeated over subsequent decades, but with successful programmes not being followed up by increased investment, as might logically be expected, and CBC entering another trough. Instead of increased confidence in the safety and economic benefits of CBC, periodic scare stories about the potential risks involved, distrust of its apparent primitive or simplistic technology in a world of increasing sophistication and, of course, unpredictable political manoeuvrings, have all contributed to general unease about this management strategy. If this is transmitted to donors and decision makers, then funding for new CBC initiatives becomes problematic. The following points summarize the present situation and future prospects of CBC of invasive alien weeds.

What have we learned?

- That the central pillar, supporting the PRA for weed CBC agents, host-range testing, is extremely robust. Some would argue that it is too robust and that useful agents could be rejected (Wapshere, 1989). The rubber-vine weed case study (Section 9.2.6) illustrates this point perfectly. What we now know is that false positives are not uncommon in centrifugal phylogenetic screening but that such 'negative' results need to be interpreted scientifically, based on the information presented. Whenever possible, this should be backed up with additional 'high-tech' data, for example, on host resistance mechanisms and simulated natural infection courts (wind tunnel experiments, more realistic inoculum concentrations). An informed rather than emotive decision can then be made within the context of the economics and the ecology of the exotic ecosystem into which the potential agent will be introduced. What we can take comfort in is that the current self-regulated system will detect any unpredicted, even bizarre, host shifts as in the *Cryptostegia* and *Xanthium* examples described in Section 9.2.6.
- That strain selection is crucial to success. Even though a strain exhibits high potential in artificial screening tests, unless there is a coevolved association, then this potential will not be realized in the field situation (see Section 9.2.6, rubber-vine weed). Fortunately, high tech has now been embraced by biocontrol scientists, who routinely use molecular techniques to determine the weed's centre of origin (as well as correct host identification!), in order to better access its coevolved natural enemies – especially in the rust fungi, which frequently exhibit rigid host specificity, often at the biotype–pathotype level.

What are the grey areas?

- Biosecurity is now in common parlance, even though the term is often misunderstood or misapplied (Evans and Waller, 2010). For CBC of weeds *sensu lato*, this can have biological warfare connotations and, almost certainly, there have been covert CBC-type introductions against weedy (drug) plants. Fortunately, these seem to have been based on good science,

although there is no international legislation (or international agreements) in place, apart from non-mandatory codes of conduct (IUCN, 1987; FAO, 1996, 1997; EPPO 1999, 2000), to stop individual countries from importing whatever potential CBC agents that they see fit. If high scientific standards are not maintained then 'one error resulting in crop damage could jeopardize the future of all [CBC] programmes' (Adams, 1988). Thus, for example, any country could officially import a microbial CBC agent targeted at an invasive weed through its quarantine service without the need to inform its neighbours. If this were based on poor science (i.e. not screened or non-specific), then the consequences for that region, or even that continent, could be disastrous as plant pathogens do not respect borders. This is not as far-fetched as it seems, because there is strong circumstantial evidence to indicate that at least two (unofficial) introductions of pathogens for weed control have been made into Australia, probably by disaffected stakeholders. One of these agents (blackberry rust), quickly reached New Zealand (Marks *et al*., 1984; Cullen and Delfosse, 1985; Julien and Griffiths, 1998); more recently, the officially released bridal creeper rust (*Puccinia myrsiphylli*) in Australia was also reported from New Zealand (Waipara *et al*., 2006).

- Undue delays, unforeseen bottlenecks or unnecessary bureaucracy could all contribute to stakeholder vigilantes taking the law into their own hands. For both the 'rogue' Australian introductions mentioned above, official releases were delayed pending further investigation. In the case of the blackberry rust, the confused taxonomy of the target weed (*Rubus fruticosus* agg.), plus the many commercial cultivars, complicated the science and, at one time, up to 19 pathogen strains were being assessed for introduction (Bruzzese and Hasan, 1983). Undoubtedly, a rogue element of the farmers affected by weed infestations, and inspired by the earlier successes in Australia with rust CBC agents, conspired to release a European strain of the pathogen. Fortunately, no commercial soft fruit growers have been affected by the introduction.
- Recently enacted legislation in various countries – with the Rio Convention on Biological Diversity (CBD) as the catalyst – under the banner of the International Regime on Access and Benefit Sharing (Cock *et al*., 2010), could halt or seriously impede the implementation of CBC (see also Thornström, Chapter 20, this volume). This international regime and the supporting national legislations are directed at commercial research (bioprospecting) and biopiracy, and are designed to stop the unregulated movement of genetic material across national borders, as well as to share the benefits between exporter and importer. CBC does not fall into this category, because it is non-commercial research with no patents to protect: 'The benefits accrue to all, not the implementing agency or the government or group that paid for the research' (Cock, 2010). However, a number of countries have already introduced laws on access and benefit sharing which are tailored as 'anti-biopiracy' legislation, and so are full of highly conservative safeguards. If these are also applied to CBC, then exploratory surveys and the export of CBC agents will effectively be stopped or, at the very least, severely impeded. Often, the designated bureaucracies empowered with administering the laws do not understand them, especially when microbial CBC organisms are involved. In other countries, there are simply no bureaucrats in a position to make such decisions, but they interpret the CBD to the full, in the sense that their biodiversity cannot be exported. It has even got to the absurd state where samples of the invasive alien plant *Mikania micrantha* in India could not initially be obtained for screening, because the Indian quarantine authorities considered it to be 'part of our biodiversity' – even though the request was for an aid programme to manage a

weed threatening not only biodiversity hot spots in the Western Ghats, but also the major export crop of tea in Assam (Ellison and Evans, 2004). As Cock (2010) pointed out, the age of the hunter–gatherer in biological control has long gone. Unfortunately, the modern – some would say, more ethical (Delfosse, 2005) – CBC scientist could be faced with potentially insurmountable legislative barriers.

9.3 Inundative (Bioherbicide) Biological Control (IBC)

9.3.1 Why do we need IBC?

The motivations for IBC have been detailed comprehensively by Charudattan (2001), who pointed to changes in modern weed management practices and increasing public awareness of environmental issues. The main drivers are summarized here:

- banning or phasing out of many traditional chemical herbicides, as well as general soil fumigants;
- the high cost of research and development, as well as the registering, of new chemical herbicides;
- the scarcity of registered products for niche markets;
- increasing weed resistance to chemical herbicides;
- public resistance to herbicide-resistant transgenic crops;
- increasing public preference for organic or green produce; and
- government initiatives and, in some countries, mandates for reducing the use of chemical pesticides, of which herbicides account for almost 50% of the agrochemical market.

In even more recent times, there has been increasing public concern that some of the best-selling herbicide products are not as safe as claimed, while the ethics of the major agrochemical companies have been brought more and more into question. This has been re-enforced, in developed countries, by nostalgic or never experienced images of the diversity of the countryside before the rise of large-scale agriculture and the mass use of chemical pesticides, especially of herbicides.

What better, then, than 'natural' products such as bioherbicides to fill the chemical-herbicide void?

9.3.2 How does it work?

In theory, a bioherbicide is applied and should function in much the same way as a chemical herbicide, because the microbial agents are often selected on their ability to produce plant toxins. Thus, the farmer expects results within days rather the months, years or even decades that CBC can take. However, in practice, since the early successes in the 1970s (Templeton, 1982; TeBeest and Templeton, 1985) – mirroring those of weed CBC – IBC of weeds has been plagued by technical problems: especially problems of mass production, formulation, shelf life and inconsistent field efficacy (Auld and Morin, 1995). In short, IBC has gone through the same periodic highs and lows as CBC and, as a management strategy in a commercial market, it has generally failed to compete with conventional chemical control as a player on the bigger agricultural stage. The beginnings were promising: select a pathogen, typically an indigenous necrotrophic (readily culturable) fungus, with a restricted host range, attacking an indigenous or naturalized weed of importance in local crops, and use relatively high-tech methods – often from the food industry – to formulate the product in a form suitable for application in small-scale field plots. This R&D phase was, more often than not, part of a publicly funded research project. To scale this up into a commercial product, venture capital was needed to cover not only the production costs but also for patenting, health and safety testing and registering; and, dealing with a living organism presented all manner of technical problems, many of which have still not been resolved. For example, in order to register a bioherbicide, efficacy data have to be submitted or made available, but, frequently, this has been impossible because the results were statistically inconsistent and depended largely on critical climatic conditions (such as

length of dew period) being suitable for host infection at the time of application. Investors, with shareholders to placate, soon lost heart and IBC of weeds seems to have settled into supplying those niche markets where chemical herbicides are banned, such as in riparian habitats or public rights of way, or are ineffective.

9.3.3 Is it safe?

As with CBC, the initial policy tended towards selecting apparently host-specific strains of common plant pathogens, often designated as *formae speciales* of the target weeds based on relatively narrow host-range testing compared with that done in CBC. Some of these fungal agents, especially in the genus *Colletotrichum*, proved not to be so host specific (Weidemann 1991, 1992), although, as will be shown later, this does not present any undue safety concerns because these pathogens are an integral part of the native biota and any negative impacts will be temporary, unlike the situation with CBC. In fact, host-specificity testing has literally been turned on its head, and broad-ranging, even previously notifiable plant pathogens, have been approved or are being assessed for IBC of weeds. The PRA is based not on specificity screening but, primarily, on epidemiology data.

9.3.4 How is it regulated?

A comprehensive overview concerning the regulation of biopesticides, in general, has recently been published (Bailey *et al*., 2010). Therefore, it is only necessary to summarize the major points here and to highlight those pertaining specifically to bioherbicides that this review has not already considered.

As emphasized (Bailey *et al*., 2010), regulation of bioherbicides has taken place within a system designed for chemical pesticides. Clearly, however, this model is not appropriate for living organisms (Waage, 1997); this was a plea made much earlier when the prototype bioherbicides were testing the regulatory system in the USA (Templeton, 1982, 1992). Charudattan (2005) compared the registration of plant pathogens for IBC and CBC of weeds in the USA and found that, although the latter pathogens could, by definition, be classed as biopesticides *sensu lato*, they are regulated differently: CBC agents were handled and approved by the Animal and Plant Health Inspection Service (APHIS – equivalent to the Australian AQIS, see Section 9.2.6, rubber-vine weed), while IBC pathogens must be registered with the US Environmental Protection Agency (EPA), adding a financial burden for environmental and health checks, in addition to long delays. The situation in Europe, is even more confused, especially because individual countries within the EU seem to go their own way, despite claims of an overarching EU regulatory system (Bailey *et al*., 2010). 'However, the Commission Directive 2001/36[/EC (OJEU, 2001)], which amended the Council Directive 91/414/EEC specifically for biopesticides, is very restrictive with regards to the procedures of risk assessment, registration, and use of microbial plant-protection products: this is a further and potent reason why no microbial products are currently included as bioherbicides in the register kept by the Directorate of the Consumer Health Protection' (Vurro and Evans, 2008).

9.3.5 Case studies: regional variations

These are selected to show how various countries or regions are reacting to and have overcome or are addressing legislative and safety problems with the use of the IBC strategy for the management of weeds. The overriding theme is niche-market use, predominantly of woody invasive alien weeds: a far cry from the optimism of the 1970s when there was an expectation that there was a role for bioherbicides in the dominant agricultural crops for control of the many indigenous or naturalized annual weeds.

North America

Registration and legislation costs, combined with persistent technical problems, have condemned most of the potential and actual bioherbicides to a premature, or in the earlier

cases, a lingering grave. Indeed, it is difficult to elicit reliable information on which of the few products are actually still on the market. Canada has active IBC weed programmes in place because of the government initiative to gradually phase out the use of chemical pesticides, especially in forestry and public areas (Boyetchko, 1999; Bailey, 2010). Similarly, in the USA, the current regulatory system for 'green-labelling' protocols has tended to favour bioherbicides (Charudattan, 2001).

The Canadian forestry service has had an ongoing programme testing various fungal pathogens as stump treatments against invasive woody weeds for several decades, especially using the silver leaf pathogen, *Chondrostereum purpureum* (Prasad, 1994; de Jong *et al*., 1996; Evans *et al*., 2001a,b), and two stump-treatment products have been registered, thus far, with the Health Canada Pest Management Regulatory Agency (Vartiamäki *et al*., 2008). It is predicted that this technology will be used more widely in the future in Canada (Bailey, 2010). The demand and potential market for bioherbicides in urban situations is growing and a strain of the leaf-spot fungus, *Phoma macrostoma*, that produces novel and lethal chlorosis-inducing phytotoxins (macrocidins) has proved to be highly effective against major weeds of turf and lawns; this is currently undergoing registration in both Canada and the USA, after extensive efficacy and safety testing, including monitoring of its impact on and persistence in the soil (Zhou *et al*., 2004; Bailey, 2010).

Perhaps the most innovative niche-market bioherbicides in the USA involve non-fungal based products. Charudattan (2005) reported on the potential of tobacco mild green mosaic tobamovirus to control a highly invasive toxic weed, tropical soda apple (*Solanum viarum*), in Florida, and the efforts to register a product from this. The virus is not vectored – so weed management is linked with prior wounding through pressure sprays – and, although several solanaceous hosts of agricultural significance are susceptible, the PRA details the safe distances that the virus can be applied from such crops. This is becoming an ever-familiar theme with bioherbicides. The product has now been approved by the US EPA and is being manufactured in a small production unit on a university science park (R. Charudattan, University of Florida, Gainesville, personal communication). Similarly, off-target movement of a bacterium, *Xanthomonas campestris* pv. *poannua*, and its fate in the soil after application as a bioherbicide, has been closely monitored around golf greens where annual bluegrass (*Poa annua*) is a major problem (Neal *et al*., 2004). The results show that persistence in and movement from the target site is minimal and that the product is safe to use within the IPM (integrated pest management) strategy for golf turf. A Japanese product, Camperico, incorporating a local strain of the same bacterium, has been registered for some time in Japan for the control of annual bluegrass (Imaizumi *et al*., 1997).

Europe

In contrast to North America, there have been few or no EU or individual country-government incentives for bioherbicide development (Vurro and Evans, 2008). These authors stress that no bioherbicides are currently registered in the EU Directorate. However, at least one, BioChon, had been registered and sold in the Netherlands as a wood-rot promoter rather than a bioherbicide (Ehlers, 2008; M.D. de Jong, personal communication). This followed groundbreaking research to obtain epidemiological data on the fungal pathogen that used conceptual and simulation models for the risk assessment (de Jong *et al*., 1990, 1991; de Jong, 2000). The product's 'active ingredient' is the plurivorous fungus, *Chondrostereum purpureum*. This is a pathogen of many species of deciduous trees, and the causal agent of silver leaf disease of rosaceous fruit trees, but it also kills and prevents resprouting of the invasive American black cherry, *Prunus serotina*. The data presented on inoculum movement to the Dutch Plant Protection Service showed that although the risk to non-targets is high at up to 500 m from the application source, it is negligible at 1500 m. Approval for its use stipulated that BioChon should not be applied within 500 m of commercial stone-fruit orchards, and that this warning should be shown clearly on the product label. While

the product has recently been withdrawn from the market (Ehlers, 2008), similar initiatives are currently underway in other EU countries with native strains of *C. purpureum*, especially targeted at *R. ponticum* in the UK and the Republic of Ireland (see Section 9.2.5), as well as against forestry weeds in Scandinavia (Vartiamäki *et al*., 2008).

Africa

The main player here is South Africa, which has a long tradition of CBC for the many invasive alien weeds of the country. Nevertheless, IBC has also seen several innovative products being applied in the field, which are produced at the cottage-industry level, are usually government sponsored and free of charge (Morris *et al*., 1999). It would appear that legislative requirements have been minimal and that the PRA has been self-regulated by the scientists themselves. For example, a stump-treatment, non-commercial preparation (Stumpout) – based on a plurivorous, wood-rotting fungus (*Cylindrobasidium laeve*) – has been used successfully in a national Working-for-Water campaign to control water-depleting, invasive Australian *Acacia* spp. (Morris, 1991).

Similar examples of aid-funded agencies attempting to apply the IBC strategy for the control of both indigenous and alien weeds are reported for other African countries. The targets have been invasive water hyacinth – with local strains of *Alternaria* – and parasitic witchweeds (*Striga* spp.) that take a heavy toll of subsistence crops. Following several decades of research on the use of a pathotype or *forma specialis* of the wilt fungus *Fusarium oxysporum* as a seed treatment against witchweeds for subsistence farmers (Elzein *et al*., 2006), it seems as though Africa could have its first registered bioherbicide. According to the latest developments (CGIAR, 2010), Kenya has been selected as the pilot country to register and commercialize the bioherbicide because of its existing registration protocol established by the Kenyan Pest Control Board. Because the fungal strain is from Ghana, a material transfer agreement has been signed to launch the initiative, presumably, after a PRA had been approved by the receiving country. Undoubtedly, the molecular characterization of this new pathotype (*f.sp. strigae*) should help to facilitate its introduction by the regulatory authorities (Elzein *et al*., 2008).

Asia

Research on bioherbicides for use against invasive weeds such as water hyacinth has been reported from India and China, as well as for the management of grassy weeds in rice in the Philippines (Watson, 1993), but no commercial products seem to have made it to the marketplace and legislative and safety regulations in this region are a grey area. As mentioned previously (see North America above), a bacterial-based bioherbicide has been developed commercially in Japan, specifically for use on golf greens.

Australasia

Only New Zealand appears to have invested significant funding to assess IBC as a management approach for its invasive alien weeds. In addition to *C. purpureum*, another infamous plurivorous fungal pathogen, *Sclerotinia sclerotiorum*, is being tested for safety and efficacy as a bioherbicide for pasture weeds (Hurrell *et al*., 2001; Bourdôt *et al*., 2008). As in previous endeavours (see above, North America and Europe), distances of application from susceptible crops will be the key criterion in any PRA.

9.4 Overview: Where Are We?

'The over-riding concern, to both regulatory bodies and the general public alike, in using plant pathogens for weed control is their potential threat to non-target plants' (Evans, 2000). This will always be the case for CBC of weeds: thus, only highly specific (coevolved) agents will ever be considered. Here, the PRA rests, predominantly, on the results and interpretation of the tried and tested centrifugal screening protocol, although data concerning the taxonomy and biology of the pathogen, as well as analyses on any potential ecological, economic or sociological impacts should also be taken into account. After all, CBC involves the introduction of yet another

alien organism – in this case, a microbial pathogen (!) – into an ecosystem: anathema to some, including organizations such as the World Wide Fund for Nature (WWF) and, at times, ecologists (Howarth, 1991; Louda *et al*., 2003; Pearson and Callaway, 2003). However, if all these environmental concerns were addressed, especially indirect multi-trophic interactions, the ecological impact studies alone would put a CBC programme far out of reach of traditional donors. Such in-depth studies have not even been undertaken for any invasive weed, let alone a CBC agent, although some regulatory authorities are now recommending that additional environmental data be collected, as shown by a request from the UK Department of the Environment, Food and Rural Affairs (Defra) to assess any potential impacts by the psyllid CBC agent, *A. itadori* (see Section 9.2.5), recently introduced against Japanese knotweed (R.H. Shaw, personal communication). CBC scientists may also undertake environmental impact studies on target weeds to strengthen their funding bid or to reinforce their argument for sustainable management. For example, monitoring the invertebrate fauna under monocultures of the UK's tallest annual Himalayan balsam, *Impatiens glandulifera* (Fig. 9.4), has revealed significant

Fig. 9.4. Himalayan balsam, *Impatiens glandulifera*. (A) heavily infested river system in Devon (SW England), showing monoculture of young plants with inset of flowering plants later in the season – the first frosts kill the plants and expose the banks to erosion (R. Tanner); (B) natural habitat in an upland meadow in the Himalayan region of Pakistan where surveys of natural enemies were undertaken (R. Tanner); (C) stunted and deformed young plant in the Indian Himalayas infected with the rust fungus *Puccinia komarovii*, with gross swelling of lower stem (arrow) caused by the systemic sexual stage (R. Tanner); (D) close-up of swollen stem containing the distinctive aecial cups of the sexual stage (R. Tanner); (E) leaf infection later in the season caused by the asexual, dispersal stage (R. Tanner). This UK-funded programme is nearing completion of the risk assessment phase for the rust fungus, and is on course to be the first to release of a CBC microbial agent for the management of an invasive alien weed in Europe.

negative shifts in their diversity and population structure (R. Tanner, in press). As Vurro and Evans (2008) conclude, somewhat cynically: 'if all the environmental concerns and risks involved in undertaking a motorized shopping trip were analysed as critically, supermarkets would go out of business'. Therefore, CBC weed programmes must continue to be self-regulated by scientifically responsible biocontrol practitioners. The resultant PRA can then be peer reviewed by the relevant quarantine authorities for any amendments, before being put in the public, and at times, political arena for debate, especially on the perceived cost–benefits.

For IBC of weeds, concerns about non-target effects have been modified and addressed over time, because, invariably, the agent is an indigenous plant pathogen. As shown, we have moved from host screening being the central pillar of the PRA to that role being taken by epidemiological–environmental studies. Thus, if it can be demonstrated that the microbial active ingredient does not persist in the area of application and has restricted movement from it, then as long as the label details these data, the product can be used safely. The grey area continues to be registration, especially in Europe, and specifically the regulations and costs involved in this, because these regulations were designed for synthetic (chemical) pesticides and not for biopesticides per se (Bailey *et al*., 2010). Recent attempts to introduce hypervirulent genetically modified pathogens into the equation will only muddy the legislative waters and increase public concern about the environmental safety of bioherbicides (Amsellem *et al*., 2002).

In summary: 'Appropriate regulations are necessary to encourage research and development of plant pathogens as [bio] mycoherbicides and classical biocontrol agents. Scientists need peer assurance that field test protocols are as nearly risk-free as practical. Society needs assurance that the potential benefits from natural controls are greater than any risks to humans, their animals, or the environment. The critical issue [still] is what regulations are appropriate to satisfy the needs of scientists and society' [?] (Templeton, 1992).

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10 Plant Growth Promotion with Microorganisms

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10.1 Introduction

Plant growth promotion by application of microorganisms has been a part of agricultural systems since at least Roman times. Ancient farmers were implored by Virgil (70–19 BC) to reinvigorate their fields by rotation of cereals with legumes such as lupins or vetches (Hamblin, 1998). These legumes became naturally nodulated by soil-borne root nodule bacteria, and the ensuing symbiosis allowed the fixation of inert atmospheric dinitrogen gas to amino acids in the nodule, with subsequent export to the legume cells. Nitrogen fixation by nodule bacteria is an example of plant growth promotion, as most soils are nitrogen deficient for plant growth and fixed nitrogen overcomes this deficiency. The nodule bacteria remain the most successful of the plant growth promoting (PGP) microorganisms and provide an excellent model to explore legal and ethical issues surrounding their usage.

However, a wide range of other PGP microorganisms are now in commerce, and this chapter attempts to address the issues raised by their exploitation. It should be noted that the overwhelming majority of PGP microbes in commerce have been developed directly from the environment, without genetic manipulation. Hence, we are not

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concerned in this chapter with the law as it relates to the release of genetically modified organisms. The key issues that do arise are: unintended manipulation of natural microbial biodiversity by the introduction of exotic genes; quarantine transgressions (as non-sterile carriers are traded globally); ownership of improved strains and intellectual property (IP); the human health questions invoked by the use of a bacterial genera linked to the incidence of human disease; and the uncertainty surrounding the efficacy of the various PGP microorganisms. This chapter will briefly outline the diversity of currently utilized PGP microorganisms and their mechanisms of action, evaluate potential risks from their use, and assess to what extent general regulations concerning their spread or protection of biodiversity can affect their utilization.

10.2 The Definition and History of Plant Growth Promotion by Microorganisms

Microbes are widely deployed in the food, mining and agricultural industries. The agriculturally significant organisms can be classified into eight functional groups (Howieson, 2007), with the PGP microbes forming a subset of these:

1. Plant microsymbionts such as nodule bacteria and mycorrhizal fungi
2. Plant-associated microbes (i.e. those which elicit or potentiate a positive reaction or effect when in intimate proximity to a plant) such as cereal endophytes
3. Rumen organisms
4. Biocontrol agents (pathogens of weeds, fungi, insects or nematodes)
5. Pathogens of plants or animals
6. Agents for nutrient solubilization, bioremediation or biodegradation
7. Agents for the production of biofuels
8. Agents facilitating DNA or gene transfer.

A microbial genus may be represented in more than one group, e.g. *Agrobacterium* is a common laboratory agent for transferring DNA (functional group 8), but also a common root pathogen (functional group 5).

This chapter will focus on the microbes in functional categories 1, 2 and 6, which elicit a growth-enhancing response when placed in close proximity to the leaves, seeds or rhizosphere of the target plant, or which are contained within the plant itself.

10.2.1 Nodule bacteria (functional group 1)

The root nodule bacteria (RNB) are found on all continents and nodulate the *Leguminosae* (*Fabaceae*), which is one of the largest families of flowering plants, with more than 18,000 species classified into 650 genera (Sprent, 2001). The RNB, as microsymbionts of legumes, were almost certainly the first group of PGP microbes to be studied at the microscopic level, and this was in the same decade (in 1883) that proof emerged that microbes such as *Vibrio* were the causative agents of serious human and animal illnesses, such as cholera. RNB were, in fact, manufactured as agricultural amendments within a few years of Beijerinck isolating and growing the bacteria in the Netherlands, and of the groundbreaking work of Hellriegel in Germany who identified their role in legume nodulation and nitrogen fixation in 1887 (O'Hara *et al*., 2002). This was only 6 years after Koch first cultured bacteria on gelatine.

Early attempts at inoculation with RNB were rudimentary, such as moving soil from fields growing well-nodulated legumes to legume-free fields (Fred *et al*., 1932). In Canada, in the early part of the 20th century, farmers were encouraged to inoculate legume seed with a mixture of sieved, air-dried soil from fields containing well-nodulated plants of the target species. Within a couple of years, cultures of rhizobia were available in the marketplaces of Europe for farmers to inoculate a range of species, and inoculation was recommended in Australia (Guthrie, 1896). The first inoculant industries for RNB developed in the 1920s (Deaker *et al*., 2004). Thus began an unprecedented anthropogenic alteration of the soil microbial biota on the planet.

The symbiotic association between RNB and legumes is considered to be a vital biological pillar in any terrestrial ecosystem (Reeve *et al*., 2010). This symbiosis annually

reduces approximately 100 million t of atmospheric dinitrogen into ammonia (Herridge and Rose, 2000). Global inoculation of legumes with RNB is valued at more than 10 billion US$ annually (calculated on the basis of the cost of replacing fixed N with manufactured N; Herridge, 2008). The manufacture of inorganic fertilizer N contributes significantly to global greenhouse gas emission via the Haber–Bosch process, with each tonne of urea manufactured consuming 1.4 t of oil equivalents (Peoples *et al*., 2009). So inoculation of legumes with PGP microbes can be seen as an environmentally friendly undertaking, and this is an important consideration when weighing the potential negative effects of their global deployment, which we discuss later in this chapter.

10.2.2 Vesicular arbuscular mycorrhizae (VAM) and ectomycorrhizae (functional group 1)

Concomitant with the isolation of RNB from nodules, the understanding of the diversity of microbes interacting in symbioses with plants was expanded with the discovery of the relationship between certain fungal hyphae and plant nutrient acquisition. Frank (1885) originally described the fungus–root interaction with mycorrhizae, and it is now realized that about 95% of all vascular plants are involved in symbiotic associations with fungi. The most notable of the roles played in this relationship is in the acquisition of phosphate (P).

Mycorrhizae can be either endophytic (exist within cells), such as the vesicular arbuscular mycorrhizae (VAM), or they may grow between the cells of plant roots, such as in the ectomycorrhizae that Frank described. As the fungal hyphae extend well out of the plant root cells and into the soil, both patterns of development can be viewed as providing an extension of the plant root systems for the purpose of exploring a greater soil volume for nutrient uptake (Smith and Read, 2008). Mycorrhizae and their interactions profoundly affect forest site productivity through the capture and uptake of nutrients, protection against pathogens, maintenance of soil structure and buffering against moisture stress.

However, it is for alleviating deficiencies of P that mycorrhizae have proven efficacious as PGP microbes. Where soil total P levels fall to 1 or 2 ppm, plant growth is usually constrained. Unfortunately, many heavily leached soils have this level (or below this level) of P (Uehara, 1978), and it is in these ecosystems, as well as in severely eroded regions, that applications of mycorrhizae have been commonplace. Although VAM are difficult to culture, they are the preferred type of inoculant, so we see cottage industries in tropical and subtropical countries where soils containing VAM are used to inoculate trees in nursery situations (Lukiwati and Supriyanto, 1995). When planted out into degraded lands, the VAM-inoculated seedling trees have a distinct advantage over uninoculated trees. In Europe, companies producing and/or developing mycorrhizal fungal technology have formed a consortium (FEMFip; see www.femfip.com) to advance the development and uptake of this PGP. VAM utilization has not spread to broad-acre crops for two main reasons. First, it is difficult to inoculate crops with soil containing VAM over wide acreages and, secondly, up to now, inorganic P fertilizers have been used as an effective replacement for VAM. Despite this, VAM are *bona fide* PGP microbes in horticulture and forestry applications and rehabilitation exercises, and may well gain prominence in agriculture as readily available world sources of rock phosphate decline.

10.2.3 Rhizosphere PGP microbes (functional groups 2 and 6)

It was not long into the 20th century before the role of the soil microbiota in the development of plant disease and also in nutrient cycling in the soil ecosystem could be quantified. The concept of the rhizosphere (that portion of the soil in direct contact with and under the influence of the root) and its role in plant growth was described in the 1960s (Rovira, 1973). Soon after this, the capacity for rhizosphere organisms to affect plant growth was discovered, and a new industry was born.

China, India and the former Soviet Union have since created a strong history of experimenting with, reporting and even

manufacturing microbes that may be classified as PGP organisms. This is now becoming a fertile market in Western countries. The microbes utilized form close associations with plant root systems and include those from the bacterial genera *Pseudomonas* and *Bacillus*, actinobacteria such as *Streptomyces*, and fungi from a wide range of genera such as *Trichoderma*, *Penicillium* and *Piriformospora*. Bacterial examples currently in the marketplace are from *Bacillus*, *Burkholderia*, *Delftia* and *Achromobacter* genera. Other fungi that are commercially promising for their PGP activity include some isolates of *Phoma* and *Penicillium*.

10.2.4 Plant-associated endophytes (functional group 2)

The latest phase in the discovery of microbes as plant symbionts is in their role as endophytes. Living within (or between) plant cells, endophytic microbes elicit plant responses from secondary metabolites produced by the endophytes. The best described of these associations is that of *Acetobacter diazotrophicus* in Brazilian sugarcane systems. This bacterium is able to grow prolifically in the presence of the abundant carbohydrate, and has the capacity to provide N in excess of 30 kg ha^{-1} to its host (Boddey *et al*., 1991). The grass endophyte *Neotyphodium coenophialum* is a fungus that has long been known to produce toxic alkaloids in the forage grass tall fescue (*Schedonorus phoenix*). These toxins deleteriously affect animal health (Christensen *et al*., 1998). When breeders removed the fungus from fescue, stand production and persistence was found to decrease, presumably because of the loss of the PGP benefit. This was overcome by selecting toxin-free endophytes and re-introducing them to fescue. There are current attempts to transfer these same endophytes to the *Lolium* genus.

Another set of endophytes recently described are the *Micromonospora,* which appear to co-inhabit legume nodules alongside their nitrogen-fixing bacteria. *Micromonospora*, previously familiar to microbiologists as the N-fixing nodule occupants of non-leguminous actinorrhizal plants such as *Coriaria myrtifolia* (Trujillo *et al*., 2006), have now been described co-existing with RNB in nodules of *Pisum, Lupinus* and *Medicago* (Garcia *et al*., 2010; Trujillo *et al*., 2007). The PGP role of these intra-nodule endophytes is yet to be fully elucidated (Hirsch and Valdez, 2010).

10.3 Mechanisms of Action of PGP Microbes

The mechanisms for the growth response to inoculation with PGP microbes vary. They may be straightforward and direct, as for the microsymbionts in functional group 1, where the association supplies otherwise limiting macronutrients such as P and N. But for microbes in functional group 2, the associative, endophytic or rhizosphere microbes, the classically described mechanisms do not always apply. As a result of a plentiful supply of nutrients exuded from the roots in the rhizosphere, the rhizosphere-bound PGP microbes have the capacity to grow prolifically, to a density in excess of 10^8 cells g^{-1} of root tissue. In these large numbers, they can produce metabolites in sufficient localized concentration to affect neighbouring plant cells. These metabolites may be enzymes, such as ACC (1-aminocyclopropane-1-carboxylate) deaminase, or hormones such as indoleacetic acid (IAA, which directly affects root growth, branching and hair formation). The bacterially derived ACC deaminase is an excellent example of a complex, indirect mechanism of action on plant growth. Glick and co-workers (1998) suggested that ACC deaminase cleaves the plant-produced ethylene precursor ACC into ammonia and α-ketobutyrate. From a plant growth-promoting perspective, it is theorized that in response to bacterial sequestration of ACC, the plant exudes increasing amounts of that compound. This is proposed to result in reduced internal ACC concentration and, consequently, a reduction in plant ethylene levels, as high concentrations of ACC induce synthesis of ACC oxidase, which converts ACC to ethylene (Glick *et al*., 1998). While there are few data on the transport or diffusion of ACC in plant cells or across the cell wall, some research seems to corroborate that

ACC deaminase-producing PGP microbes can reduce the ACC concentration in canola roots (Penrose *et al*., 2001) and in canola seed and seed exudates (Penrose and Glick, 2001). The result of decreased ACC concentrations in the roots is proposed to be a reduction of the ethylene-dependent inhibition of root elongation. Furthermore, because ethylene is implicated as a major factor in plant stress responses, it is speculated that ACC deaminase-producing PGP microbes may alleviate some of the reduced yields associated with ethylene production under stressful conditions, such as salinity (Mayak *et al*., 2004) and nickel toxicity (Burd *et al*., 2000). PGP microbes may even be essential in assisting plant performance in micro-gravity environments, such as space stations, where stress hormones accumulate (Ferl *et al*., 2002).

The solubilization of micronutrients to benefit plant growth is a further mode of action of some PGP microbes (functional group 6). The micronutrient iron is limiting to plant growth in some soils because it is often complexed into stable oxides, as which it can become unavailable. Some microbes have the capacity to exude siderophores that solubilize and complex iron to make it plant available (Dilworth *et al*., 1998; Jin *et al*., 2006). Microbes such as bacteria and fungi are also reported to solubilize P by two mechanisms: some produce phosphatases that solubilize organic P compounds; and others produce organic acids, freeing up orthophosphate for plant use (Kucey *et al*., 1989). Fungal PGP organisms are being developed from the genera *Phoma* and *Penicillium*. These have been shown to induce a systemic plant resistance against invasion by fungal pathogens such as *Colletotrichum orbiculare* (Meera *et al*., 1994).

With the cloning era, it has become possible to more elaborately investigate the relationship between PGP microbes and their host plants, and it is becoming obvious that some of the relationships are established by a complex pathway of low molecular weight biochemical signals that control gene expression. This is now very well described for the nodule bacteria (Geurts and Bisseling, 2002). However, in many other PGP relationships with microbes, these mechanisms are unknown or poorly described.

10.4 Issues for PGP Microbes and the Law

The application of PGP microbes in industries such as agriculture and horticulture raises several legal and moral questions. These include:

- Who owns the microbes if they have been developed from nature? Does ownership reside with the 'inventor' or with the sovereign country from whose environment the PGP microbe was isolated?
- Because the microbes can be widely distributed and are relatively easy to culture, and can mutate at high frequency, how is the IP surrounding their development protected?
- What are the implications of unintended alteration of natural microbial biodiversity by the introduction of exotic genes through the process of inoculation?
- What are the quarantine implications if PGP microbes are moved across borders in non-sterile carriers (such as peat or clay) that are traded globally?
- Are there health risks associated with the use of microbial inoculants from a genus associated with human, animal or plant pathogenicity?
- What is the efficacy of many of the less researched microbes?

10.4.1 Ownership

All of the common PGP microbes belong to relatively ubiquitous species and it is possible to find them in gardens, yards, farms, fields and forests, as well as in the remaining undisturbed areas of the globe where they evolved and remain to this day relatively undisturbed as a component of the natural biodiversity. Isolation of the microbes from their natural environment (their *in situ* location) is the first step in their exploitation, and it is perhaps in this action that 'ownership' may be borne, or at least contested.

In their *in situ* repositories, the microbes are probably dependent upon some form of host interaction for their survival and multiplication – plant, animal, insect or other

microbe. Few PGP microbes are competent saprophytes in isolation from their hosts. Once taken from their natural habitat, cultured and stored, microbes become *ex situ*. *Ex situ* repositories are usually in the control of an institution or an industry that exploits them. The major difference from *in situ* repositories is that in these *ex situ* collections, the microbe is usually cultured in pure form, in the absence of any host, and the full metabolic requirements of the microbe must be met from artificial sources. *In situ* repositories are, of course, relatively inexpensive to maintain; however, there are substantial sociological, legislative and community consultation procedures to work through to ensure they succeed because they generally occupy land owned by the state. *Ex situ* collections are the converse – of relatively low diversity, expensive to maintain and in private ownership.

The Convention on Biological Diversity (CBD) of 1993 sets out a series of articles that assign principles of conservation of genetic resources and rights of ownership, which are recognized in international law. The CBD puts forward the argument that conservation of genetic resources, including microbes, is 'a common concern of humankind'. It also sets principles for the fair and equitable sharing of the benefits arising from the use of genetic resources, notably those destined for commercial use. Importantly, the CBD is legally binding and countries that join it are obliged to implement its provisions.

The CBD is somewhat contentious in relation to PGP microbes in that it assigns sovereignty over natural resources to states (see Thornström, Chapter 20, this volume). The CBD also suggests that scientific experiments should be undertaken within the country of origin of the genetic resources, where possible. This is likely to be a difficult or impossible undertaking with PGP microbes, because response to inoculation is likely to be species and environment specific and not all countries have the necessary research infrastructure for experimentation.

Hence, the issue of ownership of PGP microbes has its roots in the origin of the microbe according to the CBD. The *in situ* repositories may be tapped only after establishment of a memorandum of understanding and a material transfer agreement with the authorities in the sovereign state. Subsequent to 1993, to otherwise exploit these repositories is an act of biopiracy. Microbes isolated before the 1993 agreement was enacted are not protected and this, combined with their ubiquitous nature, raises the issue of tracing the history of microbes and the date they were first identified or isolated (see Thornström, Chapter 20, this volume).

Differentiating strains of PGP microbes

While species of PGP microbes may be nearly ubiquitous, strains vary considerably. As an example, strains of RNB that belong to a single species and that nodulate a single species of legume can differ greatly in their nitrogen fixation and ecological properties. Molecular techniques, usually based upon some form of PCR (such as PCR-RFLP – PCR restriction fragment length polymorphism) can reliably differentiate microbial species at the strain level (Lee *et al*., 1997). However, there are obstacles to the application of PCR. First, not all microbes are readily amenable to PCR because specific primers are often required. Secondly, as we mentioned in the previous section, not all laboratories have the expertise to conduct this form of research. Techniques for reliably differentiating strains within the broad suite of PGP microbes (fungi, bacteria, archaea, viruses, algae, etc.) need to be developed, standardized and broadly adopted.

As with differentiating microbes, despite the wealth of molecular tools available, microbial taxonomy is in a state of rapid flux as we learn more about the lateral transfer of genes on mobile genetic elements (Boucher *et al*., 2007). There is little consensus among microbiologists on how to reliably classify many microbes below the genus level, particularly the bacteria (e.g. Young, 2010). Also, nomenclatural changes have the potential to unwittingly confuse the origins of some PGP microbes.

Microbes replicate very quickly and the conditions under which they are cultured can lead to genetic change (drift), mainly through the loss of plasmids or DNA units bearing non-essential genes. Although bacteria and fungi can be readily freeze dried or

lyophilized in glass ampoules for storage to minimize the opportunities for mutation, this is not always practicable. If a 'protected or owned' PGP microbe has undergone genetic drift, then perhaps it cannot be considered the same organism that was originally described in any invention.

10.4.2 Protection of intellectual property (IP) and the inventive use of PGPs

Protection by first to market

The contemporary attitude to protection of ownership of highly developed PGP microbes is to adhere to the principle of 'first to market'. Often, the new PGP microbe, its mode of application and the acquisition of data that testify to its efficacy, conditions for manufacture and pathway to commercialization takes several (3–10) years. The protection of the innovation by being first to market is considerable, and this is becoming a more popular approach compared with patent protection.

Protection by patent – products or processes?

Microbes isolated from the environment cannot be patented per se because they are not considered an invention (Stix, 2006). In contrast, a genetically modified microbe may be granted a product patent, because it can be argued that its modification is an invention. Thus, modifying *Escherichia coli* by insertion of genes to produce insulin in batch fermentation is patentable.

However, patents have been granted for inventions based upon microbiological processes, where the microbe produces a new or superior outcome, and these are referred to as 'process patents'. So a brewer may patent the application of a specialized yeast strain to deliver particular outcomes (e.g. a special flavour; a high level of alcohol). A fungus that produces a unique antibiotic may be patented under the process that delivers or purifies this new compound (e.g. US Patent 3,169,100). Similarly, a new microbe isolated from the environment and not previously described, but which can be used in a process for the treatment of plants can now be counted as an invention, and therefore can be patented. Examples in the RNB world are plentiful, with novel isolates being described almost daily. Ardley *et al*. (2011) described novel species of *Microvirga* isolated from the nodules of the legumes *Lupinus texensis* and *Lotononis angolensis*. These legumes, when taken from their natural environments (Texas and Angola, respectively) and introduced to agricultural environments, do not grow well without the application of their specific nodule bacteria, because of nitrogen deficiency. The application of these inoculants to the legumes to enhance growth and nitrogen fixation is theoretically patentable.

In the case of the associative microbes, a process patent can also be considered when the application of the microbe alters the composition of the plant physiologically and allows it ultimately to express different traits. For example, patent number 4,878,936 lodged in 1989 in the USA covers the application of 'helper bacteria' to increase the nodulation of legumes. This patent covers a method of inoculating legumes with *Bacillus cereus* ATCC 53522 and its enhancing mutants. Included in the patent is a method of placing the nodulation-enhancing bacteria in the vicinity of the legume roots by coating the seed with the nodulation enhancing bacteria and an inert carrier. This helper bacterium results in increased nodule weights, numbers, size and longevity. More recently, a bacterial co-inoculant has been described that enhances the rate of nodulation by RNB on a common pasture species, *Medicago truncatula* (Fox *et al.,* 2011). The earlier nodulation results in increased shoot yields and total N and could theoretically be patented as a process for increasing nitrogen fixation.

In the case of the exploitation of the toxin-free grass endophytes described earlier, there is IP protection surrounding the technology to remove the toxic strains of the fungus from fescue, and techniques to reintroduce new fungal strains into the seed, which then colonize the plant when it grows (Australian patent AU2003267883B2). In the case of this PGP, IP protection is based around the techniques of removal of one fungus and the introduction of its replacement. In the attempts to transfer the fungus to a new grass

genus, *Lolium*, this association is not found in nature and therefore also satisfies the 'invention' criteria.

10.4.3 Alteration of natural microbial biodiversity by application of PGPs: does covering the globe with a few strains threaten biodiversity?

Without doubt, the greatest global change in the distribution of microbes has come about through human exploration of the world since the 16th century. Feedstuffs carried for animals on ships contained, for example, RNB and legume seeds, which were distributed widely around the planet. Ships sailing from the Mediterranean basin introduced these legumes and their associated microbiota to sub-Saharan Africa, on to Australia, and more recently to both South and North America. Since then, the deliberate use of RNB as inoculants for legumes, particularly in the 20th century, has continued to perturb the natural distribution of PGP microbes. Massive changes have occurred in the tropics, subtropics and warm temperate zones of Africa, Asia and America, where *Glycine max* (soybean) inoculated with *Bradyrhizobium japonicum* now dominates grain legume production. There are nearly 70 million t of inoculated soybeans produced annually in the USA, in addition to 34 and 53 million t in Argentina and Brazil, respectively (Hungria *et al*., 2006). The RNB inoculants for this crop have thus been distributed over more than 150 million ha of the American continent in the last 30 years. There is now a similar attempt to introduce soybean to Africa (Giller *et al*., 2010), and plantings across the globe have been almost entirely inoculated with less than five strains – the *B. japonicum* serogroups associated with USDA123, USDA110, CB1809 and, more recently, *B. elkanii* strains SEMIA 597 and SEMIA 5019.

As for soybean in the Americas, large tracts of land have been cleared of their native vegetation in central Asia, temperate America and southern Australia, and planted to cool season forage legumes from two main genera, *Trifolium* (clovers) and *Medicago* (medics). Again, the majority of these legumes have been inoculated with RNB at some stage in their production. The perennial forage crop *M. sativa* has wide adaptation to soil and climate and, because of this, through human activities, it has spread from its centre of origin (believed to be in the temperate zones of Persia) to become the dominant forage on all continents, carrying its RNB with it. Annual clovers and medics were established across 25 million ha of arable land throughout southern Australia over the last 150 years of the 19th and 20th centuries, with RNB inoculants available since 1896. As in the case of legumes in the tropics, this represents a massive global change in the distribution of RNB.

It is of concern that there are perhaps only 50 species of forage legumes and less than 15 species of grain legumes in transglobal commercial trade (Kelley *et al*., 2000), inoculated accordingly with a narrow suite of RNB. Is it prudent, from a gene conservation perspective, to cover the globe so completely with only relatively few strains of RNB? We have evidence that these inoculants displace the original RNB, because of host preference for matched inoculants (Stepkowski *et al*., 2005). Not only may introduced inocula displace natural biodiversity, but we have recently learned that introduced PGP microbes may exchange their (exotic) DNA with indigenous microbes and, by doing so, greatly alter the properties of the recipient microbe. The recently described phenomenon of an exotic RNB inoculant strain which transferred its 'symbiosis island' to resident, but non-nodulating, microbes, concomitantly allowing these resident bacteria to become nodulating organisms (Nandasena *et al*., 2009), serves as a strong reminder of how easy it is to unwittingly alter the dynamics of the natural microbiota. What is the impact on the *in situ* conservation of microbial biodiversity in this example?

10.4.4 Quarantine transgressions

The supply and distribution of PGP microbes is becoming a transglobal trade, as fewer and larger companies dominate the industry, and oceans and authorities are no longer a substantial barrier to trade. Although

the distribution of PGP microbes is subject to a wide range of regulations and laws that specify product integrity and phytosanitary conditions – which are administratively tedious – these conditions are actually able to be met quite simply in a biological sense: the paperwork must only specify the genus and species of the traded microbe. While some PGP microbes may be distributed as spores (e.g. *Bacillus*) or as dried powders (e.g. yeasts) for multiplication in the hands of a secondary manufacturer, most PGP microbes are distributed in a hydrated, functional form (i.e. as vegetative cells) in carriers that sustain them. These carriers, such as finely ground and ameliorated peat, are very difficult to sterilize, and although methods such as autoclaving or exposure to gamma radiation are invoked, carriers are rarely sterile. In fact, the quality assessment (QA) applied to RNB in many countries acknowledges that contaminants must be less than 1000 cells g^{-1} (Deaker *et al.*, 2004). Thus, trade in PGP microbes is often also trade and distribution in associated microbes of generally unknown form or function.

10.4.5 Efficacy and attempts to regulate PGP microbial products

PGP microbes that stimulate plant growth have gained substantial acceptance in the rural communities of many developing nations, despite a lack of clarity about their efficacy. It is speculated that the reason for this is that, in many rural settings, it has been normal practice to gather and distribute animal wastes across fields to improve plant growth. These wastes contain decomposing microbes, as well as abundant N and P. The efficacy of this recycling is not disputed. However, the application of pure cultures of PGP microbes is seen by many as a sophisticated extension of the spreading of farmyard wastes (Dr Rosalind Deaker, University of Sydney, 2011, personal communication).

Because PGP microbes have become a large and lucrative market in broad-acre agriculture, they are a favourite target for misleading claims. There are several ways to protect against false claims for the efficacy of PGP microbes, such as QA registration processes and the development of industry standards and best practice, but the current proliferation of dubious products indicates that the issue is not resolved. One of the major difficulties is that applications of PGP microbes carry the uncertainty of a complex biology with them, and quite often they simply do not work. The multiple interactions between the genotype, or genetic make-up, of the target plant (G), the genotype of the PGP microbe (g) and the environment (E) in which it is applied can mitigate against success. There are many environmental factors that can reduce the efficacy of PGP microbes. Examples are hostile soils (dry or acid) in which a seed-applied PGP microbe cannot survive in sufficient numbers to colonize the rhizosphere. The endophytes are more resilient in this regard because they can be protected within the seed, rather than on its surface. There are also examples in which a minor change in G can have a major change in efficacy. The natural loss of DNA from a symbiotically sensitive region in RNB is a notable example of such an alteration that completely removes the PGP effect (O'Hara *et al.*, 2002). So, it is relatively common for PGP microbes to fail, even with the most efficacious of relationships, such as the legume–RNB association.

For the associative relationships, which are less stringent and in some ways less reproducible, efficacy is much harder to prove (and police). Some contemporary products of liquid PGP microbial applications claim the presence of more than 20 species of microbe (e.g. SC27 Soil Inoculant and Super Microbial Organic Complex), often in addition to extracts of exotic plants such as seaweed. To reproduce statistically sound experiments to test these products under a wide range of G × g × E is very difficult, and expensive, so claims of efficacy are not always verifiable.

Because of this uncertainty, there have been attempts to 'legislate' and 'register' PGP microbes in the past, and this provides the opportunity for legal redress. Legislation is often under the Fertilizer or Pesticide Acts, and registration is policed under consumer protection acts, or similar, depending on

the country. In Canada, PGP inoculants are regulated under the Fertilisers Act (Louter *et al*., Chapter 11, this volume) and administered by the Canadian Food Inspection Agency, which demands proof of efficacy as part of the registration process. This offers some assurances to consumers that the product works as claimed by the manufacturer. However, the most effective contemporary form of protection seems to be adherence to quality standards. Membership of a manufacturing group that adheres to this standard, identifiable by an emblem or statement, can imply that a product is quality assured. This places the responsibility more upon the user to undertake some form of due diligence before purchase. In Australia, a 'Green tick' logo is applied to inoculants which have:

1. labelling that shows the plant host for which the inoculant should be used;
2. an explanation of application method;
3. described storage conditions;
4. an expiry date/shelf life;
5. a guaranteed number of live RNB at point of sale; and
6. a batch number.

A further layer of confusion in relation to the efficacy of PGP microbes is imposed by the fact that they are live cultures, and therefore storage and shelf life become confounding considerations in their performance (i.e. the E component in G × g × E). Given that a sufficient number of microbes need to be present for most products to give effective results, this is an area where quality control programmes can be valuable. An example of this is the RNB inoculant industry, which has used research programmes to increase bacterial numbers in the inoculants, and then to marry them with carriers that provide a resilient shelf life to ensure the success of these inoculants. These quality control programmes can be supported by legislation, such as in Canada and France, or they can be voluntary, as in Australia and New Zealand (Herridge *et al*., 2002). Involvement of both the public and private sectors in establishing and managing a quality control programme for RNB inoculants has proven extremely successful in Australia, and may provide a good model for PGP microbes in other countries. In Canada, over-regulation was found to have a negative impact on the development and deployment of biopesticides, whereas streamlining the process improved uptake (Bailey *et al*., 2010).

10.4.6 Human health and environmental risk

The use of PGP microbes on a commercial scale seems to have largely escaped the regulatory scrutiny of microbes used in other areas, such as biocontrol agents (biopesticides) in functional group 4. This is somewhat ironic given that the microbes involved are often similar species. Perhaps this is a reflection of the fact that the first PGP microbes were direct, growth-enhancing, nodulating bacteria, and these have traditionally been considered non-pathogenic.

The Organisation for Economic Co-operation and Development (OECD) has established a steering committee to help identify the issues associated with the use of microbials as biocontrol agents and to give guidance to member regulatory agencies with regard to the identification of microbes and appropriate risk assessment procedures (see Meeussen, Chapter 21, this volume). Microbes registered for use as biopesticides, such as the entomopathogenic fungus *Metarhizium*, are subject to risk assessment processes similar to those used for new chemical pesticides. In the USA, the US Environmental Protection Agency (EPA) now regulates these microbes through the Federal Insecticide, Fungicide and Rodenticide Act (see Jaronski, Chapter 7, this volume). Similarly, in Australia, biopesticides are regulated by the Australian Pesticides and Veterinary Medicine Authority (APVMA), and are also subject to registration and risk assessment processes.

While, as far as can be ascertained, there has been no directive from the OECD on the use or assessment of PGP microbes, there are examples in which some are assessed under existing regulations. For example, in the USA, microorganisms that fix nitrogen with legumes and thereby promote growth are regulated under the Toxic Substances Control Act (TSCA), and it would be reasonable for

other PGP microbes to be included under this same guideline. The Canadian approach is more advanced, with all microbial inoculants submitted to the Canadian Food Inspection Agency subject to a science-based evaluation of product safety information, efficacy and labelling (Leggett *et al*., 2003). Similarly, in Australia, biological products require registration as they fall under the provisions of the Agricultural and Veterinary Chemicals Code Act 1994 (AgVet Code). By definition, any product deemed to alter the physiology of a plant (i.e. promote plant growth) is registered as a chemical agricultural product. European legislation is country specific but there are generally two routes to registration – as a fertilizer or as a plant protection product. Registration in the latter category is necessary if the PGP is listed in Annex I (Active substances authorized for incorporation in plant protection products) of the European Union (EU) Council Directive 91/414/EEC (OJEC, 1991). Registration as a fertilizer must be accompanied by data from at least ten efficacy and phytotoxicity experiments. A greater emphasis is placed on non-target behaviour and residual activities in the EU legislation (Directive 91/414/EEC) than in Australia, or in the Americas (Hauschild *et al*., 2011).

One difficulty with safety assessment for these organisms is the complication that arises from imprecise identification and description of different species of microorganisms, particularly the bacteria, as alluded to in Section 10.4.1 (Differentiating strains of PGP microbes). A relevant example is that of the early approval of several *Burkholderia cepacia* strains as biocontrol agents of fungal diseases and nematodes in the late 1980s. *B. cepacia* is commonly isolated from the sputum of patients with cystic fibrosis, but is generally considered to be a colonizing bacterium rather than an infecting bacterium. However, a low proportion of patients colonized with *B. cepacia* may succumb to the 'cepacia syndrome', which leads to death, within weeks or months of colonization. At the time of the approval, the authorities were supplied with extensive biochemical and taxonomic characterization data which classified the strains into groups that were considered as environmental strains and therefore non-pathogenic (Parke and Gurian-Sherman, 2001). As bacterial taxonomy progressed, data became available that one of these strains actually possessed a DNA region known as the *B. cepacia* Epidemic Strain Marker (BCESM), which had been used to distinguish between the pathogenicity of clinically isolated strains. This put some doubt on the original approval and raised other questions as well, which resulted in the inoculant being voluntarily removed from sale. At what point can we be assured that we know enough about a microbe so that there can be reasonable certainty about its safety? It is worth remembering also that many inoculant delivery methods, or carriers, of PGP microbes, such as peat and clay, have a very pronounced fine fraction that readily becomes aerosolized.

The contemporary increase in genome sequencing capacity allows some positive progress on this front. As *Burkholderia* strains have recently been discovered as nodulating organisms of the forage legumes in South Africa (Garau *et al*., 2009), it is likely they will be developed as inoculants for new agricultural legumes in this decade (Howieson *et al*., 2008). We now have the ability to screen whole genomes for genes associated with pathogenicity and mammalian colonization (see Wassenaar and Alter, Chapter 13, this volume). In fact, this was recently achieved for four *Burkholderia* strains and the process has identified those which may be suitable for use as inoculants, with minimal human or environmental risk (Hirsch *et al*., 2009). This sequencing technology represents an opportunity to take an important step towards mainstream acceptance of microbial inoculants by reducing the uncertainty that often accompanies new products.

10.5 Conclusions

The PGP microbes are a fundamental asset in many primary production settings, and they have been exploited for thousands of years. However, there exists a lack of clarity or focus on impediments relating to their continued refinement, such as quarantine transgressions, ownership, IP protection and alteration

to microbial biodiversity in the sovereignty in which they are applied. This is perhaps a reflection of the relative invisibility of many of these microorganisms, as much as a reflection of their biological complexity. Disease-causing agents, by way of contrast, have received far more attention in research and policy, as crop losses and other detrimental effects are more visible, and economically quantifiable.

It is contended that the uptake of these beneficial products will be expedited by developing and applying benchmarks, such as expectations of efficacy, accepted principles of ownership and QA standards in their manufacture. The 1993 Convention on Biological Diversity (CBD) makes a start in dealing with issues of ownership and exchange of germplasm. However, we acknowledge that not all PGP microbes are efficacious in all settings, because of the vagaries of biological interactions when dealing with live products, and consumers and regulators must be aware of this.

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11 Regulation of Microorganisms Used for Bioremediation, Biorefinery and Other Bioindustrial Applications in the USA and Canada*

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11.1 Introduction

Microorganisms (such as bacteria and fungi) are naturally occurring, ubiquitous and necessary for our environment to function. The vast majority of these microorganisms are fundamentally harmless to humans, animals and the environment. Clearly, there are several potentially harmful bacteria and fungi that do present a public health or environmental risk (e.g. *Staphylococcus*, *Escherichia coli*, *Salmonella*, *Fusarium graminearum*), but it is easy to forget that most microorganisms are beneficial and necessary for our healthy

* The views expressed in this chapter are those of the authors and do not necessarily reflect the views or policies of their respective organizations.

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existence and survival. It is estimated that there are between 500 and 1000 different species of bacteria in and on our body, each hard at work digesting our food, outcompeting potentially pathogenic microbes, producing the vitamins needed to keep us healthy, and boosting our immune system to help ward off illness. In fact, over 90% of the cells in a normal healthy human are bacterial cells! This is a good example of a symbiotic relationship whereby beneficial microorganisms contribute to the metabolism of food in the gastrointestinal tract and provide essential vitamins and nutrients, as well as performing the vital functions that keep all of us fit and well.

Away from our own bodies, microorganisms work to maintain critical life cycles in nature. They break down organic materials (e.g. in the compost heap), help plants to grow and thrive, and some even degrade toxic chemicals. As our understanding of the benefit of microorganisms has grown, scientists have discovered ways of harnessing the capabilities of these microorganisms to perform various tasks: among others, to ferment wine, break down man-made waste, and enhance the effectiveness of cleaning and odour-control products. As a result, there has been an increase in the inclusion of such microorganisms in both industrial and consumer products. In parallel with this trend by commercial interests to increase the use of microorganisms in products, and the desire by consumers for 'natural' or biologically based products has come increasing awareness of the need for preventing, where possible, any adverse effects to humans or the environment that may arise from their use. Regulatory authorities are already in place in the USA, Canada and a number of other countries to assess the potential risks.

The uses of microorganisms for industrial applications such as biodegradation, bioremediation or biofuel production are just a few of the many possible uses for microorganisms outside the agricultural and food realms. In the USA and in Canada, regulation addresses *any* use of a microorganism that is not already regulated under a comparable, 'commodity'-specific regime (such as food or a pesticide use).

In the USA, an important (but not the only) criterion for regulation is whether or not the microorganism concerned has been produced using gene sequences from different taxonomic genera. Naturally occurring and intrageneric microorganisms are implicitly on the Toxic Substances Control Act Chemical Substance Inventory and thus are not subject to pre-manufacturing review. The USA also has authority to regulate existing microorganisms already on the Inventory.

In Canada, there is no implicit listing of microorganisms on the equivalent list (the Domestic Substances List). Therefore, the range of microorganisms (naturally occurring and genetically modified) that are subject to 'New Substances' review is greater. As in most countries, this regulatory difference reflects a policy approach based on differing scientific, historical and social factors. In addition, in Canada, microorganisms that were in use between 1984 and 1986 (i.e. so-called 'legacy' strains) were considered to be existing and were exempt from the Regulations concerning new substances. In Canada, these are now in the process of being assessed for possible adverse effects.

The major use of microorganisms in industrial and commercial applications has been for the production of microbial fermentation products such as fine and speciality chemicals, predominantly enzymes. Enzymes are used as processing aids and catalysts for a variety of commercial and industrial applications including laundry detergent, textiles, food, beverages, feed and biofuels. Viable microorganisms are also used in a wide variety of industrial and consumer applications, including waste-water treatment, bioremediation, drain cleaners, grease-trap interceptors, floor cleaners, septic treatments, odour control, organic matter degradation and enhanced nutrient utilization.

In this chapter, the authors will discuss various attributes of the regulation of such bioindustrial microorganisms, including bio-based consumer products in the USA and Canada, beginning with a few examples and proceeding to a description of the regulatory systems and assessment processes in the two countries. A commercial perspective of the need for good quality control of bioproducts will complete the chapter.

11.2 Some Examples of Bioindustrial Uses of Microorganisms

An overview of the various uses of microorganisms is given in another chapter (Sundh *et al*., Chapter 1, this volume). However, a few of the industrial applications of microorganisms that deserve special mention here are their use in bioremediation and in biorefineries.

11.2.1 Bioremediation

The use of microorganisms to clean up environmental contamination by harmful chemicals is a decades-old application. In many cases, the practice has been to monitor a polluted site and let nature 'take its course' (*natural attenuation*) or to physically or chemically augment the growth of endemic, naturally occurring microbes at the site of the contamination (*biostimulation*). When microorganisms are intentionally grown (produced in a fermenter) and added to the contaminated environment for clean-up, it is called *bioaugmentation* (Drahos *et al*., 1992). At this point, depending on the jurisdiction and the organism, the activity may be subject to regulation at the federal or local levels of government.

A newer approach to the use of microorganisms has involved genetically engineered endophytic microorganisms as a component of phytoremediation (the use of plants to enhance the breakdown of contaminants). Field trials of such endophytic bacteria (Weyens *et al*., 2009) have taken place in Europe, and similar trials are also expected in the USA.

11.2.2 Biorefineries

The integrated biorefinery may prove to be the largest new development in industrial microbiology in decades. The concept is to utilize the metabolic properties of microorganisms, often genetically engineered, to substitute for organic chemical processes that often have required use of problematic chemicals and significant energy input. Through this development, not only traditional fine and speciality chemicals can be made, but commodity chemicals can be produced in volume as well.

One important stimulus for the expansion of the integrated biorefinery has been the development of the biofuel industry in the last decade. Initially, yeasts were utilized to produce ethanol, with fungi employed to pretreat biomass to enhance fermentation. In recent years, genetic engineering of these microorganisms has dramatically enhanced yield and efficiency, and permitted the use of bacteria as ethanologens (to ferment lignocellulosic feedstocks to ethanol) as well (Merino and Cherry, 2007; van Maris *et al*., 2007; Quinlan *et al*., 2010).

The future of this industry, however, may not be limited simply to ethanol production. Bacteria and algae are being engineered to produce a range of fuels from biodiesel and jet fuel to 'green' gasoline (petrol). Hydrogen gas is also being investigated as a fuel. The production of these commodity chemicals may not necessarily be the prime economic driving force for the further development of industrial microbiology. Rather, many are evaluating ways to redirect metabolic flux to make high value 'co-products', the production of which may be more profitable than that of high-volume fuels. A single facility can thus be flexibly used to make high-volume fuels, plastic monomers or low-volume high-value chemicals depending on the microbes used, or the conditions of fermentation. The use of synthetic biology is a technique that may rapidly advance this industry.

Most of the microorganisms now being considered for applications of the integrated biorefinery are subject to existing laws and regulations as described below. Some will present unique safety and environmental review scenarios, such as the use of algae in ponds, or of microbes made primarily through synthetic biology, but the procedures are in place to perform these reviews in the USA and in Canada.

11.3 Legislation and Regulation

11.3.1 The USA

The US federal government established a Coordinated Framework (OSTP, 1986) for the oversight of biotechnology in 1986,

confirming that regulatory authorities existing at that time were sufficient for this task. In this context, the Toxic Substances Control Act, 1976 (TSCA, 1976), which is administered by the US Environmental Protection Agency (EPA), is the primary statute for oversight of biotechnology applications in the general industrial and commercial sector. Microorganisms and their DNA molecules are 'chemical substances' under Section 3 of TSCA, and therefore are subject to all the provisions of TSCA, except to the extent they are manufactured, processed or distributed in commerce for use as pesticides, foods, food additives, drugs, cosmetics and medical devices that are subject to review by other federal agencies as authorized by separate statutes, including those for tobacco, nuclear materials, pharmaceuticals and cosmetics, and pesticides (but not pesticidal intermediates). However, the range of potential applications of biotechnology beyond these excluded categories is large, and TSCA was seen as filling large gaps in oversight that the other established federal authorities could not cover. Examples of these uses include: biofuel production, biomass conversion, waste treatment, bioremediation, biomining, mineral leaching, oil recovery, desulfurization of fossil fuels, nitrogen fixation, biofertilizers, biosensors, cleaning, and closed-system fermentation for the production of enzymes and speciality chemicals. In 1997, a 'Biotechnology Rule' (US EPA, 1997) was published that formalized the application of TSCA to commercial and industrial biotechnology as envisioned by the Coordinated Framework of 1986. Thus, through the use of the Coordinated Framework and the formalization of TSCA biotechnology regulations, lead authority for the review of non-agricultural industrial biotechnology was placed in the hands of the US EPA. The oversight roles in the US federal government are not entirely exclusive; for example, potential overlap exists for some agricultural uses. Governmental units with regulatory responsibility must still comply with the statutory mandates that they administer. But there is a clear delineation of lead authority and appropriate agency interaction has taken place when rare instances of overlapping responsibility have been found.

TSCA regulation of microorganisms

The majority of US EPA oversight of industrial biotechnology microorganisms occurs through the pre-manufacturing review authority in Section 5 of TSCA (15 U.S.C. §2604). Regulations implementing the TSCA Section 5 reporting requirements for microorganisms are limited to those microorganisms that are considered 'new'. These implementing regulations are contained in Part 725 of Title 40 of the US Code of Federal Regulations (CFR). Another part of TSCA, Section 8(b) (15 U.S.C. §2607(b)(1)), contained in Part 710 of CFR Title 40, requires that the EPA maintains a TSCA Inventory of Chemical Substances ('TSCA Inventory'), which includes all such substances in commerce. Any such substances not listed are, by definition, 'new'. Simply put, new substances are those not listed in the TSCA Inventory (similar to Canada's use of the Domestic Substances List). The EPA also has authority under TSCA (e.g. Sections 4, 5 and 6) to regulate existing microorganisms that are already on the TSCA Inventory.

Included within this definition of 'new' are microorganisms that result from the deliberate combination of genetic material from organisms classified in different taxonomic genera – termed 'intergeneric'. Also included in intergeneric microorganisms is a microorganism constructed with synthetic genes that are not identical to the DNA that would be derived from the same genus as the recipient (the majority of the genome) cell. The production and use of microbes that are 'new' must follow the requirements of TSCA Section 5, which mandates pre-manufacturing notifications to the EPA that enable review before a microorganism can be listed on the TSCA Inventory. Exemptions from these reviews include naturally occurring microorganisms, as they are considered to be implicitly listed on the TSCA Inventory, genetically engineered microorganisms other than those that are intergeneric (e.g. intrageneric, physical or chemically mutagenized microorganisms), and intergeneric microorganisms resulting only from the addition of well-characterized, non-coding regulatory regions.

Categories of pre-manufacturing oversight: the microbial commercial activity notice (MCAN)

Before the manufacture, processing or importation of an intergeneric microorganism, companies must make an appropriate submission to the EPA under Section 5 of TSCA contained in 40 CFR, Subpart D of Part 725 of the Biotechnology Rule, which establishes the reporting programme for new microorganisms. Persons intending to manufacture or import intergeneric microorganisms for commercial purposes in the USA must submit a Microbial Commercial Activity Notice (MCAN) to the EPA at least 90 days before such manufacture or import, unless the organisms are specifically exempted. The EPA then reviews the submission in order to determine whether the intergeneric microorganism may present an unreasonable risk to human health or the environment. If the EPA makes that determination, it may impose appropriate regulatory restrictions on the microorganism. If the EPA makes no determination within the 90 day time frame, the subject microorganism is eligible to be placed on the TSCA Inventory, manufactured, used or imported. Brief extensions of the time frame may be negotiated to permit gathering and review of additional relevant information and data, but these are not commonplace.

Exemptions from the MCAN pre-manufacturing notification

RESEARCH AND DEVELOPMENT EXEMPTION. One exemption from MCAN reporting is the Research and Development (R&D) Exemption for certain R&D activities that are conducted in contained structures and are subject to regulation by another federal agency. Applications that meet these criteria are exempt from EPA review, reporting and record-keeping requirements for contained research conducted by researchers who are required to comply with the National Institutes of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (NIH, 1994). All other manufacturers conducting contained TSCA R&D activities may qualify for a more limited exemption. This exemption for R&D in contained structures specifies factors that a Technically Qualified Individual (TQI) must consider in selecting the appropriate containment. A structure is defined as a building or vessel which effectively surrounds and encloses the microorganism and includes features designed to restrict the microorganism from leaving. In proposing the Biotechnology Rule, the EPA envisioned that this exemption would most likely apply to research performed in contained structures such as pilot fermentation plants, greenhouses, laboratories and certain bioreactors used for waste treatment. However, other forms of structures could be used. The EPA's approach relies on the experience and judgement of the TQI, recognizing that many different kinds of microorganisms displaying a wide range of characteristics could potentially be used in research. It also recognizes that appropriate types of controls (e.g. procedural, mechanical, and/or engineering) will vary with the microorganism and type of research. The EPA expects that the TQI will be cognizant of these factors when selecting containment and inactivation controls appropriate to the microorganism(s) being utilized. The TQI is required to keep records to document both compliance with the containment requirements and compliance with the notification process for employees involved in the R&D process.

TSCA EXPERIMENTAL RELEASE APPLICATION (TERA). The TSCA Experimental Release Application (TERA) under TSCA Section 5, described in Subpart E of Part 725 at 40 CFR §725.238, is an exemption from MCAN reporting for commercial R&D involving an intentional environmental release of an intergeneric microorganism. This is likely to be a common activity for many bioremediation uses, as these generally involve some release of subject microorganisms. The TERA is essentially an abbreviated MCAN for a field test application with a shortened review period of 60 days, although the EPA may extend the review period for good cause. The EPA must approve the test before the researcher may proceed, even if the 60 day period expires, and approval is limited to the conditions outlined in the TERA notice or approval.

A significant difference between the requirements for an R&D exemption and the need for a TERA is the consideration of the use of a structure for containment. As indicated above, the EPA may interpret the definition of a structure broadly, using the experience and judgement of the TQI to select appropriate containment measures tailored to the needs of the microorganism and the research. The intent of the R&D exemption is to freely permit research with contained microorganisms that meet the criteria of the Rule, while the intent of a TERA is to provide oversight of research having some expected release of the microorganism. This takes into account the reality that a released microorganism cannot be recalled. Therefore, the EPA encourages potential researchers who wish to perform their research in atypical contained structures to confer with the EPA before initiating their efforts to confirm whether or not a TERA application is required.

TIER I AND TIER II EXEMPTIONS. There are exemptions from MCAN reporting for certain microorganisms which are manufactured within closed systems and which meet several other criteria. These Tier I exemptions require meeting three basic categories of criteria: (i) employment of a microorganism with a history of safe use; (ii) criteria that ensure the safety of the introduced DNA; and (iii) use of one of the ten recipient organisms listed at 40 CFR §725.420 of the Biotechnology Rule that have undergone categorical risk assessment as a species, or as a group of strains within a species, or any such microorganism subsequently listed through a petition process described in 40 CFR §725.67. A manufacturer is not required to wait for EPA approval before commencing manufacture when all these conditions are met, but must provide a simple notification within 15 days of commencing manufacture. A manufacturer, who otherwise meets the conditions of the Tier I exemption, may utilize modifications to the specified containment restrictions, but must submit a Tier II exemption notice that allows the EPA to review those modifications.

TEST MARKETING EXEMPTION (TME). An additional exemption from MCAN reporting requirements is the Test Marketing Exemption (TME) noted at 40 CFR §725.300. Test marketing activities usually involve limited sale or distribution of a substance within a predetermined period of time to determine its competitive value when its market is uncertain. This exemption has rarely been used for biotechnology applications. Most manufacturers who intend to test market a new microorganism file a MCAN rather than request a TME. However, there may be situations in which this exemption is appropriate, such as for microorganisms which were previously reviewed by the EPA at the R&D stage, but are not ready for full commercialization. In addition to the general administrative requirements, certain technical information is required for each TME submission, making the review similar to an abbreviated MCAN review.

11.3.2 Canada

The Government of Canada issued a statement in January 1993 announcing the 'Federal Regulatory Framework for Biotechnology'. Among the principles of this framework was one that expressed that existing legislation and institutions would be used to clarify responsibilities and avoid duplication. The Minister of the Environment at that time indicated his intention to work with other Ministers to ensure that appropriate environmental assessments would be conducted for all products of biotechnology. This intention found its expression in the 1999 Canadian Environmental Protection Act (CEPA, 1999) which created a mechanism and criteria for environmental and human health assessment before import, manufacture or sale. If other legislation met the same pre-market assessment criteria, products assessed under that legislation would be exempted from assessment under CEPA. Such statutes were listed in Schedule 4 of CEPA and included the Seeds, Feeds, Fertilizers, Health of Animals and Pest Control Products Acts. In the case of microorganisms, if the use of the microorganism does not fall within the scope of these acts, it is subject to the 'New Substances' provisions in CEPA and its New Substances Notification

Regulations (Organisms), or NSNR (O) (Department of Justice, 2005). Examples of such uses include those that fall under TSCA in the USA (including intergeneric microorganisms used in biofuel production, waste treatment, bioremediation, biomining, oil recovery and desulfurization of natural gas) but also include naturally occurring microorganisms that are 'produced' for use in these applications. In Canada, biofertilizers are regulated under the Fertilizers Act. The relationship among these Canadian statutes is illustrated in Fig. 11.1, using a filter column as a metaphor for their interaction.

The scope of substances considered to be 'new', and so subject to the NSNR(O), is determined by whether or not the substance is present on the Domestic Substances List (DSL). The DSL lists all those substances, including certain microorganisms, that were in commerce between 1984 and 1986, and functions in a manner similar to the TSCA Chemical Substance Inventory in the USA in that these organisms are exempt from the new substances regulations because they were in use before those regulations came into effect in 1997. In a process that began in 2004, these 'legacy' microorganisms (about 65 strains) are being assessed by the Government of Canada in order to determine whether or not they present a risk to human health or the environment and whether they should, therefore, be subject to risk management actions.

The NSNR for products of biotechnology were first published in1997 and slightly revised and renumbered in 2005, at which time they became known officially as the NSNR (O) (Department of Justice, 2005). The regulations specify, in one of five Schedules, the information to be provided depending on the activity proposed. Table 11.1 provides a short title for each Schedule and gives the duration of the assessment period.

In the event that a notifier believes that the proposed activity is such that some information elements (normally data elements) are not needed (or cannot be obtained) to complete the assessment, then a request for a 'waiver' from providing that element can be made. The acceptance of such a request will be published in the *Canada Gazette* (the official Government of Canada publication for legal notices and proposed and final regulations).

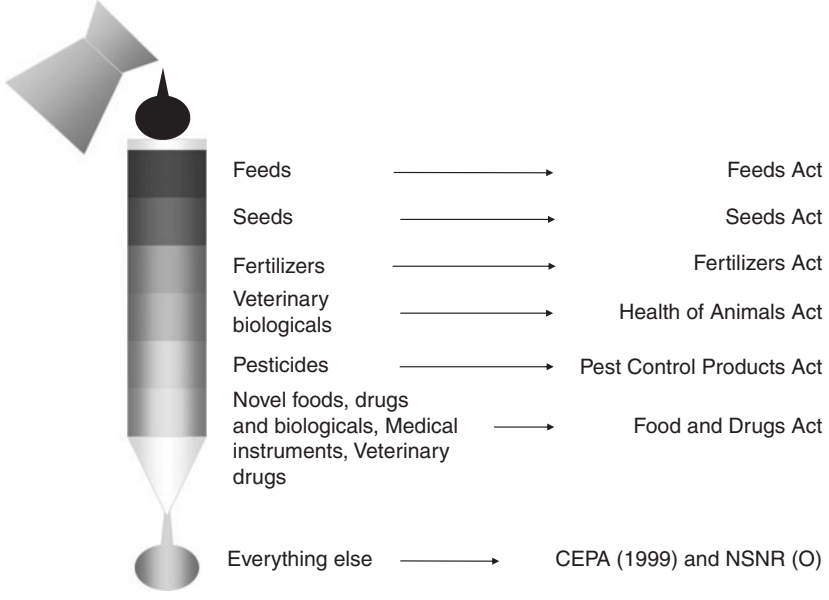

Fig. 11.1. Regulation of various bioproduct types and the legislation that governs them in Canada. CEPA, Canadian Environmental Protection Act; NSNR (O), New Substances Notification Regulations (Organisms) (of CEPA).

Table 11.1. Schedule numbers and titles of regulatory groupings within the Canadian New Substances Notification Regulations (Organisms) and the maximum assessment period for each.

Schedule number	Description	Time (days)
1	Introduction of a microorganism anywhere in Canada	120
2	Microorganism not for introduction outside a contained facility/export only	30
3	Microorganism for introduction in an experimental field study	90
4	Microorganism for introduction at the same site where isolated and manufactured	30
5	All organisms other than microorganisms	120

When the information in the notification package is deemed complete (contains all of the prescribed information or accepted justification for waivers from certain information elements), evaluators from the government authorities Environment Canada and Health Canada conduct a joint risk assessment to determine whether or not the organism is – or is capable of becoming – 'toxic' ('toxic' is a prescribed term in CEPA, Section 64; it includes potential adverse effects on the environment or on human health).

This risk assessment must be completed within timelines prescribed in the Regulations, which range from 30 days for a contained activity with a microorganism to 120 days for an activity resulting in full release of the microorganism into the Canadian environment (Table 11.1). Both direct environmental effects and indirect human health effects are taken into consideration when arriving at the final assessment conclusion. Import or manufacture of the organism may only begin once the assessment period has expired and any necessary risk management measures for organisms found to be toxic are in place.

Exemptions to the NSNR (O) regulations include those activities covered by one of the Acts cited in Fig. 4.1, and also organisms that are in transit – loaded on a carrier outside Canada and moved through Canada to a location outside Canada, whether or not there is a change of carrier during transit, Subsection 2(2) of the NSNR (O).

Subsections 2(3) and 2(4) exempt microorganisms and higher organisms that meet the definition of an R&D organism. This definition states that a R&D organism that is undergoing systematic investigation or research by means of experimentation or analysis other than test marketing, whose primary objective is any of the following: (i) to create or improve a product or process; (ii) to determine the technical viability or performance characteristics of a product or process; or (iii) to evaluate the organism prior to its commercialization, by pilot plant trials, production trials, including scale-up, or customer plant trials so that technical specifications can be modified in response to the performance requirements of potential customers.

For microorganisms, containment guidelines such as the Public Health Agency of Canada's Laboratory Biosafety guidelines, or Appendix K of the NIH Guidelines (NIH, 1994) are recognized as acceptable for adequate containment when applied correctly based on the risk level of the microorganism(s). Thresholds in volume are also in place; in order to be exempt from notification, the regulations require that: (i) import volumes into a contained facility be in a quantity of less than 50 ml or 50 g; (ii) manufactured quantities of the microorganism at any one time be less than 1000 l in volume when the organism is considered to be biosafety level 1 (as determined by the Public Health Agency of Canada); (iii) where there is a requirement for containment level 2, manufacture and presence at any one time in a contained facility is less than 250 l in volume; or (iv) if the organism is a human pathogen and a level 3 or 4 containment is required, manufacture or presence of the microorganism at any one time in a contained facility is less than 250 l in volume and a permit or an approval in writing to transfer has been granted under the Human Pathogen Importation Regulations.

11.4 The Risk Assessment Process

11.4.1 The USA

Under the pre-manufacturing review programme authorized by Section 5 of TSCA, within the time period specified by rule or statute, which varies according to the type of submission, the EPA conducts a risk assessment on the 'new' microorganism, under the paradigm that 'Risk is a function of Hazard × Exposure'. Separate assessments for various elements of risk are integrated into a final risk assessment. The components include:

1. a verification of the identification of the subject microorganism;
2. a human health hazard assessment;
3. an ecological effects hazard assessment;
4. a report that analyses the construction of the microorganism and summarizes the pertinent chemical information and production volume;
5. an analysis of the genetic construct that evaluates any potential hazards associated with the genetic modifications and the potential for horizontal gene transfer;
6. an engineering report that assesses manufacturing processes affecting worker exposure and microbial releases to the environment through manufacturing or during field applications; and
7. an exposure assessment that evaluates the potential for survival, reproduction and spread of the microorganism, and the exposure of environmental receptors and of the general population.

Unlike review under CEPA in Canada, there is no specified schedule of information elements under Section 5 of TSCA for reviews of new substances. Rather, submitters must provide to the EPA all relevant data and information in their possession or reasonably ascertainable. These data must be sufficient to enable the EPA to complete a risk assessment. If a submission of any type contains insufficient information to proceed with a review, the EPA may request the submitter to allow the review time to be extended in order that they may provide the necessary information. The EPA also has risk management options that may be employed to mitigate the effect of uncertainty due to data or information limitations, as described in the last paragraph of this section.

Because TSCA is a risk–benefit statute (unlike CEPA in Canada), the risks of using the microorganism determined in the assessment are weighed against the benefits to society (which are evaluated in an economics analysis) to arrive at the final risk management decision. Several outcomes are possible as a result of the review process. Under the pre-manufacturing review programme authorized by TSCA Section 5, the EPA may determine that there is sufficient information to conclude that the microorganism presents 'no unreasonable risk of injury to human health or the environment', in which case the Agency takes no regulatory action and the company may take action (e.g. commence manufacture, begin research or test the market) after the regulatory time frame expires. The time frames are firm, so that no action may take place until the time has expired, even if the EPA finishes its review early. In the case of the MCAN, if the EPA does not otherwise notify the submitter by the end of the 90 day period, the latter may commence its commercial activity without constraint.

Another outcome may be that there is sufficient information to determine that the microorganism presents 'an unreasonable risk of injury to human health or the environment'. In this case the EPA has a wide range of means to prohibit or restrict the production or use of the microorganism, from limits to size or method of production, to a complete ban on production and use. A last category of outcomes includes the case previously mentioned where there is insufficient information to determine for certain that the risk is 'unreasonable', but there is sufficient information to imply that possibility exists for unreasonable risk and/or substantial/significant exposure. In such cases the Agency may negotiate a Section 5(e) Consent Order to restrict the use of the microorganism and to specify the data needed to lift the Consent Order. If the submitter does not agree to negotiate with the EPA, the Agency may impose restrictions based on the information available and the submitter may choose to use the courts to

seek relief. However, this last option has never been considered by any manufacturer seeking to produce a biotechnology-derived microorganism subject to TSCA. The need for Consent Orders has also proved to be a rare occurrence.

11.4.2 Canada

The risk assessment process, outcomes and risk management measures described in this section are applicable to all microorganisms subject to the NSNR (O). The initial source of information used in the risk assessment is that provided by a proponent through the notification package (schedule as dictated by the proposed activity; see Table 11.1), but evaluators also use in-house information and any additional information available in the public domain. This information helps to assist the evaluators in determining both potential hazards associated with the organism and potential pathways of exposure to them, in order to estimate risk and the likelihood that an organism is – or is capable of becoming – 'toxic' as defined under Section 64 of the Act. Therefore, Section 64 effectively provides the end points to be addressed through risk assessment. If necessary, evaluation teams may contact external experts (both in Canada and abroad) to further inform the risk assessment without divulging any confidential business information. These consultations are generally driven by specific technical or scientific questions posed by the evaluation teams and are only used when in-house expertise cannot adequately address uncertainties.

The information elements listed in each schedule provide the minimum information that must be provided by the proponent so that evaluation staff may begin the risk assessment. In broad terms, the information required to be provided in a notification package includes information in respect of: (i) the identification and characteristics of the organism (including any genetic modifications); (ii) the manufacture and import details of the organism; (iii) the introduction of the organism into the environment (including any containment or other measures in place to prevent introduction into the environment); (iv) the environmental fate of the organism; (v) the ecological effects of the organism; and (vi) the human health effects of the organism. Note that there is no assessment of benefits or evaluation of performance claims under this act and regulation.

The evaluation and risk assessment is a joint responsibility between two Ministers: from Environment Canada, which conducts an assessment for environmental effects, including any effects on biodiversity; and from Health Canada, which conducts an assessment for indirect human health effects (that is, exposure resulting from the environmental release of the organism). As mentioned earlier (Section 11.3.2), direct human health effects may be assessed under other legislation, such as the Food and Drugs Act, as appropriate. The ability to import or manufacture under CEPA does not exempt the notifier from any other laws or regulations that are in force in Canada and that may apply to the organism or activities involving the organism.

Currently, a memorandum of understanding exists between Environment Canada and Health Canada for the risk assessment of organisms contained in products regulated under the Food and Drugs Act. Such assessments, conducted by Health Canada's Environmental Assessment Unit, assesses environmental risk under CEPA on behalf of Environment Canada for organisms that have been notified under the Food and Drugs Act.

The Framework for Science-Based Risk Assessment of Microorganisms outlines the systematic steps used by the Health Canada and Environment Canada substances assessment programmes to conduct science-based risk assessments of microorganisms (Environment Canada and Health Canada, 2011). The assessment is based on whether the microorganism is toxic as defined under Section 64 of CEPA. In other words, it describes a *framework* of the basic concepts considered when conducting this assessment based on available information on hazard and exposure for both 'existing' (i.e. those on the Domestic Substance List) and 'new' microorganisms (i.e. those notified under the NSNR (O)). The approach is

structured into a series of steps to ensure that microorganisms undergo a rigorous, consistent and expeditious analysis to determine whether or not they are toxic, as defined under Section 64 of CEPA, using the paradigm that risk is proportional to the product of hazard and exposure.

$$\text{Risk} \propto \text{Hazard} \times \text{Exposure}$$

During risk assessment of DSL microorganisms, the identification and determination of the severity of hazard are based on an exhaustive literature review and other sources, such as experimental data from completed and ongoing research projects and decisions from other jurisdictions. For 'new' microorganisms, risk assessment is based on the information provided, as required under the NSNR (O). The determination of exposure sources and characterization of exposure levels are based on available information describing current known use patterns, proposed uses or other potential uses. This risk is characterized depending on the hazard severity and potential for exposure, and taking into consideration the weight of evidence and scientific uncertainties.

Possible risk assessment outcomes and risk management measures

There are three possible outcomes as a result of a risk assessment. First, if there is no suspicion that the organism is toxic or capable of becoming toxic, then no further action will be taken by the government and the activity can proceed.

A second possible outcome is that both Ministers (from Environment and from Health) determine that the organism is 'not toxic' for the notified activity (use), but that one or both Ministers suspect that the organism may become toxic should it be used for an activity that is significantly different from that which has been proposed and assessed. In other words, a 'Significant New Activity' (SNAc) with the organism could change the exposure and result in a different risk conclusion. In the case where a 'new activity' is proposed, the government has the authority to require re-notification and assess the new information before allowing the import or manufacture of the microorganism for that new use. The original risk assessment provides the basis for the publication of a 'SNAc Notice' in the Canada Gazette. In addition to identifying the organism to which the SNAc applies, the notice typically also includes a description of what constitutes a significant new activity with respect to the assessed organism. Should someone decide to request a new activity for that microorganism, the notice will also generally include the new information requirements for the 'Significant New Activity Notification' (SNAN) and the timeline within which the assessment would take place.

The third possible outcome of a risk assessment is a suspicion by either Minister that the organism is or may become toxic with respect to human health or the environment. In response to this outcome, control measures may be applied to minimize any risk to the environment, biological diversity or human health. These may include the ability to manufacture or import subject to any conditions that the Ministers may specify, the adoption of 'best practices' or a pollution prevention plan, but could include prohibition from manufacture or import. In the latter case, the development of specific regulations for the organism within 2 years is required, or the prohibition may be in effect until supplementary information or test results have been submitted and assessed. Generally, the choice of the instrument chosen is the result of consultation with the affected notifier. In the case of a prohibition, and the creation of a new regulation, then a socio-economic analysis, will also be required to accurately determine the cost of the regulation to the notifier and to justify this instrument choice in the context of the risk identified in the assessment.

Microorganisms that have been assessed under a full release scenario (Schedule 1) may be eligible for addition to the DSL unless conditions are in place on the use of the organism. Once on the DSL, notification is no longer required in advance of import or manufacture unless this is proposed for a significant new activity as specified (or flagged) on the DSL.

11.5 Non-regulatory Programmes

11.5.1 The USA

While reviews based on TSCA regulations provide the dominant oversight mechanism for many microorganism-based products, the EPA does occasionally evaluate the health and environmental effects of viable naturally occurring microorganisms for non-regulated purposes. In the USA, some unregulated microbial products are seen as potentially more environmentally beneficial than existing synthetic chemical-based products.

A number of third party certifying organizations have been established to fill this gap for unregulated products. These organizations include (but are not limited to) Design for the Environment (DfE), Green Seal and EcoLogo. They evaluate a wide range of products claimed to be more sustainable and less hazardous than existing products that use problematic chemicals, including a number of products that employ microorganisms. These third party certification agencies evaluate a number of parameters such as a product's individual formulations/components as well as its efficacy to perform as claimed.

As part of the DfE programme, producers of bio-based products may seek a review from the EPA's DfE Division in order to achieve a DfE certification and permission to use the DfE logo on the approved product. The applicants for DfE microbial products receive a health and environmental review similar to those under TSCA, except that most such microbes are naturally occurring, so the evaluation of the genetic construct is not required.

11.5.2 Canada

The Canadian Environmental Technology Verification Program (ETV) (http://www.etvcanada.com/) is the independent verification organization which supports the implementation of innovative environmental technologies in Canada. It aims to provide reliable independent verification of the environmental performance claims of innovative technologies, processes and products. The ETV Program is being delivered by The Bloom Centre for Sustainability (http://www.bloomcentre.com/) under a licence agreement with Environment Canada; more information on this organization is available at its web site.

The EcoLogo Program (http://www.ecologo.org) began in Canada but is now worldwide; it is managed by TerraChoice. This programme is designed to assure customers that EcoLogo-labelled products meet stringent standards of environmental leadership that reflect the entire life cycle of the product, although there are often some performance criteria that must be met as part of that standard.

11.6 Industrial Practices for Production of Viable Microorganisms: Microorganism Production and Quality Control

Quality control practices in the production of microorganisms are critical to the safety and performance of the products manufactured. The use of validated, consistent assays for critical parameters, e.g. microbial counts, is required to ensure product purity and performance. Microbial identifications are used to ensure that the organism contained in the product is the correct strain. This is of particular importance, as the fermentations used to produce viable microorganisms are inherently susceptible to contamination, and in extreme cases even to producing a material that contains none of the intended species. Such a situation would, of course, have major implications for product performance but, even more importantly, for the safe use of the product. Additional quality control checks are also required during post-fermentation product manufacturing, such as during downstream processing, formulation, etc. Due to the importance of these practices, a system of monitoring, training, product tracking and compliance is used, such as is required under ISO certification.

The identification of microorganism strains is of great importance to industry, academia and regulatory agencies. Because a

microorganism's toxicological risk is often initially assessed on the basis of its taxonomic classification, this situation may be problematic. Taxonomic identification has been carried out by numerous methods over the years, and may result in divergent names being assigned to the same organism. Today, the most generally accepted microbial taxonomy procedure is via 16S rDNA sequencing; however, other molecular methods may be more important for certain species and other technologies or approaches are continuously developed (see Bergmans, Chapter 12, this volume). From an academic standpoint, a multiphasic approach using 16S rDNA sequencing and other data is needed to accurately identify a microorganism. When referencing a microorganism's genus, species and strain, it is recommended to note the specific taxonomic procedure used to derive said name.

Microorganism-based products can comprise multiple microorganism species or strains. In general, each strain must be produced independently. The production of multiple microorganisms in the same fermentation is generally not recommended owing to the extreme difficulty of maintaining consistent product quality using this method. The use of independent fermentations enables the manufacturer to ensure that each target microorganism has been produced successfully and without contamination. In the formulation process, the individual strains can be blended to achieve the specific microorganism requirements and activity rate (colony forming units, cfu) for the product.

11.7 Conclusion

Science-based regulation in the USA and Canada ensures that robust risk assessment has been conducted with respect to environmental and human health safety of bio-based products for industrial and domestic use. In addition, these governments have ensured that voluntary programmes are in place so that the claims of such products are verified. These steps ensure that the future of bio-based products looks bright. In addition, the marketing of products as 'natural' will increase with the perception that natural products are better for the environment. However, in the USA and Canada the claim of 'no harm to the environment or to people' is validated by risk assessment, as embodied in regulation, where as claims made for performance may be verified by independent bodies.

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12 Determining the Safety of Microorganisms – Introduction and Overview

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12.1 Introduction

The use of supposedly beneficial microbial strains in settings where they may or will be in direct unrestrained contact with humans, animals, plants or other microorganisms in the environment poses questions about their safety. The practice of 'biosafety' may in general be defined as endeavours to protect humans and the environment from harm that may arise from exposure to biological agents. Biosafety considerations for a given microorganism focus on the interactions that may occur between the microorganism and 'target' as well as 'non-target' organisms in the environment. These interactions may be beneficial or neutral, or they may lead to adverse effects (where adverse effects on target organisms may be intentional). The aim of this chapter is to give an overview of how to determine whether a microorganism is safe for use – that is, whether its interactions will be expected not to lead to unintended adverse effects on humans and (organisms in) the environment, and also to briefly introduce the regulatory systems that play a role in safety assessment and marketing.

The concept of biosafety is traditionally applied first and foremost to the safe use in laboratory settings of microorganisms that may be pathogenic, i.e. that may cause disease to humans (see, for instance, the laboratory

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biosafety manual of the WHO, 2004). This manual, like other similar manuals, focuses on good laboratory practice when working with known pathogens at the necessary and mandatory biosafety level. The lowest risk group of microorganisms consists of organisms that are unlikely to cause human or animal disease. Activities with these organisms should be performed under conditions of GMT (good microbiological techniques). GMT has two aspects: not only safeguarding laboratory workers from exposure to the microorganisms, but also safeguarding the microorganisms from harm caused by the environment, especially from contamination with other microorganisms (including viruses). The last aspect is of prime importance, and applies also when work is done with microorganisms that are supposed to be safe, or are even beneficial.

As a general rule, it is not possible to show that a microorganism is completely 'safe'. What can be shown with reasonable certainty is only its lack of specified *un*safety: a microorganism can be presumed to be safe if it has been proved that it is *not* to be considered pathogenic. Neither should the microorganism be toxigenic, and it should also be free of known allergens. In some fields of application of microorganisms, it is essential to demonstrate their safety, for instance in their industrial use as food or feed additives or for the production of food, feed or chemicals, e.g. in fermentation processes. The discussion document on the concept of the Qualified Presumption of Safety (QPS) of microorganisms, introduced by the European Food Safety Authority (EFSA, 2005; Leuschner *et al*., 2010), shows how such an approach can work in practice. A QPS evaluation of a microbial species takes into account the body of knowledge that is available: safety-related data on the species that are available from the scientific literature and from scientific databases, its history of use, e.g. in industrial applications, and what is known about its ecology as well as clinical aspects. The evaluation is done in a tiered approach, first taking into consideration whether the species has a known history of safe use under the circumstances of the intended use. If this does not yield enough data to draw a conclusion on the safety of the species, its taxonomy may be taken into consideration to check whether there are known pathogens among taxonomically related microorganisms (usually at the taxonomic level of the species or genus). If that is the case, the presence or absence of known virulence factors of these pathogens is considered to further underpin the status of the species as pathogenic or non-pathogenic. If strains are directly released into the environment, ecological considerations have to be taken into account, such as the survival and persistence of the microorganism in the environment.

This overview chapter will first discuss the role of taxonomy in the determination of the safety of microorganisms. The determination of the presence of virulence factors will be the second topic, and safety considerations for ecological (environmental) applications of microorganisms will be the third. The chapter will focus on the determination of safety of bacterial strains, but similar considerations apply to new strains of other groups of microorganisms.

12.2 The Role of Taxonomy and Related Subjects in the Determination of the Biosafety of Microorganisms

12.2.1 The role of taxonomy

The OECD monograph on the use of taxonomy in risk assessment of microorganisms (bacteria) (OECD, 2003; see also OECD, 2008) states that: 'the taxonomic identification of a subject microorganism is a key element in any risk assessment for a biotechnology product. The use of taxonomy in risk assessment may be seen as having two components, 1) providing a common frame of reference and 2) use in predictive analysis. In order that predictive analyses can take place, good identification of both the subject and a comparison microorganism is needed. Inferences derived from a comparison bacterium's characteristics may be used to help formulate questions for risk assessment of the subject microorganism. Data for subject or comparison bacteria may be acquired directly through testing, or

indirectly via interpretation of published, or otherwise available, information relevant to the issues of the case at hand. Selection of a comparison bacterium may be complex, but they can be used in risk assessment, given a good understanding of bacterial systematics and the relationships between the comparison and subject bacteria'.

Although no formal methodology for bacterial taxonomy exists, *Bergey's Manual of Systematic Bacteriology* (Bergey's Manual Trust, 2011) is considered authoritative for the classification of bacterial strains. In the recommended approach, the relatedness of bacterial strains is primarily described on the basis of comparisons of their 16S RNA gene sequences, as well as on basic descriptive data such as microscopic and biochemical observations, and general genomic data such as GC content and DNA/DNA hybridization data. Konstantinidis and Tiedje (2005) have taken this approach an important step forward towards what they call a genome-based taxonomy for prokaryotes; this is based on pairwise comparisons of whole genome sequences of bacterial strains that are now rapidly becoming available. These approaches are sufficient for establishing the taxonomic position in, for instance, the QPS approach. For the use of bacterial taxonomy in *predictive* analysis, it is, however, key to understand how genetic and phenotypic differences between bacterial strains come about. This requires, inter alia, a good understanding of the clonal relationship between bacterial strains.

12.2.2 Clonal relationship between bacterial strains

As bacterial reproduction is clonal, all offspring bacteria derived from a single original bacterium should in principle be genetically identical, except for spurious mutations that occur (Barrick *et al*., 2009). There is ample evidence that such clonal relationships are found under natural conditions in specific niches that we can recognize and where selective pressure is high. Such clonal derivatives may usually be recognized and discriminated from other clones by similarities and differences in their 'housekeeping' genes and enzymes, identified by using multilocus sequence typing (MLST) techniques (Maiden, 2006).

Clones will tend to be stable because of selective pressure in their niche that acts against loss of functions through mutations. Where selective pressure is strong, we may expect to encounter bacterial populations that have a clearly clonal structure. Pathogenic bacteria are one example. Pathogens live in niches, in their hosts' bodies, where they have to escape continuous attacks by the defence mechanisms of the host that constitute the selective pressure. It even appears, particularly in pathogens, that the selective pressure may lead to the development of so-called genetically monomorphic clones (Achtman, 2008). In these clones, genetic diversity is so low that this becomes a problem when typing them. Wirth *et al*. (2006) make a convincing case on how different genetic mechanisms may structure populations of *Escherichia coli* as they shift from avirulent commensal strains to virulent to epidemic strains and vice versa. Two mechanisms are considered: the occurrence of mutator strains that have enhanced levels of mutation, and horizontal gene transfer of virulence characters. Enhanced frequency of mutation will be a negative trait in populations of avirulent *E. coli* that are not under high selective pressure. Once an avirulent strain has picked up a virulence factor, for instance by horizontal gene transfer (HGT), it will interact more closely with the host and its defence systems. Under these circumstances, genetic diversification may play a more comprehensive role, and mutator strains may be more prevalent. Further virulence factors may also be picked up by HGT, leading to some strains becoming epidemic; these may be even more reliant on genetic diversification. Indeed, sudden bursts of genetic diversity are observed when epidemic strains surface in bacterial populations (Maynard Smith *et al*., 1993).

From a taxonomic point of view, when we try to interpret bacterial diversity as the result of clonal descent and the occurrence of mutations, combined with exchange of traits that may occur through various processes of HGT (Heuer and Smalla, 2007), we have to rely on our knowledge about what has

happened to the individual bacterium in a population. There is a host of typing techniques that may help us here, to the point where some scientists (Achtman, 1996) speak of YATM (yet another typing method), or even TBCA (totally boring clonal analysis). In the present 'postgenomic' era, the possibilities for typing individual bacteria in populations and following their genetic fate have exploded. In a review of the situation, Medini *et al*. (2008) make an excellent point for the application of genomics and other related available techniques to study bacterial populations.

12.2.3 Taxonomy: regulatory considerations

The amount of data that is becoming available from the plethora of typing techniques that exist pose a significant problem: which of this knowledge is 'nice-to-know' and which is 'need-to-know' as relates to the taxonomy of a strain from a regulatory perspective? The answer very much depends on the case under consideration. A new strain that has been freshly isolated from the environment, and subsequently studied and possibly actively modified in the laboratory by traditional strain improvement processes or by genetic modification, which will then be reintroduced into the environment, must of course be typed in order to determine to what genus and species it belongs. From this typing, probably by means of 16S RNA, and by comparisons with properties of its close taxonomic relatives, a first prediction can be made about the potential pathogenicity of the strain. This will also allow an evaluation of the possibilities for HGT to close relatives of any new genes and traits that have been introduced into the strain. Strains that have been isolated from sources where pathogens are expected, for instance isolates from patients, will need to be fully typed, even further than at the species level, e.g. at the serotype level, or still further, at the molecular level, in order to predict their pathogenic status. In any case, strains derived from such sources will not be regarded as beneficial without rigorous testing. It is sometimes suggested that a full sequencing and analysis of the genome of a strain should be required for this purpose. It is, however, questionable whether such a comprehensive approach will provide interpretable knowledge about the strain, as the exercise may lead to more 'nice-to-know' questions than 'need-to-know' answers. One reason to follow the full genomic approach could be to have a reference available, if, unexpectedly, a problem turns up during the intended use of the strain. For instance, an attenuated derivative of an originally pathogenic strain that is used as a vaccine strain could revert to a pathogenic state in the process. In that case, it could be useful to trace how this reversion has occurred at the molecular level by comparing the genomes of the attenuated and the newly pathogenic strain. For such a case, however, the sequence data would not need to be available proactively; it would be sufficient to have a non-mutated sample (e.g. one kept at –80°C) of the original strain available that could be sequenced as need be.

12.3 Virulence Factors: 'Dominant' Traits in Regulatory Considerations

A major question in the determination of the safety of a microbial strain is whether the strain is pathogenic, or may become pathogenic during its use (for an extensive discussion of the role of virulence factors in regulatory considerations, see OECD, 2011). In probably all regulatory systems, e.g. for microbial biocontrol organisms, occupational safety and microbes used in food/feed or genetically modified organisms, questions will be asked about the virulence of a strain in order to establish the safety of its use. In that sense, we could call virulence a 'dominant' trait in regulatory considerations. It is, therefore, important to understand the concept of pathogenicity and its correlative 'virulence'.

12.3.1 Understanding the concepts of pathogenicity and virulence

Pathogenicity is the potential of a microorganism to live and replicate in the body of its host, and to cause harm, i.e. disease, resulting from

this 'lifestyle'. Virulence is a (semi-) quantitative measure of the degree of pathogenicity of a strain. Although the use of beneficial microorganisms might pose environmental risks, human health regulations are most prominent, and these require that the *lack* of pathogenicity of microbial strains is proved. In general, these health regulations require that, in order to implement adequate safety measures, the virulence of a microorganism has to be ascertained. This requires that microbial virulence is defined in an unambiguous manner – a matter that is, however, not straightforward because for every general statement on virulence, numerous exceptions can be noted. Microbial virulence is taken as the sum of a variety of factors, including attachment, local proliferation, tissue damage, invasion, replication and dissemination. Many bacterial attributes may contribute to virulence, e.g. fimbriae, flagellae, toxins, immune-modulating factors and lytic enzymes. As a matter of fact, any definition of bacterial virulence is a simplification of reality (Sparling, 1983; Brubaker, 1985; Maynard Smith *et al.*, 2000; Wassenaar, 2001; Wassenaar and Gaastra, 2001; McClelland *et al.*, 2005; Brown *et al.*, 2006; Casadevall, 2006). At the end of the 19th century, Koch postulated criteria to establish a bacterium as the causative agent for a disease: (i) the bacteria must be present in every case of the disease; (ii) the bacteria must be isolated from the host with the disease and grown in pure culture; (iii) the specific disease must be reproduced when a pure culture of the bacteria is inoculated into a healthy susceptible host; and (iv) the bacteria must be recoverable from the experimentally infected host. In a 'molecular' analogy of Koch's original postulates, bacterial virulence is defined by the cumulative activity of bacterial genes and gene products that enable the bacterium to cause disease in living organisms (Fredricks and Relman, 1996; Wassenaar, 2001; Wassenaar and Gaastra, 2001; Falkow, 2004). Removal or inactivation of such genes leads to loss or attenuation of virulence, rendering the bacterium less virulent or even avirulent. Restoration of the loss of gene function or reactivation of that function leads to the re-establishment of virulence potential. In this way, there may be various lines of evidence that a gene encodes a virulence factor. The evidence that a gene product acts as a virulence factor may be phenotypic, e.g. inactivation or restoration of the gene results in loss or restoration of virulence; it may be immunological, e.g. presence of antibodies against a gene product results in immunity from the disease, or infection results in the production of antibodies against the gene product; or there may be arguments from comparative genetics, e.g. homology with known virulence genes, or homologous genes displaying antigenic variation within or between strains.

The molecular definition of virulence characters should be handled with care in the establishment of the safety of bacterial strains. In principle, one could correlate safety and lack of safety with the absence or presence of virulence characteristics, i.e. with the absence or presence of expressed virulence genes (see Wassenaar and Alter, Chapter 13, this volume, which presents an in-depth investigation of the presence of virulence genes in bacterial genera used in food/feed cultures or as probiotics). However, the genes that are determined according to the molecular rules to encode virulence determinants may be classified in different classes (Wassenaar and Gaastra, 2001). 'True virulence genes' code for gene products that are involved in interactions with the host, are directly responsible for pathological damage during infection and are absent in avirulent strains. The class of 'virulence-associated genes' encode virulence factors that, for instance, regulate expression of true virulence genes, or are required in any way for their post-translational activation. Then there are 'virulence lifestyle genes' which encode factors that are essential for interaction and survival in the host, but which are not directly responsible for pathogenesis – for example colonization factors, factors needed for intracellular survival or for evasion of the host immune system, or factors that employ host factors necessary or beneficial for survival in the host.

12.3.2 Virulence in a broader context

We may ask ourselves whether all classes of virulence genes play an equally important role in the determination of the safety of a

strain. Clearly, the 'true' virulence genes are important, but virulence-associated genes need the presence of true virulence genes in order to affect safety, and the activity of virulence lifestyle genes should also be scrutinized in order to determine their role in the safety characteristics of a strain. Interesting examples are the so-called housekeeping genes that are necessary for virulence, but are commonly present in both non-pathogenic and pathogenic strains. The *aroA* gene, which codes for the enzyme 5-enolpyruvylshikimate-3-phosphate synthetase, is widely present in bacteria and performs an essential step in the pathway that produces aromatic amino acids. Virulent strains of *Salmonella* can be intentionally attenuated by inactivation of the *aroA* gene (Stocker, 2000), and this strategy is widely used for the construction of vaccine strains, in Gram-positive pathogenic bacteria (Bronze and Dale, 2010) as well as in Gram-negative bacteria (*Salmonella*). Clearly, *aroA* fulfils the 'molecular' criteria of a virulence gene, but it should not be considered as a virulence factor on its own, and the presence of the gene in an otherwise non-pathogenic strain will clearly not lead to specific virulence characteristics.

By the way they are defined, the presence of 'virulence' factors is restricted to pathogenic organisms. In our anthropomorphic view of pathogens, we tend to think that they cause harm to their hosts on purpose, but the view of pathogenicity as the result of a pathogenic lifestyle, as an adaptation to life in the host, is probably more realistic. It has been pointed out that factors similar to virulence factors are typically also found in soil bacteria, where they enable the soil-bound lifestyle of these bacteria (Casadevall, 2006). Casadevall argues that 'each soil-dwelling microbe possesses a unique combination of characteristics, including some held widely in common such as the ability to form biofilms, acquire iron, or produce proteolytic enzymes'; these are all factors that can also contribute to the virulence of pathogenic bacteria. The picture that emerges is that throughout the environment, including the environment of human and animal hosts that we normally take into consideration when defining virulence factors, there is an enormous set of traits that are important for the fitness, or virulence, of bacteria, that are dependent on their lifestyle. Where these bacteria are in close contact, a rapid exchange of these fitness/virulence factors may occur. This may lead to a change from a non-pathogenic to a pathogenic lifestyle, or to a change in the host of a pathogen because it has become adapted to the situation and challenges that occur in another host. Such a host jump may even occur between the kingdoms of living organisms. The possibility of host jumps is a major concern in the risk assessment of viruses. The possibility of cross-kingdom host jumps in bacteria is less well known, but is an important issue in the safety assessment of bacteria (Van Baarlen *et al*., 2007a).

An example of host jumps is found in the *Burkholderia cepacia* species complex, with *B. cepacia* as a type species. The genetic diversity in this complex is very large and it has been termed a 'multireplicon complex' (Mahenthiralingam *et al*., 2005). Formerly belonging to the genus *Pseudomonas*, *Burkholderia* is commonly found in soil, e.g. in the plant rhizosphere and water. Members of the *B. cepacia* species complex have been identified as plant commensals, where they may even be endophytic, when they do not only contribute to nitrogen fixation, but also have a role as opportunistic plant, animal and fungal pathogens. A common factor among human pathogenic *Burkholderia* species is the production of toxin-like virulence factors, such as the pectinolytic enzymes that are found in the original onion pathogenic isolates but not in non-pathogenic representatives (Gonzales *et al*., 1997). Additional factors that are associated with virulence are the Gram-negative bacterial endotoxins, different porins, the acyl-homoserine-L-lactone involved in quorum sensing and other regulatory processes, and factors that promote the utilization of nutrient sources in the host (Baldwin *et al*., 2006).

The species *B. cepacia* is only one of a number of examples of microorganisms that apparently have made a cross-kingdom jump, a topic that is summarized by Van Baarlen *et al*. (2007a). These authors provide an overview of the factors that are probably prerequisites for cross-kingdom jumps to occur. In the

first place, there should be a close proximity, e.g. some sort of symbiotic contact, of the microorganism and its future host. Then, the microorganism should be able to use a future host as a source of nutrients. One of the most important nutrients in this respect is iron. The dearth in the environment of iron in forms that can be utilized can be limiting for growth, and microorganisms produce factors that enhance iron utilization, such as haemolysins and siderophores, which act also as virulence factors. The factors that promote infection should have a broad specificity, e.g. use target components in the future host that are of a type that is largely conserved between kingdoms. Additionally, the microorganism should be able to deal with immunity systems in the host. The innate immune systems of organisms show remarkable similarities across kingdoms, relying largely on the recognition of molecular patterns that are common to microorganisms. The potential of a microorganism to overcome such shared defence mechanisms appears to coincide with their potential to infect multiple hosts (Van Baarlen *et al*., 2007b). Self-protection plays an important role. An example is the formation of melanin, which may protect microorganisms, for instance, by scavenging the reactive oxygen that is produced in host defence reactions. The other way around, many Gram-negative bacterial pathogens employ type III secretion systems that inject bacterial effector proteins into host cells; these type III systems thereby act as virulence factors, because mutants that do not produce the type III secretion system lose their virulence. Type III secretion systems are, however, also used by free-living non-pathogenic microorganisms, and presence of a type III secretion pathway as such is no indication of a pathogenic lifestyle. In general, pathogenic microorganisms have close relatives that are free living. The success of a cross-kingdom host jump, or of a formerly free-living microorganism becoming a pathogen, depends on the initial possibilities for the microorganism to be able to grow and colonize in the new host to an extent where further evolution into a full-blown pathogenic lifestyle may occur.

From a regulatory point of view, the question arises as to what extent the possibilities for exchange of (potential) virulence factors between microorganisms is a point of concern in risk assessments. This depends very much on the organisms' role and native ecology in their natural habitats. Although all kinds of speculations could be made about future developments, it is clear that those shifts in lifestyle from free living to pathogenic that have occurred can be recognized in retrospect, but would have been difficult to predict specifically in a prospective scenario of risk assessment. A proactive approach for identification of potential changes in lifestyle would be needed to obtain more insight into this question. The US Centers for Disease Control and Prevention (CDC, 2011) recently initiated the 'One Health Office', which could be an important step in this direction, as it 'facilitates and coordinates program activities that seek to attain optimal health for people and animals by promoting global collaboration between human and veterinary medicine while engaging the principles of public health and ecosystem health'. Another example is the development of a general method for early warning and surveillance systems of emerging zoonoses described by Havelaar *et al*. (2010).

12.4 Environmental Applications of Bacteria/Microorganisms

Microorganisms are employed in the environment for a variety of processes: most prominently for agricultural purposes, as growth enhancers and for phytosanitary uses as disease suppressors; also for geochemical processes such as bioremediation, mineral leaching and other mining uses; and, more recently, for the bulk production of chemicals, biomass and biofuels. Where these applications are under any form of regulatory oversight, a safety assessment of the organisms will have to be done. On the one hand, this requires, good insight into what the protection goals are and, consequently, an insight into what potential adverse effects of the microorganisms on the environment could be envisaged. On the other hand, an adequate performance of an environmental risk assessment can only be done on the basis of a good insight into

how microorganisms actually 'work' in the environment, how they accomplish their expected beneficial activities, and how they could accomplish the postulated potential adverse effects. The role of a microorganism in ecological systems can only be understood if both the fundamental biological properties of the organism, and its ecological properties and interactions are understood. The importance of understanding the role of a microbe and its interaction with other members of microbial communities, working together in consortia, cannot be overemphasized. For example, the processes necessary for successful bioremediation, and the ways in which individual microorganisms contribute to these processes, can only be understood in this way (de Lorenzo, 2008; see also Section 12.4.2, Bioremediation). Obviously, these considerations are also of paramount importance in identifying the potential hazards of a microorganism, as they may also occur in a similarly complicated way through the effects of the microorganisms on the entire consortium of that organism.

12.4.1 Safety evaluation of environmental applications of microorganisms: protection goals and adverse effects

The microorganisms that are used in environmental processes are, in principle, beneficial and, in any case, they should be non-pathogenic, and in general non-hazardous, to non-target organisms (including humans). In addition, they should not have adverse effects on the structure and dynamics of soil microbial populations. This last issue is most prominent in legislation for environmental releases of genetically engineered microorganisms, where effects on biogeochemical cycles are a concern – particularly effects on carbon and nitrogen recycling – through changes in the decomposition of soil organic material (see for instance OJEC, 2001a, 2002). To express this specific protection goal in legislation is one thing, but to make the goal operational in the practice of environmental risk assessment is by no means straightforward. In general, one may state that protection goals are the maintenance of biological diversity and assurance of the life support functions of the soil and of aquatic systems. This requires an extensive knowledge of the role of microorganisms in the environment, and the development of assessment concepts for the protection of soil systems (Breure *et al.*, 2005). This knowledge is also required in risk assessment/management to evaluate whether an observed effect on the microbial composition of a soil ecosystem constitutes an adverse effect that could be seen, under the circumstances where it occurs, as an acceptable risk. As an illustration, Mensink and Scheepmaker (2007) have proposed a risk decision tree, with examples of how an environmental safety evaluation of microbial biocontrol agents could take into account data on the characterization of a microorganism, its efficacy, emissions, exposure to and environmental effects on non-targets and the environment.

A fundamental factor that complicates the development of suitable assessment concepts is our lack of understanding of the microbial environment. Ever since it became apparent, in the early 1990s (Torsvik *et al.*, 1990), that there are many more varieties of microorganisms in the environment than we have seen in isolation in the laboratory, there have been extensive studies to fill this gap of knowledge. One reason for this lack of knowledge is the fact that it is difficult to culture many of the microorganisms in the laboratory. This is partly because of our poor understanding of the correct culture methods (for instance, the development of culture media that have a suitable composition), and of other prerequisites for growth of the organisms in the laboratory. This is one reason why many environmental microorganisms appear to be 'viable but not culturable' (VBNC). However, the VBNC status may also be due to more fundamental processes in the physiology of the organisms that are necessary as a response to certain environmental stresses in order to allow an organism's survival (Oliver, 2010).

Many techniques have been developed and applied to determine the population structure of microorganisms in soil. This has resulted in a plethora of data that should be subject to a meta-analysis in order to obtain

an overall picture of soil microbial communities, and, in the end, to be able to determine whether a disturbance of the population may result in extensive and permanent adverse effects. This is an important question, for instance, in releases of microbial biological control agents that are intended to have adverse effects on pathogenic soil microorganisms, but which could also be expected to have similar adverse effects on (beneficial) non-target organisms (Brimner and Boland, 2003; Winding *et al.*, 2004). A first prerequisite for performing a risk assessment of such cases is a good understanding of how the available data should be interpreted in the context of the environment, e.g. whether they reflect the presence of just live organisms or also the remains of dead organisms, as is the case for many DNA-based methods, or whether they reflect actual activity of live organisms, as is observed in many RNA-based techniques or in direct measurements of physiological activity, such as soil respiration (OECD, 2004; Boersma and Van Elsas, 2009).

12.4.2 Environmental safety evaluations: two case studies

Predisposed as we are to laboratory studies in which we mostly work with pure cultures of microorganisms, we may look at the microbial environment as a place where specific roles are played by separate microorganisms, albeit in constant interaction with each other. This may be true for some processes where we employ microorganisms for specific, beneficial processes, such as energy conversion and carbon capture in photosynthetic microorganisms. However, it is certainly not true for other processes, which require physiological processes in the soil where metabolites are converted in several steps by different organisms, as in bioremediation.

Photosynthetic and carbon capture processes

In the research and development of sustainable processes aimed at reducing our dependence on fossil fuels for energy production, and at the direct reduction of carbon dioxide in the atmosphere, microorganisms, in particular micro-algae (including eukaryotic unicellular algae as well as prokaryotic *Cyanobacteria*) have come up as organisms that are potentially very useful (Brennan and Owende, 2010; Sayer, 2010; Wijffels *et al.*, 2010). Micro-algae can also be used as a source of biomass, e.g. for food or feed production (Kay, 1991).

Micro-algae have simple growth requirements (light, CO_2, minerals) and may be cultured in defined or undefined media, e.g. waste water, in various forms of bioreactors under contained use or under confined conditions in the environment, e.g. in so-called raceway ponds. The advantages and disadvantages of various systems have been addressed by the EPOBIO project, a project funded through the European Union's Sixth Framework Programme (FP6) to realize the economic potential of plant-derived raw materials (Carlsson *et al.*, 2007).

Environmental risk assessment of these types of applications of microorganisms will probably have to cover a variety of aspects, e.g. the production of toxins in relation to worker protection and food/feed safety. A less obvious but notable concern is that the use of micro-algae to sequester carbon from the atmosphere, for instance by growing and then burying algal biomass, would at the same time lead to serious depletion of organic matter and minerals from the agronomic environment.

Recently, it has become clear that genetic modification can now be used to redirect algal metabolism in 'designer strains' made for optimal production of biofuels, or for the production of, for instance, pharmaceutical products. This is expected to 'fuel a biotechnology revolution' (Rosenberg *et al.*, 2008). If such strains are employed in production systems that are in open contact with the environment, this will spur discussion on the potential environmental impacts of such strains that may lead to harmful effects. This discussion will be difficult, as demonstrated in Section 12.4.1. For a rigorous process of environmental risk assessment, it will be necessary to make a thorough evaluation for these types of applications with genetically modified microorganisms, in analogy with what Raybould (2006) has suggested for the environmental risk assessment of transgenic crops.

Bioremediation

Bioremediation is the exploitation of biological activities for the mitigation and, wherever possible, the complete elimination, of the noxious effects caused by environmental pollutants in given sites (de Lorenzo, 2008). It is a good example of a process that relies on the activity of a large number of microorganisms, each of which harbour part of the metabolic processes involved, as well as on the free exchange of metabolic intermediates, and on quite a number of physico-chemical conditions in the environment: O_2 tension, availability of electron acceptors and water, temperature, granulation of the matrix, etc. The scientific study of bioremediation therefore requires a comprehensive insight into these factors, and into how bacteria interact under these circumstances. Bioremediation of a pollutant usually requires a number of subsequent steps, which not only require various enzymes, but also various physico-chemical conditions. de Lorenzo (2008) has drawn a convincing picture of how this problem may be approached from the viewpoint of systems biology. He draws attention to the dimensions of the 'bioremediation space'. There are three dimensions to the effectiveness of any bioremediation process: (i) the catabolic landscape that comprises the biological entities involved; (ii) the chemical landscape, e.g. (eligible) nutrients, electron donors/acceptors and stressors; and (iii) the relevant factors of the abiotic landscape, e.g. humidity, temperature, redox (O_2) status, matrix conditions, etc.

The biological dimension, the microorganisms that are involved in the various processes, shows considerable complexity. For each step in the catalytic pathway for a pollutant, there may be a collection of enzymes available, a so-called pan-enzyme, located in different organisms. These organisms form an intricate network in a landscape where they possess a non-diffusible part of their metabolism that is not secreted, as well as other metabolites, such as amino acids, that can diffuse readily in and out of the cells, but will usually be used in the cell's metabolism as soon as they are available. Other secreted compounds that are metabolized more slowly may also diffuse in and out, but are more readily available to other members of the community. Pollutants and their degradation products will fall into this class. In this way, the degradation of pollutants requires a concerted activity of a microbial consortium that comprises the pan-enzymes necessary for complete degradation of a pollutant, and offers the necessary physico-chemical conditions required for all intermediate reactions.

The intricate structure of an environmental process such as bioremediation makes the environmental risk assessment of such a process equally complex, and this will be the same for many other environmental processes. If de Lorenzo (2008) is right in his expectation that 'systems and synthetic biology will be translated into more vigorous biological agents that, once deliberately entered in the target site, perform the cleanup with high efficiency', then we will need an approach to the risk assessment of such deliberate releases that takes into account aspects of systems and synthetic biology. This will require novel paradigms for environmental risk assessment.

12.5 Concluding Remarks

The risk analysis of environmental applications of beneficial microorganisms is a field for which little straightforward guidance is available. One area for which some regulatory guidance is available is with microbial plant protection agents (e.g. see OJEC, 2001b; OJEU, 2005). More information and a critical discussion of the current requirements for microbial biological control agents have been given by the REBECA (Regulation of Biological Control Agents) project (Ehlers, 2011). Guidance also exists for applications of genetically modified microorganisms for food and feed purposes (EFSA, 2011) because of the clear need to ensure that the organisms used are safe. Pathogenicity and virulence are areas where guidance is manifold, but they are mainly focused on the identification of virulence factors.

In environmental risk assessment of beneficial microorganisms, however, the present focus is on showing that a microorganism is non-pathogenic, and hence free of virulence-related genes (OECD, 2011). But, even if a microorganism contains virulence-related

genes, the question will be whether these virulence factors can cause pathogenicity. A non-pathogen (for instance harmless *E. coli* strains) may still contain virulence-related genes.

The environmental risk assessment of beneficial microorganisms in environmental applications confronts us with the problem that it is not at all clear what damage microorganisms may cause. Any deliberate release of microorganisms will inevitably cause environmental effects. Indeed, it is the aim of the release to cause a beneficial effect. In order to approach the risk assessment for this situation in a rational way, more risk assessment research is needed on the environmental effects of microorganisms as well as on the baselines concerned: what are the functions of the microbial environment that are known to be *vital* for our protection goals, how are they kept balanced and what is the robustness of this balance? The microbial environment is under constant pressure from various changes, not only from natural causes but also from human activities. From all the available knowledge, it appears to be quite robust and flexible, and able to adapt to various environmental stress factors without noticeable adverse effects. Any environmental risk assessment of a potential environmental impact caused by deliberate release of a microorganism should take this into consideration as a background and baseline.

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13 Virulence Genes in Risk Assessment of Beneficial Microorganisms: What Do Genome Sequences Tell Us?

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13.1 Introduction

Risk assessment of beneficial microorganisms depends on proper characterization of the genetic potential of the organisms in question. In this chapter, probiotics and starter cultures are used as an example to summarize the molecular information needed from these organisms for safety assessment. Probiotic bacteria have a (proven or assumed) beneficial effect on the host they colonize; starter cultures are deliberately added during food production, for instance for fermentation purposes.

Virulence properties can be assessed by *in vivo* animal models of virulence, which can be based on LD_{50} studies or on disease severity scores, *in vitro* models using cell lines to assess specific virulence mechanisms (invasion, toxicity, macrophage survival, etc.), or genetic characterization to determine the presence or absence of virulence genes.

The chapter deals with the genetic and genomic evidence for the absence of virulence properties in bacteria that are intended for beneficial use, with emphasis on probiotic and starter cultures that are suitable for human consumption. Legislation issues

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related to the safety of probiotics are not treated here, as this topic has been reviewed previously (Wassenaar and Klein, 2008), and is also dealt with elsewhere in this book (von Wright, Chapter 3, this volume). In the past, virulence characterization was based on active searches for the presence of known virulence genes, for instance by PCR analysis. The shortcoming of this approach was that the presence or absence of virulence genes could only be demonstrated for those genes that were actively being searched for; there would be no information available about other genes that might be present. This drawback has been resolved by the increasing availability of complete genome sequences.

In the era of genomics, the first step in the genetic characterization of (potentially) beneficial bacteria should be to determine the DNA sequence of the organism's complete genome. All genes present in the strain of interest can thus be identified and categorized. The logical next step would be to predict any recognizable potential risk related to any possible virulence genes identified. Such predictions depend on inference from previous knowledge. A number of genome sequences have so far been obtained from organisms that have been given Generally Recognized As Safe (GRAS) status in the USA. For substances to obtain GRAS status, their use in food has to have a proven record of safety based either on published scientific evidence or on a long history of use (Mattia and Merker, 2008). Well-defined starter cultures and a number of probiotic bacterial strains have received GRAS status, e.g. *Bifidobacterium lactis* strain Bb12 or *Streptococcus thermophilus* strain Th4 (GRAS notice number 49) (US FDA, 2011). European regulation of the use of bacteria in the food chain differs to that in the USA, and the two regulatory systems have been compared elsewhere (Wessels *et al*., 2004). Starter cultures and probiotic bacterial strains will be used as examples of safe organisms, and because the distinction between them is not always clear (a number of probiotic bacteria are also in use as starter cultures), both types of bacteria are collectively described here as 'beneficial bacteria'.

The genomes of safe bacteria, an increasing number of which are completely sequenced, can serve as a reference for genes or gene combinations that are unlikely to pose a risk to human health. Even more genome sequences are available from (entero) pathogenic bacteria, so that, in theory, a comparison of pathogenic and beneficial bacterial genomes could identify those genes that are found exclusively in pathogenic organisms; these would be more likely to contribute to virulence. The challenge would be to identify the critical combination of these genes that would result in pathogenic potential. Such a comparison might also identify genes that are exclusively found in beneficial organisms, and these, in genomes that lack virulence genes, could in theory provide a 'genetic signature' for safe use.

Actual practice is more recalcitrant and this chapter discusses some of the advantages and difficulties related to safety assessments of beneficial bacteria based on genome sequences. First, we consider the quality requirements of a genome sequence for this approach to be of use.

13.2 Quality Requirements of Complete Genome Sequences

13.2.1 Quality of genome metadata

A bacterial genome sequence comprises both chromosomal DNA and any plasmid DNA; the term 'genome' is only synonymous to the term 'chromosome' when extrachromosomal autonomously replicating DNA is absent. Recently, the number of publicly available, completely sequenced bacterial genomes exceeded 1000, but when these sequences were reviewed, it was concluded that large differences exist in genome sequence quality as well as in their annotation (Lagesen *et al*., 2010). The quality of a published sequence depends on the number of ambiguous sequences it includes; a published bacterial genome can still contain unacceptably large numbers of these (represented as 'N' rather than one of the four DNA nucleotides). Another factor determining the quality of a

genome sequence is how well the sequence was assembled from short reads; in quite a few instances, such assembly has introduced mistakes which are sometimes corrected in follow-up studies. Genome annotation, which describes the function of the predicted genes, varies in quality depending on how the genes were identified, and how careful their functional prediction (which is mainly done automatically) was manually checked.

There is no internationally accepted standard for the quality assessment of bacterial genome sequences, though efforts are made to standardize the metadata, i.e. the relevant information about the strain that was sequenced. To this extent, the minimum information about a genome sequence (MIGS) that is ideally to be provided with the sequence has been specified (Field *et al*., 2008). Nevertheless, a brief review of some of the Microbial Genome Projects at the NCBI (the US National Center for Biotechnology Information) web site (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) illustrates that essential information on metadata is frequently incomplete or incorrect. For instance, the metadata summarized in the table 'Organism info' on the NCBI web site is too incomplete to be of any use. As an example of incorrect information, in the table of 'Genomes in progress', under Project Identification Number (PID) 46979, *Enterococcus faecalis* PC4.1 is listed, but the organism from which this sequence was derived is *E. faecium* PC4.1. Such mistakes are a nuisance, as the incorrect information is easily incorporated into automatically extracted data, and is not likely to be picked up unless all data are reviewed manually. It is anticipated that the mistake will be corrected when the genome sequence reaches completion; however, a number of genome sequences remain in public databases as 'in progress' for a long time, possibly forever. The oldest records of 'Genomes in progress' were last updated in 2002; these include a *B. longum* genome (a look at the GenBank file of this genome revealed it was last updated in 2003, but that information was not incorporated into the summarizing table at NCBI).

Even if the metadata information is correct, it may be of limited use. For a genome sequence of an *E. faecalis* strain isolated from urine and that most likely caused an infection, it will be stated that the genome was derived from a pathogen; but in another individual this strain may behave as a commensal inhabitant of the gut. The same might apply to an *E. faecium* isolated from the blood of a bacteraemic patient with severe underlying conditions – a healthy individual could be colonized by the same strain without any symptoms. Whether such opportunistic pathogens cause infection depends on host factors as well as on bacterial factors. This will have consequences for the way we interpret their gene content, as will be discussed below.

13.2.2 Quality of raw sequences and assembled genomes

At the time of writing, there are three commonly used platforms for 'next-generation' sequencing of bacterial genomes: 454 pyrosequencing (Roche Diagnostics), Illumina (Solexa) sequencing, and ABI SOLiD sequencing (Life Technologies). Recently, the first bacterial genomes have been sequenced using 'third-generation' technology, which is based on single-molecule sequencing, and this holds great promise for the future, as it uses very little material, is inexpensive, produces quite long read lengths, and can be stunningly fast, as was recently illustrated during the 2010 Haiti cholera outbreak (Chin *et al*., 2011).

The raw sequence data for the 'next-generation' sequencing methods consist of short reads: 100 nucleotides (or less) for Solexa and the ABI-SOLiD machines; and around 500 nucleotides or a bit longer for the 454 machines. These short pieces must be assembled into larger pieces or 'contigs' (i.e. contiguous pieces of DNA, where overlapping reads are combined like pieces in a puzzle). The sequence coverage for many of these machines can easily be sufficient (40-fold, in some cases) to help minimize the number of assembled contigs. Assembly can be done with standard software such as Velvet or other methods for the short reads (Solexa and ABI-SOLiD; Miller *et al*., 2010), or with Newbler, which can be used for the 454 reads. Often, the high-throughput coverage of

Solexa is combined with the long reads of 454 to help in assembling genomes. Whereas complete assembly to a fully closed circular chromosome would be ideal, assembly gaps due to repeated sequences, notably those related to multiple rRNA loci, are inevitable. Gaps are also frequently the result of low sequencing coverage of particular DNA fragments, but this is less of a problem in third-generation sequencing technology (Nagarajan *et al*., 2010). Although it is possible to assemble a small bacterial genome into one piece based on a single run, this is usually the exception, rather than the rule. In practice (at present), the assembly of raw sequencing data from next-generation sequencing often results in over 100 contigs for many bacterial genomes, which means that numerous genes are possibly incompletely sequenced or even missed. A genome sequence can be established in draft form within weeks or even days, but closing all gaps can be demanding in terms of time and resources (Nagarajan *et al*., 2010). Finishing a genome sequence by closing all gaps will not only provide a complete picture on gene content, it also enables the correction of errors in contig assembly that occur quite frequently (Nagarajan *et al*., 2010). Nevertheless, it may not be necessary to close all gaps. Even though there are no regulations about what is acceptable, as a rule of thumb we propose to work with genome sequences containing as few contigs as possible. Moreover, the number of ambiguous nucleotides should be minimized. The cut-off depends on the genomes being used and, of course, on what is needed, but it is advisable to exclude contigs shorter than 1 kbp, which is approximately the average length of a bacterial gene. Gene finding on such short contigs becomes problematic because the chance of detecting multiple open reading frames (ORFs) increases with decreasing sequence length, and such ORFs are frequently artefacts.

13.2.3 Quality of annotated gene files

The next step is to generate an annotated genome, in which the protein coding and non-translated genes have been identified. One popular method for finding proteins from a bacterial genome DNA sequence is Prodigal software that was developed at Oak Ridge National Laboratory, Tennessee (Hyatt *et al*., 2010). This method is quite fast, and in general can give annotations quite close to those found in annotated bacterial genomes in GenBank (the US National Institutes of Health genetic sequence database at NCBI), so that the resulting annotation file does not require too much manual curation. For a complete genome annotation, the non-translated genes should also be included. Transfer RNAs (tRNAs) can be found with the program tRNA-Scan-SE (Schattner *et al*., 2005), and rRNAs can be found with the program RNAmmer (Lagesen *et al*., 2007). Currently, finding other small non-coding RNA genes is more difficult. While programs to identify them exist, these genes are not yet part of standard genome annotations.

13.3 Definition of Virulence and Other Risk-related Properties

The key information relevant for microbial risk assessment based on complete genome sequences of beneficial bacteria, especially those aimed for human consumption, is whether virulence genes are present. If they are, the risk that these genes pose in terms of consumer safety needs to be estimated. The way virulence genes (which code for virulence factors) are usually defined and identified has been extensively reviewed (Wassenaar and Gaastra, 2001; Wassenaar, 2004), and this information will not be repeated here. Of the possible methods to identify virulence genes, the one approach relevant for this contribution is identification by sequence homology with known virulence genes.

One complication in the definition of virulence in general is that rather than describing a specific gene functioning in a specific virulence pathway of a particular pathogen, virulence cannot always be separated from niche adaptation/survival strategies. One of the few distinctions between these two is active invasion, which has been shown for a number of pathogenic intestinal bacteria, but is never employed by commensal gut

bacteria. However, not all (enteric) pathogens are invasive. Many of the gut commensals use the same strategy of attachment (adhesins, fimbriae/pili) and motility (flagella, type IV pili) to survive in the gastrointestinal tract as is employed by pathogens. Although in pathogens any of these mechanisms may contribute to pathogenicity (and would result in attenuation upon inactivation), for commensals they contribute to colonization fitness. For example, the genome sequence of *Lactobacillus rhamnosus* GG revealed the presence of mucus-binding pili, which are more typically found on pathogenic bacteria (Kankainen *et al*., 2009). The genes responsible for such actions may be called 'virulence' genes when they reside in pathogens, but are colonization genes when present in commensals. The subtlety of this distinction is lost in gene annotation, and from sequence similarity it cannot be identified whether the gene in question is 'good' or 'bad', as it depends on the context of the rest of the genome. Would it be easier to reason the other way around, and propose that a gene found in a commensal with no known virulence should not be called a virulence gene? Obviously, this proposition would not be compatible with the view that the function of a gene is dictated by its sequence and not (also) by its genetic background – a view that is still at the basis of molecular genetics. Furthermore, this pragmatism will not work for the opportunistic pathogens, which contain genes that function in pathogenicity only under certain circumstances, and under other conditions contribute to a commensal lifestyle. Putatively benign organisms are unlikely to be true pathogens, but they might behave as opportunistic pathogens in certain conditions (Wessels *et al*., 2004; Koretz, 2009; Verma *et al*., 2010), so the distinction needed in microbial risk assessment is that between true commensal organisms and mostly commensal organisms, which can, under exceptional circumstances, behave as opportunistic pathogens. How then, do we interpret the genes that report significant homology both to genes found in a commensal and, with equal significance, to a virulence gene in a pathogen's genome? Unfortunately, there is no simple answer to that question. Gene function depends on context, and the challenge is to predict the correct function of such a gene in that particular organism, set in the context of the complete genetic content of that organism. The problem is even more complex, because the outcome of colonization is an interplay between the microorganism and the host.

Probiotic activity is the combined result of interaction between the host, the intestinal microbiota present and the probiotic strain; similarly, a pathogenic phenotype is defined by the combination of host, residual microbiota and virulence properties of the pathogen. In other words, pathogenicity is not an objective criterion but depends on the circumstances. Even non-pathogenic species can, under very specific circumstances, cause disease, such as in severely immunocompromised individuals or in critically ill patients (Koretz, 2009). Ingested probiotic bacteria can in principle translocate to cause infections, though this has rarely been observed in healthy individuals (Liong, 2008). Despite the broad usage of *Bifidobacterium* spp. as probiotics in the food industry, infections by single *Bifidobacterium* species have been described, and this is mostly restricted to *B. dentium* (Meile *et al*., 2008). Further, while *B. dentium* is being recognized as a potential oral pathogen involved in caries, the majority of these bacteria live in a non-pathogenic relationship with their host (Lee and O'Sullivan, 2010). Then again, a recognized pathogen will not harm an individual who has fully developed immunity against it, no matter how many virulence genes it carries. Most intestinal pathogens can produce a range of conditions, from asymptomatic colonization to severe disease, depending on the overall health of the host, the current immune status, the administered dose and, possibly, the residual microbiota. Therefore, it is impossible to objectively define the exact virulence potential of a given organism, as virulence depends on the circumstances.

Given this difficulty, it is challenging to prove whether one is dealing with a pathogen, an opportunistic pathogen, a commensal or a (potential) beneficial organism, when the proof is based only on a bacterial genome sequence. The absence of virulence genes is

no guarantee of a non-pathogenic phenotype, and the presence of virulence genes is no guarantee of pathogenicity. For example, haemolysin A, a well-recognized virulence gene in pathogenic *Escherichia coli*, is present and even weakly expressed in an *E. coli* strain that has been in use as a probiotic for decades (Willenbrock *et al*., 2007). Clearly, the presence of this single virulence factor does not cause pathogenicity, and even after knowing the complete genome sequence, we would still not be able to predict virulence, or absence thereof. Another example is given by the genome sequence of the probiotic strain *E. coli* Nissle 1917, which was found to be very similar to that of the uropathogenic *E. coli* CFT073 (Grozdanov *et al*., 2004; Hancock *et al*., 2010); not knowing the strain's properties and based on the information from its genome sequence only, one would judge Nissle as a potential uropathogenic *E. coli*. At present, if a genome sequence is the only available source of information it would be difficult to accurately predict whether an *E. coli* isolate would be safe for human consumption.

Apart from virulence genes, the presence of antibiotic resistance genes is considered undesirable in probiotic or otherwise beneficial bacteria, and an antibiotic profile should be established in a safety assessment (Bernardeau *et al*., 2008; Wilcks and van Hoek, Chapter 4, this volume). The main reason that these genes are considered undesirable is not because they would hamper treatment following an infection (probiotic bacteria should not be able to cause an infection in the first place), but that they could donate these resistance genes to potential pathogens. However, not all antibiotic resistances are transferable, so in addition to an antibiogram, gene transferability should also be determined. The probability of DNA transfers between species, or even between genera, is smaller than transfer within a species, but it may not be negligible. Moreover, probiotics may be used during or shortly after an acute infection, so the presence of pathogenic bacteria is likely, and these can then serve as potential acceptors of resistance genes. Intake of probiotic bacteria may even be advised during an antibiotic course to minimize the side effects that the antibiotic can have on the residual gut microbiota. This advice can be questioned, because first of all susceptible probiotic bacteria will not be able to colonize, but will be killed by the antibiotic and, secondly, because the procedure may select for probiotic bacteria that have acquired resistance as a result of the selective pressure. To reduce the side effects of antibiotic use, a probiotic should be taken after the course was terminated.

A final risk may be perceived from genes that provide the capacity for DNA transfer, irrespective of the nature of the genes that could be transferred by this capacity. Transfer of plasmid or viral (phage or prophage) DNA occurs frequently between bacteria. DNA uptake from the residual microbiota to the probiotic bacteria would be undesirable if this could result in pathogenicity or antibiotic resistance – though it should be realized that in this scenario, such genes must be present in the microbiota in the first place, implying that pathogenic bacteria or resistant bacteria are already in residence. DNA donation from benign bacteria to the residual biota would be less of a problem because the probiotic bacteria are unlikely to contain genes that can be harmful, even if transferred to a different genetic background. Hence, the presence of prophage DNA (bacteriophage DNA that is inserted and integrated into the bacterial chromosome) is not necessarily problematic, but can – at low frequencies – contribute to horizontal gene transfer (HGT) by transferring small fragments of chromosomal DNA to new hosts. Genome analysis of probiotic strains has revealed the presence of one or more prophage/prophage-like elements in many *Lactobacillus* spp., *Lactococcus lactis* and bifidobacteria (Ventura *et al*., 2005, 2006).

Bacterial conjugation involving conjugative plasmids might further contribute to HGT, which can assist in the dissemination of, for example, antibiotic resistance genes; interspecies conjugative transfer of tetracycline and erythromycin resistance plasmids from probiotic bacteria has been demonstrated *in vitro*, and evidence for such transfers *in vivo* is accumulating (Jones *et al*., 2010). These mobile elements carry the genes required for processing the plasmid into a transfer-competent form (*mob* genes) and

the genes involved in the formation of a *trans*-envelope machinery and a pilus structure (*tra* or *trb* genes) (Filloux, 2010).

With all this knowledge and insight, how well do the genomes of proven safe bacteria perform? Are they really free of virulence and antibiotic resistance genes? Nearly all of the beneficial bacteria that have been completely sequenced were approved for their applications before their genome sequences were available. As the next section will illustrate, these genomes sometimes contain genes whose presence might not have been expected.

13.4 Comparison of Genomes from Probiotic, Starter Culture and Commensal Bacteria

13.4.1 Summary of publicly available genomes

As of December 2010, GenBank listed 1279 complete and 3529 in-progress bacterial genomes (plus 93 complete and 82 incomplete archaeal genomes). This is a moving target, and novel genomes are being sequenced, and made publicly available, at such a rate that the information given here represents only a moment in time. The most common bacterial phyla for probiotic research are the *Actinobacteria* and *Firmicutes*. The list includes 21 completed *Actinobacteria* genomes, and 375 genomes in progress. Of these, 15 finished and 26 ongoing genome sequences belong to the genus *Bifidobacterium*. Even more genomes of *Firmicutes* are available or in progress: 316 and 1236, respectively. The most relevant genera include *Lactobacillus* (28 finished, 120 in progress), *Lactococcus* (five and three), and *Leuconostoc* (four and seven). Two genera of *Firmicutes* contain well-known benign as well as pathogenic strains or species: *Enterococcus* (two finished genomes and 108 genomes in progress) and *Streptococcus*; for *S. thermophilus* (one of the two non-pathogenic members of this genus) there are four finished genomes but none listed as in progress.

These numbers illustrate the size of the currently available data sets and this is expected to increase in the near future. Few microbiologists or risk researchers will have the computational skills and equipment to handle such large sets of data. Fortunately, some simple genome comparisons can be done using tools that are available online. Notably, the Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov) provides useful data for some quick comparisons and these were used for the analyses presented in the next three sections.

13.4.2 Some statistics of sequenced genomes from probiotic and other benign organisms

From the IMG web pages, 40 completely finished genomes of beneficial bacteria were selected. These were from five genera that mostly contain species that are frequently used as probiotics or starter cultures. Three of the listed genera contain strains that have obtained GRAS status. All these genomes are captured here under the general term of 'beneficial bacteria' (Table 13.1). For all 40 genomes, the genome length and GC content, as well as the total number of genes, were extracted from the IMG web pages, and the percentage of genes for which a function could be predicted was calculated. The percentage of genes with unknown function for which homologues could be detected, and genes that did not detect homologues in the current sequence database were also reported on. In addition, the percentage of genes that could be attributed to Clusters of Orthologous Groups (COG) categories was recorded for each genome. These findings are summarized in Table 13.1, reported as averages for each of the genera. The genome size of *S. thermophilus* is on average the smallest, while *Lc. lactis* has the largest average genome size. This difference in size is only partly reflected in the number of genes: *Bifidobacterium* species have on average the lowest number of genes, but not the shortest genomes. A function could be predicted for 66.9–73.8% of all genes. The percentage of genes for which a COG class could be identified was relatively constant, between 70.2 and 72.6%. Approximately one quarter of the genes showed homology with other genes of unknown function, whereas few genes did

not have significant homology with any other genes (singletons). The percentages do not add up to 100%, as the total number of genes given in Table 13.1 also includes pseudogenes (dysfunctional relatives of known genes that have lost their protein-coding ability or are no longer expressed).

13.4.3 Presence of putative virulence genes in selected probiotic genomes

The selected genomes of Table 13.1 were searched for genes that contained the word 'virulence' in their functional description, using the selection tools of the IMG web pages. This identified the four COG classes shown in column 1 of Table 13.2, which were represented by eight genes, five of which were found in *Lactobacillus* species as listed in the table. Notably, the genome of *Lb. rhamnosus* strain Lc705 contained two genes for helicase/virulence-associated protein e, as well as the gene for queuine tRNA-ribosyltransferase.

The semantic search performed here is rather crude and its findings are not complete; a number of genes are missing. For instance, a fibronectin-binding protein was identified in *Lb. casei* strain BL23 (Muñoz-Provencio *et al*., 2010), and although this genome was included in our analysis, the gene was not detected in our search. The reason is that its functional description in the IMG database lacks the word 'virulence', even though the gene belongs to the same COG1293 functional group as the fibronectin-binding protein found in *Lb. acidophilus* from Table 13.2, whose functional description does include 'virulence'. There is no logical explanation as to why that word is used in some but not in other functional descriptions of genes that are clearly orthologues. That this was the case was confirmed by amino acid sequence comparison using the BLAST tool (program) at NCBI to search for similar sequences in the bacterial genera of interest. A BLAST analysis with the *Lb. acidophilus* gene from Table 13.2 as the query resulted in hits obtained from genome sequences (genome

Table 13.1. Averaged statistics of 40 genomes from five genera containing beneficial strains.

Genus or species[a]	Genome size	% GC	No. of genes	% with predicted function	% with COG[b]	% with homology, no function[c]	% without homology[c]
Bifidobacterium (11 genomes from 4 species)	2,216,684	59.8	1870	66.9	72.0	28.6	0.74
Lactobacillus (19 genomes from 12 species)	2,398,851	43.0	2376	68.5	70.9	26.8	0.42
Lc. lactis (4 genomes)	2,532,267	35.5	2558	67.5	70.2	27.6	0.72
Leuconostoc (3 genomes from 3 species)	2,024,721	38.3	2076	73.8	72.6	20.0	1.76
Streptococcus thermophilus (3 genomes)	1,819,083	39.0	1944	67.8	71.6	26.1	0.70

[a]These genera or species were selected because they are frequently used as starter cultures or probiotics; some of their members have GRAS (Generally Recognized As Safe) status (not applicable to *Leuconostoc* and *Lc. lactis*). Most of the genomes selected here were obtained from strains that are used as probiotic or starter cultures. For more information, see Lukjancenko *et al.*, 2012.

[b]COG, Clusters of Orthologous Groups (of genes).

[c]The percentages in the two homology columns do not add up to 100% as the total number of genes given also includes pseudogenes.

Table 13.2. Genes reported from the 40 selected genomes containing '*virulence*' in their function description.

Gene function description	Gene identified[a]	BLAST hits in genomes with the identified gene[b]
Fibronectin-binding protein (adherence–*virulence*), COG1293[c]	1 gene in 1 *Lactobacillus acidophilus* genome (1)	20 *Lactobacillus* 4 *Leuconostoc* 4 *Lactococcus lactis* 3 *Streptococcus thermophilus*
Prophage protein, helicase/ *virulence*-associated protein e, COG5545	2 genes in 1 *Lb. rhamnosus* genomes (3)	11 *Lactobacillus* 1 *Leuconostoc* 1 *Lc. lactis*
Queuine trna-ribosyltransferase (tRNA-guanine transglycosylase) (guanine insertion enzyme)/ *virulence*-associated protein vacc, COG0343	1 gene in 2 *Lb rhamnosus* genomes (3)	9 *Lactobacillus* 5 *Leuconostoc* 3 *Lc. lactis* 3 *S. thermophilus* 10 *Bifidobacterium*
Virulence factor mvin family protein, COG0728	1 gene in 2 *Bifidobacterium longum* (4) and 1 gene in 1 *B. animalis* (5)	14 *Bifidobacterium*

[a]The total number of genomes included in the analysis for the species listed is given in parentheses.
[b]Results for BLAST hits in genome sequences only are listed; these also include incomplete genome sequences, whereas the previous column refers to finished genomes only. (See text for more information on the BLAST analysis.)
[c]COG, Clusters of Orthologous Groups (of genes).

hits are marked as such by the BLAST tool at NCBI, as opposed to hits on gene sequences that were directly submitted). These results are also presented in Table 13.2. The same procedure was followed using the other genes in that table as the query. The results illustrate that these 'virulence' genes are far more widely distributed in the organisms of interest than the original semantic search results suggested, though they are not conserved in all these genomes. The procedure we followed illustrates that: (i) functional descriptions of genes may be inaccurate and variable; and (ii) BLAST homologous searches are superior to semantic approaches. Nevertheless, semantic searches can identify genes that can be taken as a starting point for homology searches, as demonstrated here.

13.4.4 Presence of putative antibiotic resistance genes in selected probiotic genomes

A semantic search was also performed to identify genes with the word 'resistance' in their functional description. This produced an extensive list of genes, from which those that were related to multi-drug resistance, non-specified antibiotic resistance or resistance to specified antibiotics were selected by hand. Genes reporting resistance to metals, bacteriophages or other functions not related to antimicrobial resistance were removed. This resulted in a total of 189 genes found in the 40 genomes. The COG classes for which more than three genes were identified are listed in Table 13.3. Again, the data reported here are not complete. For instance, two glycopeptide antibiotic resistance proteins in *Lb. casei* strain ATCC334 are also present in *Lb. casei* strain BL23 (as confirmed by subsequent BLAST analysis whose results are not shown), but in that genome they were reported as 'hypothetical protein' which explains why they were not identified in the semantic search. Thus, while the yield of putative resistance genes is already considerable, it is still an underestimate of the true number of resistance-related genes in these genomes.

The data described above were analysed in an alternative way to identify genomes that contain few or many of the genes of interest presented in Table 13.3. Table 13.4 lists the

Table 13.3. Genes reported in >three genomes with the term '*resistance*' in their function description (selected for antibiotic resistance related function).

Function description '*resistance*'	No. of genes per genome[a]
Efflux pump antibiotic *resistance* protein	5 genes in 1 *Lactobacillus casei* (2) and 1 gene in 1 *Lactococcus lactis* (4)
Glycopeptide antibiotics *resistance* protein	1 gene in 1 *Bifidobacterium longum* (4) 3 genes in 1 *Lb. brevis* (1) 2 genes in 1 *Lb. casei* (2) 2 genes in 1 *Lb. delbrueckii* (2) 1 gene in 1 *Lb. gasseri* (1) 2 genes in 2 *Lb. rhamnosus* (3) 2 genes in 1 *Lc. lactis* (4) 1 gene in 1 *Lc. mesenteroides* (1) 3 genes in 1 *Streptococcus thermophilus* (3)
Glyoxalase/bleomycin (a glycopeptide) *resistance* protein/dioxygenase	4 genes in 1 and 1 gene in 1 *B. longum* (4) 2 genes in 1 *Lb. casei* (2) 2 genes in 1 *Lb. reuteri* (2)
Methicillin *resistance* protein	1 gene in 1 *B. animalis* (5) 4 genes in 1 and 3 genes in 1 *B. longum* (4)
Multi-drug *resistance* ABC transporter, ATP-binding and permease protein	6 genes in 1 *Lc. lactis* (4)
Drug *resistance* transporter, EmrB/QacA subfamily	1 gene in 1 *B. dentium* (1) 2 genes in 1 *B. longum* (4) 3 genes in 1 *Lb. reuteri* (2)
Multi-drug *resistance* abc transporter ATP-binding and permease protein	3 genes in 1 *B. animalis* (5) 2 genes in 1 *Lb. salivarius* (1) 1 gene in 1 *Lc. lactis* (4)
Multi-drug *resistance* efflux pump	3 genes in 1 *Lb. acidophilus* (1) 1 gene in 1 *Lb. casei* (2) 3 genes in 1 *Lb. salivarius* (1) 2 genes in 2 *Lc. lactis* (4)
Multi-drug *resistance* protein	2 genes in 1 *B. animalis* (5) 1 gene in 1 *B. dentium* (1) 4 genes in 1 *Lb. acidophilus* (1) 1 gene in 1 *Lb. casei* (2) 1 gene in 1 *Lb. helvetica* (1) 1 gene in 1 *Lb. salivarius* (1) 4 genes in 1 and 1 gene in 1 *Lc. lactis* (4)
Multi-drug *resistance* protein b	4 genes in 1 *B. animalis* (5) 2 genes in 1 *B. dentium* (1) 3 genes in 1 *Lb. salivarius* (1) 1 gene in 1 *Lc. lactis* (4)
Multi-drug *resistance* protein b, mf superfamily	5 genes in 1 *Lc. lactis* (4)

[a]The total number of genomes included in the analysis for the species listed is given in parentheses.

genomes in which the minimum number of 'resistance' genes could be detected, and those with the maximum numbers of such genes. The finding of as many as 17 resistance-related genes in a single genome was somewhat unexpected. The Genome Project web page at NCBI provides the following information on the *Bifidobacterium* strain with the highest number of resistance genes in Table 13.4: '*B. animalis* subsp. *lactis* BB-12 is a well-characterised probiotic bacterial strain. It has demonstrated probiotic effects, with anti-inflammatory and anti-pathogen properties'. Apparently, the presence of antibiotic resistance genes was not considered a risk for probiotic application; more likely though,

Table 13.4. Genomes with maximum and minimum number of resistance genes, per genus and species/strain.[a]

Genus	Min. no.	Max. no.	Species and strain
Bifidobacterium	0		*longum* NCC2705
		13	*animalis lactis* BB-12
Lactobacillus	0		*delbrueckii bulgaricus* ATCC 11842
			fermentum IFO 3956
			johnsonii NCC 533
			plantarum JDM1 and WCFS1
			reuteri JCM1112
			rhamnosus GG
			sakai 23K
		17	*casei* BL23
Lactococcus	2		*lactis cremoris* SK11
		16	*lactis lactis* KF147
Leuconostoc	1		*citreum* KM20
		4	*kimchii* IMSNU11154
Streptococcus	1		*thermophilus* CNRZ1066
		4	*thermophilus* LMG18311

[a]Note that these data are based on semantic IMG searches only, and thus may underestimate the true abundance of these genes.

their presence was not known at the time the strain was introduced as a probiotic. These genes did not draw much attention either, once the sequence was completed: the genome announcement of this sequence (Garrigues *et al*., 2010) does not mention any resistance genes. Some of these genes would be responsible for intrinsic resistance; others, such as *tetW*, are so widely present in gut bacteria that they can be considered ubiquitous and a probiotic strain carrying them would not increase any risk. However, that may not apply to all of the genes found in this brief analysis. Whether the resistance genes reported in Tables 13.3 and 13.4 could be expressed, or whether they are transferable, was not assessed here. One publication describes the experimental application of strain *B. animalis* subsp. *lactis* BB-12 together with doxycycline (a tetracycline), which resulted in the faecal shedding of reduced numbers of susceptible colonies of the strain (Saarela *et al*., 2007). The authors concluded that this phenotype was not the result of DNA uptake as the *tetW* gene, which they held responsible, was already present in the original *B. animalis lactis* BB-12. They may not have realized that this was not the only resistance gene present, and the reported results illustrate that some of these genes can, under selective pressure, result in a resistant phenotype.

Not all antibiotic resistance genes are transferable. When the antibiotic susceptibility of probiotic strain *Lb. brevis* KB290 was tested, resistance against four drugs was found, but none of these resistances seemed to be transferable (Fukao *et al*., 2009). These findings were sufficient to consider the bacteria safe for human consumption according to the European Qualified Presumption of Safety (QPS). In addition, when a resistant phenotype is detected, it may not be the result of acquired resistance. Resistance to aminoglycoside and fluoroquinolone was frequently detected in starter cultures, but these might be intrinsic properties (Hummel *et al*., 2007); as these authors pointed out, break-point values are often inadequately defined, so that phenotypic resistance testing is not without difficulties.

The *Lb. casei* strain BL23 that carries at least 19 resistance genes (including the two that were not identified in the semantic search, as discussed above) is described as follows: '*Lactobacillus casei* BL23 is a probiotic strain that was originally isolated from cheese and will be used for comparative analysis'. In its genome announcement, Mazé *et al*. (2010) remarked upon the high similarity of strain

BL23 to *Lb. casei* strain ATCC334, the only other genome available for this species at that time. A 99% similarity was identified to ATCC334, a strain that is in use as starter culture. The BL23 genome is almost 0.2 Mbp larger than that of ATCC334. A significant fraction of the accessory genome is linked to prophage insertions and insertion element sequences; other regions present only in BL23 are related to carbohydrate utilization. It was not tested here whether that similarity extends to the resistance genes identified in the first strain but not in the second by the semantic search.

The presence of all these resistance genes does not have to coincide with a multi-drug resistant phenotype, as not all genes are necessarily expressed. Presence of a virulence gene is even less likely to result in virulence, as virulence usually results from the presence and expression of multiple genes. All organisms from which these 40 genomes were derived are mostly non-pathogenic (an exception can be made for the opportunistic pathogen *B. dentium*). Nevertheless, these findings of 'risky' genes in 'safe' bacteria may be unexpected; given this finding, what can we expect to find in genomes from bacteria with unknown properties? Can safety be predicted at all, based on the gene content of a sequence genome? To address this, we will look at a comparison of genomes from both pathogenic and commensal/probiotic bacteria of the five genera so far discussed, to which *Enterococcus* is added.

13.5 Comparison of Complete Genomes from Pathogenic and Probiotic Microbes

In the introduction to this chapter, we proposed to compare genomes from beneficial organisms with those from pathogens. Unfortunately, it is not possible to extract, from genome sequence data, those genes that are exclusively found in pathogens, irrespective of their functional descriptions, and take these as predictors for virulence. In some cases, such a comparison would involve different strains within a species, for example comparing commensal and pathogenic *E. faecium* strains, with the caveat that the latter are opportunistic pathogens only. In other cases, the comparison may have to be extended to different species, as for *Streptococcus* (*S. thermophilus* as a GRAS organism versus the other, pathogenic *Streptococcus* species). It may even require comparisons between genera (*Lactobacillus*, *Leuconostoc* and *Lc. lactis* versus pathogenic *Firmicutes*). If one compares the gene content of different genomes within a species, both between species of one genus, and between genera, the degree of variation in gene content would vary several orders of magnitude, as exemplified in Fig. 13.1. The figure summarizes variation within and between genomes of different genera. For this analysis, both finished and unfinished genomes were used. Moreover, *Enterococcus* genomes were included, and pathogenic *Streptococcus* genomes (three genomes per species) were added to the *S. thermophilus* genomes to capture the gene content of this genus. In total, 81 genomes were analysed; a complete list is available elsewhere (Lukjancenko *et al*., 2012).

To construct Fig. 13.1, the pan-genome of each genus was first established; this contains all the genes that can be found in any of the sequenced genomes in this genus (Ussery *et al*., 2009). An example of a pan-genome and core genome plot of the genus *Lactobacillus* is shown to the left of Fig. 13.1. The resulting *Lactobacillus* pan-genome was used as a starting point for the comparison shown in the panel on the right. This shows the number of novel gene families found in each newly added genus as the light-grey columns. Adding the novel genes from the *Lactococcus* pan-genome to the running total results in the accumulative pan-genome (the mid-grey columns). The accumulative pan-genome of these six genera exceeds 29,000 genes. The third, darkest grey columns enumerate gene families that are conserved in all these pan-genomes, amounting to 172 gene families. It should be noted that these genes do not have to be conserved and present in every single bacterial genome that was included here; instead, they are conserved in the pan-genomes of these genera, so these 172 gene

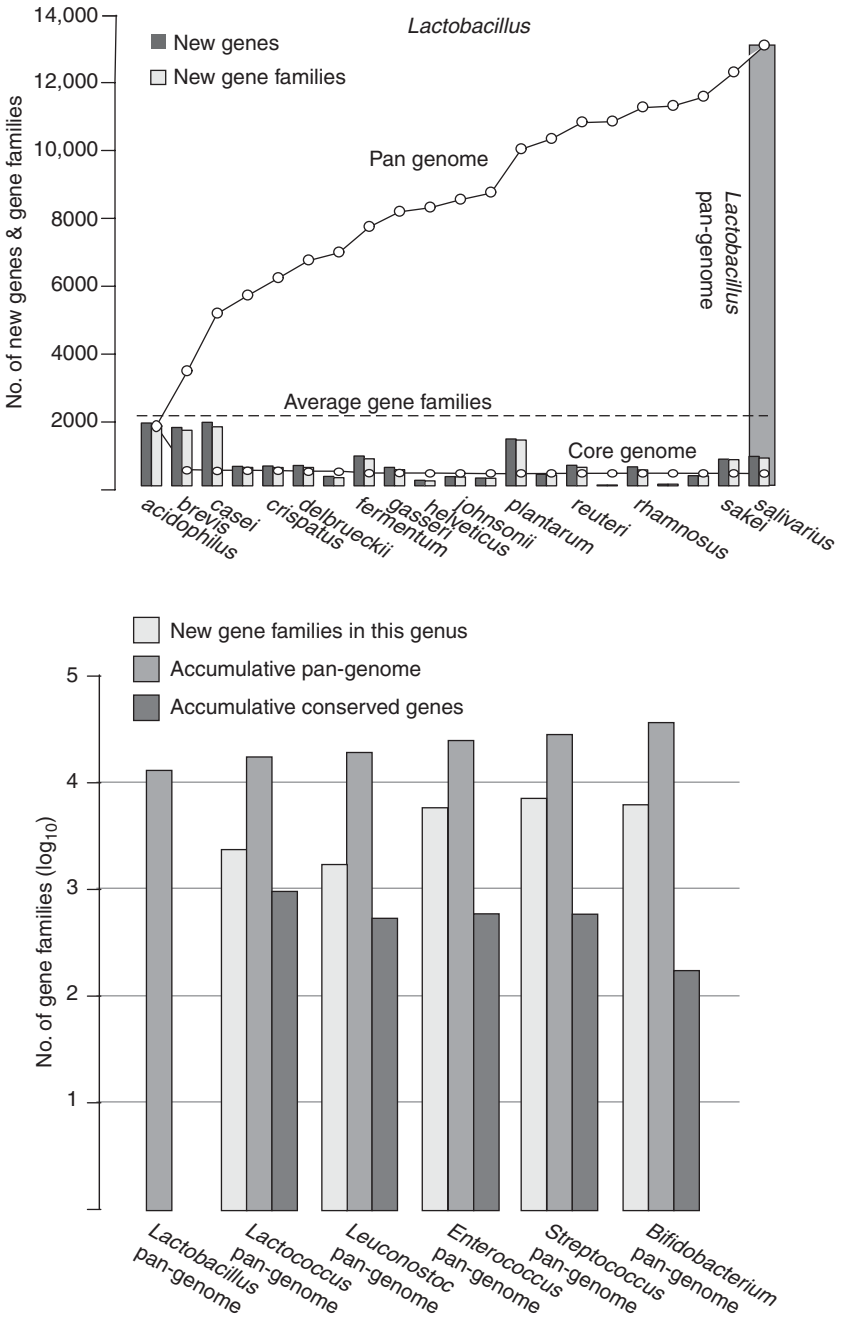

Fig. 13.1. Comparison of the pan-genomes of six bacterial genera. In the top panel, a pan-genome and core genome plot of the *Lactobacillus* genus is constructed. The horizontal broken line indicates the average number of gene families present in a *Lactobacillus* genome. The pan-genome of this genus (based on the 21 analysed genomes) is used for construction of the graph in the bottom panel. Similar pan-genomes were calculated for all six genera. These pan-genomes were added one by one and the novel genes, accumulative pan-genome and accumulative conserved genes were recorded. Note that the scale in the bottom panel is logarithmic whereas that in the left panel it is linear. Courtesy of Oksana Lukjancenko.

families are present in at least one genome per genus. Compare this with the true core genome of all 81 genomes that has been described: there are only 63 gene families that are conserved and present in all of these genomes (not shown in the figure) (Lukjancenko *et al*., 2012). The core genome of the *Lactobacillus* genus contains 363 gene families, and that of *Bifodobacterium* 725 (O. Lukjancenko, unpublished data).

With this degree of variation within a genus (as Fig. 13.1 illustrates for *Lactobacillus*) and between genera, there is too much 'noise' that hides the 'signal' of virulence genes found in pathogens and absent in probiotics. If one were to compare the gene content between, say, a probiotic *Lactobacillus* strain and a pathogenic *Enterococcus* strain, there will be many genes found in the latter that are absent in the former, but that has little to do with their difference in phenotype: these genes make the difference between an *Enterococcus* and a *Lactobacillus*.

There is a way to tackle this problem. We have split the 81 genomes of interest into two groups: in one group we combined all pathogenic *Firmicutes* and in the other all non-pathogens (*Firmicutes* plus bifidobacteria). For these two collections, the pan-genomes and core genomes were calculated. We then compared to which COG classes these pan-genome genes and core-genome genes belonged, and compared the relative enrichment (i.e. conservation) to COG classes in the core genome with respect to the pan-genome. This identified significant differences between the enriched core gene families (genes conserved in all genomes of the collection) of the pathogens compared with the non-pathogens (Lukjancenko *et al*., 2012). The analysis identified, for instance, that genes involved in post-translational modification and chaperones were over-represented in the core genome (compared with their pan-genome) of non-pathogens but not of pathogens. Conversely, genes involved in cell wall/membrane biosynthesis secretion were over-represented in the core genome of pathogens but not in that of non-pathogens. The latter finding matches our knowledge of virulence genes, which are frequently cell surface structures or secreted proteins. Thus, without specifically searching for a role in virulence, genes were identified in the core genome of pathogens that are likely to have a function in virulence. It is hypothesized that the genes identified as enriched in the core genome of non-pathogens would likewise play a role in establishing a commensal or possible probiotic relationship with the host.

Where do we go from here? The approach described above can be used as a proof of principle. When more genomes of probiotic, commensal and pathogenic strains become available, the method can be fine-tuned and optimized. That could possibly lead to a better understanding of the genes involved in either pathogenicity or a commensal/probiotic relationships with the human host. A pragmatic approach could be to accept that presence of 'risky' genes in probiotic strains with a long history of safe use is not a problem, as long as they are present in that particular species. If we accept that view, a list of genes could be produced that, in particular genomic backgrounds, do not contribute to virulence, despite the fact that they can do so in other, putatively pathogenic organisms. This gives a means to weigh the risk these genes provide, so that novel genome sequences can be interpreted for the degree of safety of the organism in question based on gene content.

13.6 Conclusion

Is it feasible that in the near future we can predict the risk to human health for a bacterial isolate, based on its genome sequence only? Even though the properties of micro-organisms would be encoded in their genes, microbes will adjust their expression profile to external conditions. Genome sequences might reveal mechanisms by which micro-organisms introduce variation into their expression profiles by genetic polymorphism, phase variation and antigenic variation, but they cannot be used to predict expression patterns under all circumstances. Currently, whole genome expression profiling and *in vivo* phenotypic tests are still needed to describe the behaviour and safety of bacterial strains. Gene expression profiling using next-generation sequencing might

ease that approach. The number of genes for which a function cannot be predicted should, it is hoped, decrease over time, at least for such genes that are conserved in multiple organisms; singleton genes with unknown function are more problematic. At present, their relevance to pathogenicity remains unknown. None the less, genome sequences can contribute to hazard identification by identifying and excluding potential pathogenic microorganisms based on the pure presence of virulence factors and to hazard characterization, by describing the microorganism's lifestyle.

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14 Occupational Safety of Microbial Agents

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14.1 Introduction

Occupational exposure to bio-aerosols containing high concentrations of fungi, bacteria and other bio-aerosol components such as endotoxin may cause various deleterious health effects dependent on the exposure levels (Rylander *et al*., 1985; Eduard *et al*., 2001). In addition to direct health effects, exposure to fungi may also have an adjuvant effect on the allergic response to other allergens. For instance, the insect pathogenic fungus *Metarhizium anisopliae* has been found to have an adjuvant effect on a standard allergen (ovalbumin) in mice (Instanes *et al*., 2006). Endotoxin, a cell wall component of Gram-negative bacteria such as *Pseudomonas* has strong pro-inflammatory properties. In the Netherlands, the recommended exposure limit for endotoxin is 90 endotoxin units m^{-3} (Health Council of the Netherlands, 2010). Non-pathogenic bacteria, including the actinobacteria (one of the dominant phyla of the bacteria), may cause respiratory symptoms by triggering immune responses in exposed individuals (Purokivi *et al*., 2001; Tlaskalova-Hogenova *et al*., 2005). Workers in a variety of occupations are exposed to microbes, which may cause allergies or occupational asthma, e.g. Godnic-Cvar *et al*. (1999) found brewery workers to be significantly more likely to react positively to moulds in skin prick tests. Many of the workers in the study also reacted positively to brewer's yeast in skin prick tests. Enzymes from microorganisms, e.g. the fungi *Aspergillus* spp. and *Trichoderma viride*, and the bacterium *Bacillus subtilis* are used in the baking and pharmaceutical industries and are associated with occupational

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asthma in workers from these industries (Lachowsky and Lopez, 2001). This subject is treated elsewhere (Flindt, 1969; Dolovich and Little, 1972; Horner *et al*., 2008), and is not part of this chapter, which reviews occupational exposure to microbial biocontrol agents (MBCAs) and also to baker's/brewer's yeast (*Saccharomyces cerevisiae*) and to *Botrytis cinerea* (used in wine production), in relation to occupational health.

The prevalence of a microorganism in its natural habitat is referred to as the background exposure level of the microorganism, and this needs to be taken into account when evaluating the environmental safety of microbial plant protection products (Mensink and Scheepmaker, 2007). In this chapter, commercial microbial biocontrol products based on MBCAs will be referred to as MBCPs. We elucidate the background exposures to different applied species of microorganisms. The focus is on MBCPs based on the fungi *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii* (i.e. *Lecanicillium* spp.), *Trichoderma harzianum*, *Trichoderma polysporum* and *Trichoderma viride*, on the actinobacterium *Streptomyces griseoviridis*, and on the bacteria *Bacillus subtilis*, *Bacillus thuringiensis* and *Pseudomonas chlororaphis*.

Directive 2000/54/EC of the European Parliament and Council (OJEC, 2000) forms the legal basis for the legislation in EU (European Union) countries concerning the protection of workers from risks related to exposure to biological agents at work. The Directive classifies microorganisms present in occupational environments according to whether the microorganism 'is unlikely to cause human disease' (Risk group 1), 'can cause disease for which there is usually an effective treatment, and is unlikely to spread to the community' (Risk group 2), 'can cause severe disease, for which there is usually an effective treatment, but there is a risk of spreading to the community' (Risk group 3) or 'can cause severe disease with usually no effective treatment, and with a high risk of spreading to the community' (Risk group 4). The Directive also lists the biological agents currently included in Risk groups 2–4. None of the microorganisms discussed in this chapter rate above Risk group 1. The Directive charges employers with assessing the risk to workers' health and safety, and taking the appropriate measures to ensure that workers are protected against exposure to biological agents.

Microorganisms selected for biocontrol and foodstuff production are supposedly not infectious in humans. However, a few case histories exist which indicate that some species used for biocontrol or foodstuff production are to some extent infectious, or at least may be prevalent in weakened or immunocompromised persons (Guiserix *et al*., 1996; Henke *et al*., 2002; Ren *et al*., 2004; Tucker *et al*., 2004; Gürcan *et al*., 2006; Oh *et al*., 2009), and a few examples will be elucidated.

Sections 14.2 and 14.3 of this chapter treat microorganisms used as MBCAs and Section 14.4 treats microorganisms used in foodstuff production. In Sections 14.2 and 14.3, exposure to aerosolized fungi and bacteria, respectively, is reviewed. The actinobacterium *S. griseoviridis* is included in Section 14.3. The first part of each of the sections reviews exposure to the applied microorganisms during different work activities, and if possible, compares it with background exposure levels of microorganisms of the same species, microorganisms of the same genera, and total fungi, actinobacteria or bacteria. Then, exposure of bystanders to MBCPs is considered, as people may work in neighbouring areas, and people may come to work in people's homes in MBCP-treated areas (e.g. home care workers). Finally, background exposures are reviewed in order to establish a frame of reference for other types of exposure. Exposures are preferentially expressed as cfu m^{-3} air (colony forming units per cubic metre of air), or as otherwise applicable; if these are available, exposures are related to epidemiological studies.

14.2 Exposure to Fungal MBCAs

Data on both occupational and background exposure to fungal MBCAs are included in Tables 14.1 and 14.2.

Table 14.1. Occupational exposure to MBCAs (microbial biocontrol agents) and to other microorganisms.

		MBCA			Other microorganisms[b]	
Microorganism	Environment	Exposure as cfu m^{-3} air[a]	Other measures of exposure	Product	Exposure as cfu m^{-3} air	Reference
Beauveria bassiana	Forest	–	50%[c]	Isolate from Japan	–	Shimazu *et al.*, 2002
Trichoderma harzianum + *T. polysporum*	Field Strawberry, outdoors	Bd	–	Binab® T Vector	9500 (3700– $3.0x10^4$)	Tendal and Madsen, 2011
T. harzianum	Greenhouse Tomato, indoors	1×10^5	–	Supresivit®	1200	Hansen *et al.*, 2010b
T. harzianum	Greenhouse Tomato, indoors	Bd	–	Supresivit®	3.7×10^4 ($4200–4.3 \times 10^4$)	Hansen *et al.*, 2010b
T. harzianum	Greenhouse Flower, indoors	3539	–	Yes[f]	9233	Li and LaMondia, 2010
T. harzianum	Greenhouse Flower, indoors	42	–	Yes[f]	5053	Li and LaMondia, 2010
Streptomyces griseoviridis	Greenhouse Tomato, indoors	Bd	–	Mycostop®	1490	Hansen *et al.*, 2010b
Bacillus thuringiensis subsp. *israelensis*	Faecal samples from greenhouse workers	–	40%[d]	Vectobac and Bactimos	–	Jensen *et al.*, 2002
B. thuringiensis subsp. *kurstaki*	Kromecote card (to measure droplet deposition), outdoors in a spray zone	–	78%[e]	Foray® 48B	–	Pearce *et al.*, 2002
B.t. subsp. *kurstaki*	Coastal area, outdoors	729 (Bd– 1600)	–	Foray® 48B	–	Teschke *et al.*, 2001
B.t. subsp. *kurstaki*	Homes in treated areas	159 (Bd– 627)	–	Foray® 48B	–	Teschke *et al.*, 2001
B.t. subsp. *kurstaki*	Greenhouse Tomato, indoors	470 (Bd– 5300)	–	Dipel®	3100 (360–7500)	Hansen *et al.*, 2010a
B.t. subsp. *kurstaki*	Greenhouse Tomato, indoors	Bd (Bd– 1400)	–	Dipel®	5.3×10^4 (Bd–5.8×10^5)	Hansen *et al.*, 2010a
B.t. subsp. *kurstaki*	Field Cabbage, outdoors	Bd	–	Dipel®	470 (240–8300)	Hansen *et al.*, 2010a

Continued

Table 14.1. Continued.

		MBCA			Other microorganisms[b]	
Microorganism	Environment	Exposure as cfu m^{-3} air[a]	Other measures of exposure	Product	Exposure as cfu m^{-3} air	Reference
B.t. subsp. *kurstaki*	Field Cabbage and broccoli, outdoors	Bd (Bd–410)	–	Dipel®	8300 ($5600–1.2 \times 10^4$)	Hansen *et al.*, 2010a
B.t. subsp. *kurstaki*	Field Celery, outdoors	Bd (Bd–160)	–	Dipel®	1.9×10^4 ($1600–2.1 \times 10^4$)	Hansen *et al.*, 2010a
B.t. subsp. *kurstaki*	Field Strawberry, outdoors	Bd	–	Dipel®[g]	Nm	Tendal and Madsen, 2011

[a]Exposure as median or average and (in some cases) as range (in parentheses).
[b]Other fungi if the organism in focus is a fungus, other actinobacteria if the organism in focus is an actinobacterium and other bacteria if the organism in focus is a bacterium.
[c]*B. bassiana* was detected 12 out of 24 months.
[d]Present in 8 of 20 faecal samples.
[e]Present on 78% of the cards.
[f]Not mentioned which products were used.
[g]Field was treated with the product the previous year.
Bd = below detection level; Nm = not mentioned.

Table 14.2. Background exposures to and frequencies of microbial species used as MBCAs (microbial biocontrol agents), other species[a] and other microorganisms.[b]

Microorganism	Environment	Background exposure to and frequency of: Species used as MBCA		Other species[a]		Other microorganisms[b]	Reference
		cfu m^{-3} air[c]	%[d]	cfu m^{-3} air	%	cfu m^{-3} air	
Beauveria bassiana	Hospital, indoors	0.2	–	Bd		(143–1192)	Rainer *et al.*, 2000
B. bassiana	Outdoors	0.2 (0.1–0.5)	–	<0.1 (0.1–0.1)		Nm	Airaudi and Marchisio, 1996
B. bassiana	Nasal mucus	–	3.4	–	0	–	Buzina *et al.*, 2003
B. bassiana	Forest	–	33	–	–	–	Shimazu *et al.*, 2002)
Trichoderma harzianum	Agricultural area, outdoors	(4–135)	–	Bd	–	Approx. (500–2000)	Das and Gupta-Bhattacharya, 2010
T. viride	Cellulose production, indoors	5 (0–24)	–	Bd	–	3×10^4 ($1900–10^5$)	Kotimaa, 1990
T. viride	Fuel chips, indoors	200 ($10–6 \times 10^4$)	–	Bd	–	$3x10^4$ ($3000–3 \times 10^6$)	Kotimaa, 1990
T. viride	Hop farms, outdoors and indoors	–	53	Bd	Bd	2.1×10^3 (0.42–9.58)	Góra *et al.*, 2004
T. viride	Settled grain dust, indoors	–	1.8	–	3.0	–	Szwajkowska-Michalek *et al.*, 2010
T. viride	Nasal mucus	–	1.3	–	0.4	–	Buzina *et al.*, 2003
Verticillium lecanii	Outdoors	<0.1 (0.2–0.3)	–	<0.1 (0.1–0.1)	–	Nm	Airaudi and Marchisio, 1996
V. lecanii	Nasal mucus	–	0.4	–	0.4	–	Buzina *et al.*, 2003
Bacillus thuringiensis	Cow shed, indoors	10^6	–	10^7	–	(10^5–10^9)	Andersson *et al.*, 1999
B. thuringiensis	Restaurant, indoors	1	–	5	100	–	Chan *et al.*, 2009
B. thuringiensis	Outdoors, outside a *B. thuringiensis* spray zone	–	9	–	–	–	Pearce *et al.*, 2002
Bacillus subtilis	Cowshed, indoors	10^7	–	10^7	–	(10^5–10^9)	Andersson *et al.*, 1999
B. subtilis	Apartment, indoors	0.19	–	1.4	–	22	Simard *et al.*, 1983

Continued

Table 14.2. Continued.

Microorganism	Environment	Background exposure to and frequency of: Species used as MBCA		Other species[a]		Other microorganisms[b]	Reference
		cfu m^{-3} air[c]	%[d]	cfu m^{-3} air	%	cfu m^{-3} air	
B. subtilis	Fibreboard, chipboard factories, indoors	–	100		100	1×10^4–2×10^4	Dutkiewicz *et al.*, 2001a
B. subtilis	Dwellings, indoors	–	1.7		53	≈440 (88–3442)	Górny and Dutkiewicz, 2002
B. subtilis	Upwind of a cattle feedlot, outdoors	492	–	95	–	Nm	Wilson *et al.*, 2002
B. subtilis	City, outdoors	≈6[e]	–	≈81[e]	–	1439 (130–2×10^4)	Fang *et al.*, 2007
B. subtilis	Herb processing, indoors	–	100		100	1.3×10^5–2.3×10^5	Dutkiewicz *et al.*, 2001b
B. subtilis	Downwind of a cattle feedlot, outdoors	2363	–	690	–	Nm	Wilson *et al.*, 2002
Pseudomonas chlororaphis	Cattle house, indoors	0.5[e]	–	266.8[e,f]	–	9773[e]	Zucker *et al.*, 2000
P. chlororaphis	Cowshed, indoors	(10^6–10^8)	–	(10^6–10^8)	–	(10^5–10^9)	Andersson *et al.*, 1999
P. chlororaphis	Flats, indoors	–	3.3		10.0	–	Górny and Dutkiewicz, 2002

[a]Other species of the genera in focus.
[b]Other fungi if the organism in focus is a fungus, other actinobacteria if the organism in focus is an actinobacterium and other bacteria if the organism in focus is a bacterium.
[c]Exposure as median or average and (in some cases) as range (in parentheses).
[d]Frequency (as %).
[e]Calculated value from paper.
[f]Other species of the family in focus.
Bd=below detection level, Nm=not mentioned.

14.2.1 Occupational exposure to fungal MBCAs and risk assessment

Beauveria

B. bassiana has been introduced experimentally to control the beetle *Monochamus alternatus* in some forests, and the frequency of *B. bassiana* in these forests is higher than in a neighbouring, untreated forest. Thus, airborne *B. bassiana* was found 100 cm above ground level in 12 of 24 months in treated forests, while it was found in only 8 of 24 months in untreated forests (Shimazu *et al*., 2002) (Tables 14.1 and 14.2). This presence is relevant in relation to inhalation, because naturally occurring *B. bassiana* has been shown to be present as particles of respirable size (Fröhlich, 2009). *B. bassiana* has also been found in sputum samples (Pore *et al*., 1970; Comstock *et al*., 1974), although investigations were not made into whether it caused any health symptoms in these cases. Furthermore, no epidemiological studies have been published concerning exposure-related health effects of *B. bassiana* used as an MBCA. However, intracutaneous skin tests of patients with recurrent complaints of bronchial obstructive symptoms showed that 6.8% of the patients had strong reactions to *B. bassiana*, while only 2.3% showed a reaction to the more common fungus, *Cladosporium* (Beaumont *et al*., 1985). Several studies have described *B. bassiana* as the causal agent of keratitis (Low *et al*., 1997; Kisla *et al*., 2000; Tu and Park, 2007; Pariseau *et al*., 2010), but c omparisons of *B. bassiana* DNA isolated from five patients with keratitis and of *B. bassiana* from two MBCPs showed that isolates were not identical and so the keratitis was not caused by isolates from the MBCPs (Pariseau *et al*., 2010).

Verticillium (Lecanicillium)

Two MBCPs based on *V. lecanii* are available on the market. However, according to current taxonomy, these two products are based on two distinct species, *Lecanicillium muscarium* and *L. longisporum*, respectively (Zare and Gams, 2001; Andersen *et al*., 2006). In this chapter we have included papers about both *Verticillium* and *Lecanicillium*.

Aerosolized *V. lecanii* (*L. muscarium*) spores are mainly of respirable size and therefore deposition in the small airways is possible (Madsen, 2011). Occupational exposure levels to *L. muscarium* or *L. longisporum* MBCPs have not been measured, but occupational exposure-related health effects have been studied. Thus, an allergological and toxicological investigation has been performed on personnel producing and manufacturing a product with *V. lecanii* (Eaton *et al*., 1986). A share of 5% of the personnel reacted positively to *V. lecanii* in an allergy test. However, no toxic effect was found on blood parameters following handling of *V. lecanii*.

In a cohort of 329 greenhouse workers, including workers handling MBCPs – Mycotal® (with *L. muscarium*) or Vertalec® (with *L. longisporum*) – a high prevalence of sensitization for both MBCAs was found and 9–21% of workers had detectable IgE antibodies to the MBCAs (Doekes *et al*., 2004). These findings indicate that use of the MBCPs may be a risk factor for occupational IgE-mediated allergic sensitization.

Cases are reported where *Verticillium* spp. have been infectious, mainly in weakened persons or immunocompromised patients (Amici *et al*., 1994; Wu *et al*., 2008), and they are known to have caused, e.g. keratitis (Shin *et al*., 2002; Yao *et al*., 2003), but in all cases the fungi have only been identified to genus level and no conclusions can be drawn concerning the virulence of *L. muscarium* and *L. longisporum*. Furthermore, the mentioned cases were not described to be related to the use of MBCPs.

Metarhizium

As an entomopathogenic fungus, *M. anisopliae* can be used against mosquitos carrying diseases, which makes indoor residual treatment a possible source of human exposure. Therefore, exposure was measured in a simulated treatment, where about 7000 spores m^{-3} air were initially found, decreasing to about 500 spores m^{-3} air within 48 h of treatment. This corresponded initially to 2% of total airborne particles observed by microscopy, decreasing to 0.1% of total airborne particles (Darbro and Thomas, 2009). As no *M. anisopliae* spores were detected in the pretreatment

sample, the post-treatment exposure is quite high. The authors have found no studies of health effects due to occupational exposure to *M. anisopliae*, although it has been reported as being infectious in humans, e.g. *Metarhizium* spp. were found to be the causal agents in 2% of the cases of fungal keratitis registered at a New York clinic (Ritterband *et al*., 2006), while a disseminated infection due to *M. anisopliae* contributed to the death of a 9-year-old immunocompromised child (Burgner *et al*., 1998).

Trichoderma

Exposure to *Trichoderma*-based products has been measured during application of MBCPs in horticultural environments. When bees were used for the application of Binab® T Vector (an MBCP containing *T. harzianum* and *T. polysporum*), *Trichoderma* was not found in the air (Table 14.1) (Tendal and Madsen, 2011). *T. harzianum* (Supresivit®) was found in air samples when its powdered formulation was prepared for application. The exposure level was 1.0×10^5 cfu m^{-3} air, which was higher than the exposure to other fungi (Table 14.1). PCR analysis confirmed that the *T. harzianum* isolates were from the MBCP (Hansen *et al*., 2010b). *T. harzianum* can persist for 9 weeks post application in the rhizosphere of greenhouse crops (Lübeck and Jensen, 2002), but no exposure to airborne *T. harzianum* was found 6 days, 1 month or 3 months after application (Hansen *et al*., 2010b) (Table 14.1). This is probably because rhizosphere material was not aerosolized during the working activities that were performed. In another study, an MBCP based on *T. harzianum* was applied in greenhouses, and high exposures to *T. harzianum* were found (Table 14.1). The exposure to *T. harzianum* was up to a maximum of 3.6×10^4 cfu m^{-3} air and constituted on average 38% of the total number of fungi (Li and LaMondia, 2010), which may be considered as high in comparison with background exposure to *Trichoderma* spp. (see Tables 14.1 and 14.2).

About half of the particles released from cultures of *T. harzianum* and cultures of the product Binab® are of respirable size and are able to penetrate into the lower respiratory tract (Madsen, 2011). In a short-term (6 min) exposure of human airways to high concentrations of *T. harzianum* (3.5×10^5 spores m^{-3} air), no more reactions were seen than during exposure to placebo in eight sensitive school employees (Meyer *et al*., 2005). The studied *T. harzianum* isolate was not from an MBCP.

T. viride has been isolated from the nasal mucus of two of 210 patients suffering from chronic rhinosinusitis and from one of 23 healthy people (Buzina *et al*., 2003) (Table 14.2). *Trichoderma* sp. was also isolated from eight of 210 people also suffering from chronic rhinosinusitis, but not from 14 healthy people (Ponikau *et al*., 1999). Case reports about *Trichoderma* infections are reviewed by Kredics *et al*. (2003). However, no scientific papers are available on occupational exposures and related health effects for MBCPs with *T. polysporum* or *T. viride*.

14.2.2 Exposure of bystanders to fungal MBCAs and risk assessment

Beauveria

In a study where *B. bassiana* was applied to a forest, the fungal conidia were dispersed by the wind; however, the density of the fungus in the air at more than 50 m from the source did not differ from the natural density of the fungus (Shimazu *et al*., 2002).

Metarhizium

Exposure of bystanders to *M. anisopliae* has not been measured directly, but exposure-related effects have been studied. *M. anisopliae* is frequently applied in sugarcane plantations, which has led to investigations of whether it might be an aetiological agent of bronchial asthma in asthma patients in those regions. Out of 50 asthma patients, eight presented strong positive reactions when submitted to a prick test with allergenic extract from *M. anisopliae*, and three showed sensitization in a bronchoprovocation test (reviewed in Barbieri *et al*., 2005). A share of 23 out of 79 atopic patients from an area with sugarcane production were found to test positive to a prick test, while only three out of 35 atopic patients from an urban area tested positive.

It was not stated whether the sugarcane area had in fact been treated with *M. anisopliae*. No one in the non-atopic control group (11 individuals) tested positive (Barbieri *et al*., 2005).

Trichoderma

T. harzianum has been found outside two greenhouses within which it had been applied (Li and LaMondia, 2010), indicating a potential exposure of bystanders. This is of relevance because a few cases have been reported in which *T. harzianum* or *T. viride* have been invasively infectious in immunocompromised patients (Loeppky *et al*., 1983; Guiserix *et al*., 1996; Chouaki *et al*., 2002). However, these cases were not related to the use of MBCPs. In another study, *T. harzianum* was not found outside the greenhouse where it had been applied (V.M. Hansen, Denmark, 2011, personal communication).

14.2.3 Background exposure to fungi

Beauveria

The entomopathogenic fungi *B. bassiana* and *V. lecanii* (*Lecanicillium* spp.) seem to be infrequently present in the air and, in general, people seem to be seldom exposed to these fungi (Madsen *et al*., 2007; Madsen, 2011). Thus, in airborne indoor dust, the concentrations of *B. bassiana* (Cheong and Neumeister-Kemp, 2005; Basilico *et al*., 2007) and *Beauveria* spp. (Garrett *et al*., 1997; Picco and Rodolfi, 2000) were low and when detected, less than 0.1% of all fungi. *B. bassiana* has been found in forest air (Shimazu *et al*., 2002), in outdoor air close to a composting facility and a waste-water treatment plant (Grisoli *et al*., 2009) and in overwintering facilities of honeybees (Sigler *et al*., 1996). *B. bassiana* has also been isolated from the nasal mucus of patients suffering from chronic rhinosinusitis (six of 210 patients) and from healthy persons (two of 23 persons) (Buzina *et al*., 2003) (Table 14.2).

Verticillium (Lecanicillium)

V. lecanii has been found in low concentration in outdoor air (Table 14.2), while exposure to *Verticillium* spp. frequently occurs in connection with the harvesting of cereals (Darke *et al*., 1976); it was found at a lower frequency of 3% of samples in a study in cotton mills (Lacey and Lacey, 1987). Exposure to *Verticillium* species has also been measured in indoor air, where the frequency of presence in samples was less than 0.1% (reviewed in Madsen, 2011). *V. lecanii* has been isolated from the nasal mucus of one of 23 healthy volunteers and of none of 210 patients suffering from chronic rhinosinusitis (Buzina *et al*., 2003) (Table 14.2).

Metarhizium

The presence of *M. anisopliae* on surfaces in an outdoor recreational environment was quantified by culturing swabs from ten locations in the park; it was found in one location at 100 cfu $5\,cm^{-2}$, which ranks it as the fifth lowest of the 19 fungal species/genera found. Tape-lift sampling was also done, but recovered no viable *M. anisopliae* spores (Sudakin and Fallah, 2008). An investigation of background exposure to outdoor airborne fungi was performed using 312 daily exposures of agar plates to outdoor air for 10 min over 13 months. Only 1 cfu (calculated value from the paper) of *M. anisopliae* was recovered, which made up 0.06% of the total number of cfu counted during the investigation (Al-Subai, 2002).

Trichoderma

Airborne *Trichoderma* species have been found in different environments and countries, and in a few studies, as the dominant taxon (Madsen *et al*., 2007). *T. viride* has often been found in the air in environments where organic material is handled (Reiman and Uitti, 2000; Dutkiewicz *et al*., 2001b; Adhikari *et al*., 2004a; Grisoli *et al*., 2009), but also in homes (Cheong and Neumeister-Kemp, 2005), and sometimes in high concentrations (Table 14.2). *T. harzianum* has only been found in airborne dust in a few studies and often at low concentrations (Adhikari *et al*., 2000, 2004a,b; Madsen *et al*., 2007). In two studies in horticultural environments, *T. harzianum* and *T. polysporum* were not found when monitored using *Trichoderma*-selective medium, (Hansen *et al*., 2010b; Tendal and Madsen, 2011) (Table 14.1).

T. polysporum seems not to have been found in airborne dust (Madsen *et al*., 2007). Sudakin and Fallah (2008) cultured swabs from outdoor surfaces in a park, and *T. viride* was detected in one sample at 10 cfu 5 cm^{-2}, which was the lowest concentration of any fungal species/genera found. Tape-lifting sampling was also done, but no *T. viride* was recovered.

14.3 Exposure to Bacterial MBCAs

Data on both occupational and background exposure to bacterial MBCAs are included in Tables 14.1 and 14.2.

14.3.1 Occupational exposure to bacterial MBCAs and risk assessment

Bacillus

Exposure to *B. thuringiensis* subsp. *kurstaki* at 5300 cfu m^{-3} air has been found for a grower applying the product Dipel® with a hand-pump. The person was exposed to slightly higher concentrations of *B.t*. subsp. *kurstaki* than to other culturable bacteria (Hansen *et al*., 2010a) (Table 14.1). Seven of the eight other growers performing other tasks in the same greenhouse were also exposed to *B.t*. subsp. *kurstaki*. However, their exposure to *B.t*. subsp. *kurstaki* was lower than their exposure to bacteria in general, although higher than background exposures to other *Bacillus* species in the same environment (Hansen *et al*., 2010a). In a greenhouse with Dipel®-treated tomato plants, the exposure of growers to airborne *B.t*. subsp. *kurstaki* reached 1400 cfu m^{-3} air during the clearing of old plants. This was lower than the exposure to the total number of culturable bacteria (Table 14.1), but higher than the exposure to the naturally occurring *Bacillus* species (median value = below detection; maximum = 200 cfu m^{-3} air) (Hansen *et al*., 2010a). Exposure to airborne *B.t*. subsp. *kurstaki* was found 52 days post application in a celery field (Hansen *et al*., 2010a), but it was not detected in the air in a strawberry field a year after application (Tendal and Madsen, 2011).

B. thuringiensis has been isolated from eight of 20 faecal samples from greenhouse personnel working with *B. thuringiensis*-treated plants. However, no gastrointestinal symptoms correlated with the presence of *B. thuringiensis* in the faecal samples (Jensen *et al*., 2002). According to a case report, a farmer developed corneal ulcers owing to Dipel® accidentally coming into contact with his eye (Samples and Buettner, 1983). Cases of hypersensitivity pneumonitis after exposure to *B. subtilis* (Johnson *et al*., 1980) and clinical infection in burn wounds of immunocompromised patients (Damgaard *et al*., 1997) have been reported, but are not related to occupational exposure to *Bacillus*.

The mean aerodynamic diameter of culturable *B. thuringiensis* particles has been measured as having different sizes in different studies, probably depending on factors such as aerosolization methods and growth conditions. This may affect their ability to stay airborne and hence also the minimum amount of time that must pass between the time that *B. thuringiensis* is applied to a crop and the time that people can return to that area, but also the possibility of the particle being inhaled. In all these studies the particles are, however, present in the respirable size fraction (reviewed in Madsen, 2011), and thus can potentially be inhaled and affect the airways. Two studies show that no occupational respiratory symptoms were associated with working with plants treated with *B. thuringiensis* (Bernstein *et al*., 1999; Doekes *et al*., 2004). However, results from studies in greenhouses suggest that regular use of *B.t*. subsp. *israelensis*. (Bactimos® and Vectobac®) may be associated with a risk of specific IgE sensitization of workers (Bernstein *et al*., 1999; Doekes *et al*., 2004).

No scientific papers are available on occupational exposures and related health effects of MBCPs containing *S. griseoviridis*, *B. subtilis* or *P. chlororaphis*.

14.3.2 Exposure of bystanders to bacterial MBCAs and risk assessment

Bacillus

B. thuringiensis can be transported by wind to areas not sprayed with the bacterium and

concentrations higher than 1600 cfu m^{-3} air have been found in areas 125–1000 m away from the spray zone (Teschke *et al*., 2001). This concentration is within the range of concentrations of bacterial cfus seen in open fields in other studies (cf. Table 14.1). After large outdoor areas were sprayed with a *B.t*. subsp. *kurstaki* MBCP (Foray® 48B), nasal swabs from children were positive for *B.t*. subsp. *kurstaki* in nearby unsprayed areas. In the sprayed zone, there was a large increase in number of positive nasal swabs following each spraying, even though the population was advised to stay indoors with windows closed during the sprayings (Pearce *et al*., 2002). *B.t*. subsp. *kurstaki* has also been found indoors in another study where it had been sprayed outdoors. Some 5–6 h after spraying, the average indoor concentration of *B.t*. subsp. *kurstaki* was measured as 245 cfu m^{-3} air, and exceeded the outdoor concentration (Teschke *et al*., 2001). This suggests that the movement of outside air to the indoors may be the result of residents entering or leaving houses. Studies have also been performed in relation to the health effects on residents in *B.t*. subsp. *kurstaki*-treated areas. According to a questionnaire study done in an area where aerial spraying (Foray® 48B) occurred, complaints of upper airway, gastrointestinal and neuropsychiatric symptoms increased significantly for residents. However, no significant increase in visits to health-care providers was found (Petrie *et al*., 2003). Another study concluded that there were no significant changes in physical health for residents in a *B.t*. subsp. *kurstaki*-treated area (Pearce and Chappell, 2002).

14.3.3 Background exposure to actinobacteria and bacteria

Streptomyces griseoviridis

Airborne *Streptomyces* species have been found in many studies in different environments, but none of them have been identified as *S. griseoviridis*; other *Streptomyces* species seem to be more common (reviewed in Madsen, 2011).

Bacillus

Exposure to airborne *B. thuringiensis* and *B. subtilis* seems to be quite common in different environments (reviewed in Madsen, 2011) (Table 14.2). They have been recovered in nasal swabs from children (Pearce *et al*., 2002), grain dust (Swan and Crook, 1998), a desert (Kellogg *et al*., 2004), a hospital ward (Augustowska and Dutkiewicz, 2006), at restaurants (Chan *et al*., 2009), at a waste incinerator (Heo *et al*., 2010) and in a cowshed (Table 14.2). The bacilli constituted 6% of all dust-borne bacteria in a grain terminal (Palmgren *et al*., 1983) and 7% of all airborne bacteria in a rural area (Lazaro *et al*., 2000).

Pseudomonas chlororaphis

P. chlororaphis is a Gram-negative, endotoxin-containing bacterium and, as mentioned in Section 14.1, the endotoxin is a strong inflammogen. There appears to be a connection between presence of *P. chlororaphis* and plant material being handled in the environment; it has been found in the air on farms during several activities associated with the processing of herbs, where it ranges from below detection to levels where it constitutes >90% of the Gram-negative bacteria (Skorska *et al*., 2005). *P. chlororaphis* was found in high concentrations both in the air and in settled dust in cowsheds, in the latter case at 10^6–10^7 cfu g^{-1} dust (Andersson *et al*., 1999), while in another study, it was detected in one of six animal houses at less than one cfu m^{-3} air (calculated value from the paper) (Zucker *et al*., 2000) (Table 14.2). *P. chlororaphis* was detected in 3.3% of more than 100 flats investigated in Poland (Górny and Dutkiewicz, 2002).

14.4 Exposure to Other Microbial Agents

14.4.1 Occupational exposure to other microbial agents and risk assessment

Saccharomyces cerevisiae

S. cerevisiae, also known as baker's yeast and brewer's yeast, is handled in many occupational settings. Inhalation allergy to

S. cerevisiae has been described (Baldo and Baker, 1988; Kortekangas-Savolainen *et al*., 1993) and allergens of *S. cerevisiae* have been characterized (Horner *et al*., 1995). *S. cerevisiae* has been found in the sputum of four of 43 cheese factory workers in an environment, where it constituted 15–17% of the airborne fungi (Guglielminetti *et al*., 2000). Four workers at this cheese factory suffered from symptoms similar to extrinsic allergic alveolitis, but these symptoms were not related to *S. cerevisiae* exposure, but to exposure to *Penicillium verrucosum*, which was also used in the cheese production. *S. cerevisiae* has been found in the air in other dairies, where yeasts constituted 30% of all airborne fungi (Cosentino and Palmas, 1991). A dairy farmer's exposure to *S. cerevisiae* has been suggested as the cause of development of hypersensitivity pneumonitis (Yamamoto *et al*., 2002). The number of brewery workers who reacted positively to skin prick tests of brewer's yeast (14%) was not significantly higher than the number of reactions of a reference group (5%) (Godnic-Cvar *et al*., 1999). In a study of 193 bakers, strong reactions were found to α-amylase from *Aspergillus oryzae* but not to baker's yeast (Baur *et al*., 1998). *S. cerevisiae* has been found growing in the lung of a healthy male working at setting up bakeries, and the infection was suspected to be caused by exposure to yeast powder (Ren *et al*., 2004).

S. cerevisiae can cause vaginitis, but this is not related to occupation in the baking industry (Papaemmanouil *et al*., 2011). The yeast has also been found in human blood but this is suspected to be caused by food and drink, and not by occupational exposure (de Llanos *et al*., 2006). *S. cerevisiae* and a subtype of *S. cerevisiae* called *S. boulardii*, which is used as a probiotic, are described as emerging fungal human pathogens; this subject has been reviewed thoroughly elsewhere (Murphy and Kavanagh, 1999; Enache-Angoulvant and Hennequin, 2005) and will not be treated further in this chapter.

Botrytis cinerea

B. cinerea is used for making sweet dessert wines (botrytized wines). In some cases, inoculation occurs when *B. cinerea* spores are sprayed over the grapes, while other vineyards depend on natural inoculation from spores present in the environment. A survey of the mycological flora of French wine cellars showed high amounts of viable, airborne spores of *B. cinerea* after grape-pressing activity, compared with storage cellars that had had no grape pressing (Simeray *et al*., 2001). Two cases have been reported of two farm workers in Austria developing hypersensitivity pneumonitis/extrinsic allergic alveolitis as a result of exposure to *B. cinerea* while working with botrytized grapes (Popp *et al*., 1987).

A high frequency of allergy to *B. cinerea* has been reported (Jürgensen and Madsen, 2009), but this has not been specifically related to working environments where people use *B. cinerea* for wine production. For example, 1% of 190 table grape workers were shown to react to *B. cinerea* in a skin prick test, while 4% of 104 chrysanthemum greenhouse workers also reacted positively (Jeebhay *et al*., 2007).

14.4.2 Exposure of bystanders to other microbial agents

Saccharomyces cerevisiae and Botrytis cinerea

We have only found one paper (Nyirjesy *et al*., 1995) reporting exposure of bystanders to *S. cerevisiae*, and exposure from eating or drinking products that contain *S. cerevisiae* or *B. cinerea* will not be treated in this chapter. We have found no papers about bystanders' exposure to *B. cinerea*. However *B. cinerea* may be carried home from work in hair or clothing, as is seen for other fungi, e.g. by farmers working in cow barns (Pasanen *et al*., 1989).

14.4.3 Background exposure to other microbial agents

Saccharomyces cerevisiae

Airborne *S. cerevisiae* has been found in many environments; it has been found in outdoor air in India in 8 of 12 months of sampling, and

constituted 2.1% of all airborne yeasts (Sandhu and Waraich, 1981), in Italian carpentry workshops where yeasts constituted 20% of all airborne fungi (Cosentino and Palmas, 1991), during fruit harvest in Canada (Adams, 1964) and in European dwellings (Górny and Dutkiewicz, 2002).

Botrytis cinerea

Airborne *Botrytis* not identified to species level has been found in many places around the world, and background exposure to it has been reviewed thoroughly elsewhere (Jürgensen and Madsen, 2009), and will only be treated briefly here. In outdoor air, *B. cinerea* has been found in a concentration of 10 cfu m^{-3} (Kemp *et al*., 2002), and to constitute about 1.5% of all outdoor airborne fungi (Çolakoglu, 2004). In indoor air, *B. cinerea* has been found to constitute about 1% (Çolakoglu, 2004), 0.3% (Basilico *et al*., 2007) and <1% (Saldanha *et al*., 2008) of the airborne fungi and was therefore not a dominating species. Furthermore, it has been found in homes with mould damage (Lugauskas *et al*., 2003), in food production places (Simsekli *et al*., 1999), in schools (Immonen *et al*., 2001), in vineyards (Magyar *et al*., 2009) and in wine cellars (Haas *et al*., 2010). High concentrations of *B. cinerea* have been found in greenhouses, (around 600 cfu m^{-3} air) (Li and LaMondia, 2010).

Compared with its low prevalence in the air in non-occupational settings, relatively many people react positively to *B. cinerea* (Jürgensen and Madsen, 2009); for example, 4.9% of 692 suspected allergic patients and 24% of 180 mould-allergic patients react positively to *B. cinerea* (Spieksma *et al*., 1987).

14.5 Discussion

People handling MBCAs in occupational settings and residents in treated areas are, in some situations, exposed to MBCAs. However, workers are not currently protected by occupational exposure limits (OELs) as there are no OELs for MBCAs; hence, there is a high motivation to perform research towards OELs for MBCAs. Similarly, many countries have no OELs for exposure to microorganisms in general. However, in the scientific literature, there are suggested OELs (expressed as units m^{-3} air) for exposure to fungi in general (Eduard, 2009). Based on a literature review, Eduard (2009) suggested an OEL of 10^5 spores m^{-3} air for various fungi. Exposure to *T. harzianum* can reach this level during the application of an MBCP (Table 14.1). The suggested OELs are mainly based on studies where exposure and symptoms of the airways have been investigated. Exposure to *B. thuringiensis* of both workers and residents in treated areas has been measured in several studies, and studies of potential health effects have also been performed. However, comparisons between studies are made difficult by the fact that exposure has been measured in different ways (e.g. as cfu m^{-3} air; as qPCR (real-time quantitative PCR) copies m^{-3} air; as presence on nasal swabs; as droplet deposition on Kromecote cards, etc.). Agreement on one method could on one hand be a basis of better comparison of different studies but, on the other hand, different methods have different advantages and thus agreement on one method could also contribute to a restricted knowledge base.

As another approach, the exposure level to an MCBA may be evaluated by comparing it with background exposure to microorganisms in general in the same environment, or with exposure to naturally occurring microorganisms of the same species as the MBCA in focus. A review found that in some investigations, exposure to MBCAs is higher than the background exposure to the same species or other microorganisms, but in ten out of 12 studied situations it was lower than the exposure to, e.g. the total number of bacteria or fungi (Madsen, 2011). *B. thuringiensis* and *T. harzianum* have been found in concentrations higher than both naturally occurring species and other bacteria or fungi in general. However, *B. thuringiensis* can also be naturally present in high concentrations (Table 14.2). While background exposure to

some microbial species used as MBCAs is well described, knowledge about other species is very limited. This might be because these species are only seldom found. The knowledge about background exposure is partly limited by the fact that in many investigations of exposure, microorganisms are not identified to species level, and sometimes not even to genus. In future exposure studies, an identification of microbial species would be useful; also, in future studies where an MBCA is monitored in the environment strain-specific markers are useful tools (Holmberg *et al.*, 2009). *Trichoderma*, for example, is often not identified to species level. Sometimes high concentrations or high frequencies have been reported of fungi from this genus, so potentially there could be a background exposure to *T. harzianum* and *T. polysporum* that is not documented. In conclusion, if the exposure to an MBCA versus background exposure to the species is to be used as a tool in the risk evaluation of using an MBCA, further studies and publications on both occupational and background exposures are crucial.

Not only morphological identification, but also additional means of characterization (e.g. at molecular level) may be important to verify that the detected microbial isolate is from the applied product and not from the background. This has been successfully done for microorganisms used in MBCPs (Jensen *et al.*, 2002; Hansen *et al.*, 2010a,b; Holmberg, 2011), as well as for yeasts (Nyirjesy *et al.*, 1995; McCullough *et al.*, 1998; Posteraro *et al.*, 1999).

As a third and more direct approach applicable for some microorganisms – measurements of the problematic component instead of the whole microorganism – can be used in the risk evaluation of the exposure. This approach can only be used if a problematic component has been identified, if a good tool for sampling and quantifying the component has been developed and if it is the only problematic component. This approach has been used in environments where microbial enzymes have been used as, for example, in the baking industry (Houba *et al.*, 1996). For applied Gram-negative bacteria, such as *P. chlororaphis*, it will be relevant to measure its contribution of endotoxin to the total exposure to this substance (Holmberg, 2011); the endotoxin is a strong inflammogen and knowledge about what exposure levels can cause health effects is known (Douwes *et al.*, 2003; Health Council of the Netherlands, 2010).

For many MBCAs, no studies on associations between exposure in occupational settings and health effects have been published. A single case study has reported an infection caused by an MBCA in an occupational setting (Samples and Buettner, 1983). Case studies indicate that some of the microbial species used as MBCAs may to some extent be infectious in weakened or immunocompromised humans. As in studies of exposure, microorganisms in case studies of infection are not always identified to species level. This weakens conclusions about the pathogenicity of microbial species used as MBCAs. For this chapter, we found eight papers reporting cases of keratitis, none of which were from Europe. One investigation, not related to the use of MBCPs, showed that 6.8% of the examined patients had strong reactions to *B. bassiana*, while only 2.3% showed a reaction to the more common fungus *Cladosporium* (Beaumont *et al.*, 1985). This indicates either that exposure to *B. bassiana* in the studied group of patients was more common than recorded in the literature (cf. Table 14.2), or the species can contain strong or possibly cross-reactive allergens. As a basis for setting OELs, it is important to perform and publish further studies concerning exposure and potential health-related effects of MBCAs.

A relatively high number of people react in skin prick tests to *B. cinerea*. Some of these investigations were performed on people working with *B. cinerea*, while others were not. The high frequency of positive reactions to *B. cinerea* may indicate that exposure to *B. cinerea* is more common than indicated from many exposure studies (reviewed in Jürgensen and Madsen, 2009), or it may contain strong or cross-reactive allergens. However, only a few cases of health effects of *B. cinerea* in occupational settings have been published. Of course, healthy worker selection may also influence this.

Occupational exposure to baker's yeast is suggested to occur when workers handle powder-formulated yeast (Section 14.4), and for MBCPs the formulation and way of applying the product influence the exposure level (Sections 14.2 and 14.3). In the development of new products with microorganisms, it is important to consider the dustiness of the products in relevant handling scenarios. In order to be able to select the least dusty product and application method, further studies of this subject with products in use would be of relevance.

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15 Model Systems for Testing Microbial Pathogenicity, Virulence and Toxicity – Introduction and Overview

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15.1 Introduction

In response to a growing interest in the introduction of new microorganisms for use in beneficial applications in agriculture, food/feed and the environment, there is a parallel demand for documentation that the organisms and products are not toxigenic or infectious to humans, or have unacceptable effects on non-target organisms in the environment. However, the testing of beneficial microorganisms for potential toxicity or infectivity is not straightforward, especially vis-à-vis toxicity, in which field the development of new methodology generally has chemical compounds in mind, and currently available methodology is poorly adapted to microorganisms. Another confounding factor when testing beneficial microbes is that it is much more difficult (and in theory impossible) to unequivocally prove the complete *absence* of infectivity or toxicity in a specific microbial isolate (compared with proving the *presence* of infectivity or toxicity). This is because it is very difficult to define which potential hazards need to be investigated, especially for newly described strains or species with no known close pathogenic relatives.

Besides the general lack of specific methodology for testing the hazards of microorganisms, the long-standing aim to replace assays using mammals (Krewski *et al*., 2009; Hartung, 2010; Liebsch *et al*., 2011) gives further impetus to the development of new, non-mammalian methodology. While it can be difficult to translate the results from an *in vitro* test to the complex *in vivo* reality, *in vivo* models using invertebrates may partly overcome this problem; in some cases, *in vitro* tests may actually have the advantage of answering a very specific

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question and may reveal the mechanism of a pathogenic mode of action.

Bioassays utilizing various types of biosensor organisms thus have a role to play in the determination of the toxicity, infectivity and sensitizing properties of microorganisms. Moreover, new approaches with invertebrates, cell lines or other microorganisms can potentially replace studies using mammals. This chapter presents an overview of some of the advantages and limitations of the available types of biosensor models for determining the toxicity or pathogenicity of microbes, and to what extent they could fulfil the regulatory requirements for new microbial products. More detailed descriptions of different types of models are given in the following chapters. Chapter 16 (Kurz and Leulier) gives an overview of the use of the nematode worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. Chapters 17–19 cover models for assessing acute cytotoxicity (Altomare *et al.*), genotoxicity (Typas and Kouvelis) and sensitization/irritation (Loprieno), respectively.

The implementation of new methods for testing microorganisms would require the establishment of common test guidelines, and so would involve international undertakings and agreements. For microbial biocontrol agents, this could be achieved within the OECD (Organisation for Economic Cooperation and Development) (see Meeussen Chapter 21, this volume), where the Bio-Pesticides Steering Group could have a role to play in adopting new and alternative methods to replace some of the animal studies currently required for testing microorganisms used in plant protection products. Also, the European Centre for the Validation of Alternative Testing Methods (ECVAM) under the EU Joint Research Centre (JRC) Institute for Health and Consumer Protection (IHCP) could be an important player.

15.2 Legislation and Guidelines for Testing Microorganisms

The growing interest in exploiting the huge potential of microorganisms has triggered the introduction of legislation on and regulatory oversight of new microbial products within, for example, plant protection products and biocides in the EU ('biopesticides' in other OECD countries), and feed additives or genetically modified microorganisms (see Bergmans, Chapter 12, this volume). The most obvious example where current legislation places strong demands for data on potential toxicity and pathogenicity is for new microbial biological control agents (MBCAs) (Hauschild *et al.*, 2011). Risk assessment is mainly based on whole-animal test systems developed for chemical pesticides and not validated for testing microorganisms. For example, the human health risk assessment of plant protection products is based on a tiered approach in the US, Canadian and European Union (EU) systems. The Tier I studies encompass a series of tests which aim at determining whether high concentrations of the active ingredients cause harm. Acute oral, pulmonary and intraperitoneal pathogenicity/toxicity is assessed using mice or rats. Acute dermal, skin and eye irritation are often assessed in rabbits, whereas guinea pigs are the animal of choice for predictive sensitization tests. If the Tier I studies result in any uncertainty, Tier II studies must be performed. These are basically the same as Tier I studies, but require multiple consecutive exposures, and these may require the use of non-rodent animal species. One of the drawbacks of the existing methods is that no microorganisms that fulfil the function of positive controls are available when testing microbes, so it is not known which real pathogenic species would give a response in the model used. For microorganisms that have already been in use as plant protection agents, much of the risk assessment of a new product using the same strain can be based on 'long history of safe use'. However, this approach cannot be used for new strains or species, so it is important to find new methods capable of predicting the potential mammalian pathogenicity of a strain of a given species.

The EU specific support action REBECA (Regulation of Biological Control Agents) recently pointed out that the availability of high-throughput and low-cost alternatives to animal test systems would encourage

the development and marketing of a new generation of MBCAs (Strauch *et al.*, 2011). Moreover, REBECA concluded that better adaptation of the test systems for microorganisms would improve the safety assessment of microbial plant protection products and biocides.

15.3 Types of Test Models Available

15.3.1 General pathogenicity and infectivity

A drawback of safety assays using animals such as rodents is that these assays are often unable to predict the pathogenicity and virulence of microorganisms to humans. This is due to the fact that some of the factors that determine whether a microorganism can cause disease or symptoms are host specific. For example, the pathogenicity of *Listeria monocytogenes* – which causes listeriosis in humans after the ingestion of contaminated food – is due to internalin A produced by the bacterium, which binds to the receptor E-cadherin present in the small intestinal tissue of humans, but not to the E-cadherin found in mice and rats (Lecuit *et al.*, 1999). Therefore, when using these rodents for assessing human pathogenicity, a false negative answer may be the result. In contrast, guinea pigs have the same E-cadherin as humans and, as a result, can be used as models for studying *L. monocytogenes* virulence (Lecuit *et al.*, 1999). This shows that it can be important to know the virulence mechanisms in order to use the right model for assessing human pathogenicity and, once again, illustrates the difficulties that can be encountered when evaluating new microbial strains or species.

Another example is the bacterium *Bacillus thuringiensis*, an MBCA widely used against, e.g. mosquito larvae. *B. thuringiensis* is closely related to *B. cereus*, a food poisoning bacterium. *B. cereus* causes diarrhoea in humans as a result of the production of enterotoxins, and it is known that many *B. thuringiensis* strains, including those used commercially, produce the same enterotoxins (Damgaard, 1995). However, it is difficult to assess this end point in rodent models. A study in which rats were given a *B. cereus* strain known previously to cause diarrhoea in humans, did not give rise to any adverse symptoms in the animals (Wilcks *et al.*, 2006).

Yet it is difficult to replace animal studies with other models when studying the infectivity and persistence of a microorganism in organs and tissues, although for microorganisms under evaluation as new MBCAs, these tests can be waived if certain prerequisites are met, e.g. if the strain is unable to grow at human body temperature (approx. 37°C), and is sensitive to conditions in the stomach (low pH, pepsin) and/or small intestine (bile acids, pancreatic enzymes).

Insects can also be used to identify pathogens, to determine important virulence factors and to understand the infection processes of pathogens and parasites as different as viruses, fungi and nematodes. A detailed analysis of the use of the fruit fly, *D. melanogaster*, is given by Kurz and Leulier (Chapter 16, this volume). Another example is the use of larvae of the moth *Galleria mellonella*, which has not only been employed for testing insect pathogens, but also bacteria and fungi pathogenic to mammals (Kavanagh and Reeves, 2004). Most of these studies have been conducted by injection of the bacteria into the larval haemocoel (which can be considered similar to the blood/lymph of higher animals), but for *B. cereus* it has been possible to use *G. mellonella* larvae as a model for oral infections (Fedhila *et al.*, 2006). *G. mellonella* is also of interest for use as a model to compare the virulence of *B. cereus* and *B. thuringiensis* strains, particularly when questions are related to their interaction with the intestinal barriers. Other advantages of *Galleria* are that it tolerates human body temperatures and is easy to maintain; the larvae also have physiological and immunity features and barriers that are similar to those of vertebrates. Thus, although this insect is not a vertebrate model, it is expected that some of the factors that permit the bacteria to overcome the host resistance immunity might be similar in an insect and in higher animal models or man (Vallet-Gely *et al.*, 2008).

The nematode *C. elegans* is a small eukaryotic organism which, during recent years, has been increasingly used for research purposes; this is also given a detailed treatment by Kurz and Leulier (Chapter 16, this volume). Its genomic DNA sequence is known, and numerous mutants are available. This worm has been used to study microorganism–animal interactions, and has increasingly been developed and used as a model to study host–pathogen interactions relevant for humans (Mellies and Lawrence-Pine, 2010).

Functional mammalian cell models use mammalian cell lines (human or animal) that can easily be cultured in the laboratory, are of relatively low cost and have no ethical issues. These are, consequently, interesting alternatives to animal models (which are expensive and have ethical issues, and demand specially trained personnel). Many mammalian cell lines are available and they all have their advantages and disadvantages. The choice of cell line is dependent on the purpose of the studies. One of the most widely used is the Caco-2 cell line derived from a human colon cancer patient, which is used as an *in vitro* model of the intestinal barrier (Sambuy *et al.*, 2005). In a recent review, this cell line and others are discussed in relation to using them as models for studying pathogen–host interactions and toxicity (Cencic and Langerholc, 2010).

15.3.2 Acute toxicity and genotoxicity

When using fungi as the active agent in plant protection products, one of the major concerns is their production of secondary metabolites that are potentially harmful to humans or animals. However, no widely accepted guidelines exist on how to evaluate the risk from the potential production of toxic metabolites. For the moment, the extent of investigations on metabolites of an MBCA depends on what is already known in the published scientific literature or becomes apparent during product development. OECD is in the process of writing an issue paper on this subject, and here the general understanding is that the requirement for metabolite testing can be waived if the toxic metabolite is only produced in the target organism and no residues are left. In specific cases though, the submission of experimental data for certain metabolites can be justified (see Meeussen, Chapter 21, this volume). In another article (Altomare *et al.*, Chapter 17, this volume), four cheap and rapid invertebrate model systems are presented that could be used for the screening of new strains or culture extracts for cytotoxicity at an early stage of development. As conveyed in that chapter, these models have been used both with pure metabolites and with crude extracts containing a mixture of metabolites, and they were shown to be predictive for toxicity when compared with, e.g. cell line studies or rodent models.

Testing for the genotoxicity of microorganisms has been a matter of discussion, as there are several questions that are still not properly answered, e.g. under what conditions should the microorganisms be tested, which fraction of the microbial formulation should be tested and which test systems would be most appropriate (see Typas and Kouvelis, Chapter 18, this volume). Typas and Kouvelis give an overview of the various *in vitro* and *in vivo* models that have been developed to test chemicals for genotoxicity, and a subset of these model systems that have been evaluated for testing metabolites of microorganisms (and occasionally crude extracts). A special concern in the case of genotoxicity is – as for general cytotoxicity – the wide array of secondary metabolites that are produced by fungi.

Waivers for genotoxicity studies of bacteria used in plant protection products are often requested by the industry with reference to the fact that no suitable methods for testing are available; an argument that is often accepted by the regulatory agencies. OECD is also aware of this problem, and internationally accepted guidelines and appropriate testing methodology and protocols should be developed (Meeussen, Chapter 21, this volume).

15.3.3 Irritation/sensitization

As previously mentioned, rabbits and guinea pigs are often the animals of choice when

testing for irritation and sensitization, respectively (see Loprieno, Chapter 19, this volume). Rabbits have been used for many years to assess the irritating effects of chemical compounds on the skin and the eye, and have also been included in testing guidelines for evaluating microorganisms. Recently, new *in vitro* methods, such as the murine local lymph node assay (LLNA) for skin sensitization or the reconstructed human epidermis (RhE) test method for irritation, have been adopted for the testing of chemicals. However, like the tests mentioned above for genotoxicity, and the *in vivo* test for irritation/sensitivity testing, they have not been validated and adopted for microorganisms.

15.4 Conclusion

We have presented an overview of available test systems that can be useful in evaluations of pathogenicity and toxicity in new microbial strains, and discussed the general issues and concerns that need to be considered. One conclusion is that no validated and widely accepted methodology exists for the toxicity testing of microorganisms, although this may also be a very difficult task owing to the wide variety of microorganisms. In future, the familiarity approach of 'long history of safe use' implemented in the European QPS (Qualified Presumption of Safety) and the US GRAS (Generally Recognized As Safe) systems (see von Wright, Chapter 3, this volume) will probably gain in importance. The QPS system was introduced by EFSA (European Food Safety Authority) to simplify the safety evaluations of new microorganisms used in the food and feed chain. If a new strain under evaluation belongs to a species that has already been given QPS status, some specific questions regarding the strain have to be answered, but a whole battery of toxicity testing does not necessarily have to be performed. When a new organism belonging to a species that has not previously been used in food, feed or environmental applications is being evaluated, a more thorough case-by-case evaluation will be required. Still, it should be emphasized that some 'common sense' has to be used, instead of blindly performing a lot of required tests that may not even be valid for microorganisms. Besides the question of whether specific assays are possible to implement for testing microbes, attention must also be given to the critical step of extrapolating the test results to actual risk for humans or the environment.

In any case, the validation of alternative test systems for microorganisms and the development of standard protocols and guidelines should be considered as important tasks. For that purpose, industry, regulatory authorities, ECVAM (European Centre for Validation of Alternative Testing Methods) and the OECD biopesticide steering group should be involved.

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16 Nematode and Insect Models to Assay Microbial Infectivity, Virulence and Cytotoxicity

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16.1 Introduction

In this chapter, we will present and comment on the use of two model invertebrates as potential alternatives to mammalian models for assaying the pathogenicity or toxicity of a given microorganism or molecule. The two models will be introduced to highlight their respective advantages and drawbacks, while specific works related to microbial infections, cytotoxicity and immunity will be detailed to illustrate the topics. Our aim is to demonstrate the potential of these model animals as alternatives to mammals by giving an objective overview of the published and unpublished work concerning microbial virulence evaluation, toxicity assays or drug screenings. We will then propose perspectives and improvements to the actual systems in order to better correspond with the requirements of safety assessment.

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16.2 Genetically Tractable Invertebrates to Study Biological Interactions

16.2.1 Physiology, life cycles and growth conditions in the laboratory

The nematode Caenorhabditis elegans

C. elegans has proven to be a relevant model for the study of different fields of biology, such as the understanding of the mechanisms of apoptosis (Horvitz *et al*., 1994), or the comprehension of neuronal function and development (Bargmann, 2006). During the last 10 years, it has also become a very useful model for the study of host–pathogen interactions (Irazoqui *et al*., 2010). Indeed, this free-living metazoan has many advantages, not the least of which are convenience, low cost and bioethical handling, making it a highly suitable model for high-throughput approaches. As an example, its small size – of around 1 mm body length – enables storage in incubators at 15–25°C, with thousands of worms being reared on a 9 cm Petri dish or hundreds in an individual well of a 96-well plate. *C. elegans* feeds on bacteria in the soil, but; in the laboratory its food source is a non-pathogenic *Escherichia coli* OP50 strain that can be cultured on Petri dishes or in liquid media. Therefore, infecting the worms with a microbe usually only requires a transfer of the animals from the *E. coli* OP50 strain to the pathogen or to a mixture of OP50 and pathogenic bacteria. When fed with innocuous bacterial strains, worms have a lifespan of 2–3 weeks, a rapid generation time of 3 days at 25°C and a high number of progeny (~300 eggs/animal). These characteristics can be used to gauge the good health of a population. In addition to the knowledge concerning worm life cycles and behaviour, the anatomy of the nematode *C. elegans* is well documented and understood, thanks to its transparent cuticle. *C. elegans* has an androdieocious reproductive system, i.e. individuals are either strict hermaphrodites or males, and populations can be composed of genetically identical individuals. Adult hermaphrodites possess 959 somatic nuclei and the developmental fate of every cell is known (Sulston *et al*., 1983). Upon feeding by the nematode, the (non-pathogenic) bacteria of the feed source are usually rapidly destroyed by an organ called the grinder. The resulting lysate then spends less than a minute in contact with intestinal cells before being excreted through the anus. Flanking the intestine are the two gonad arms, nerve cords and muscles. The worm is surrounded by a transparent, mechanically resistant cuticle secreted by the epidermis (Altun and Hall, 2005). It is important to note that there are no motile cells or a circulatory system.

In the interaction with pathogenic microbes, the majority of the infections take place within the intestinal lumen, with these microorganisms being able to remain intact and proliferate (Aballay *et al*., 2000; Labrousse *et al*., 2000; Kurz *et al*., 2003). The transparency of *C. elegans* not only allows infections to be followed directly with ordinary microscopes, but also permits the use of fluorescent techniques *in vivo*, such as the monitoring of reporter gene expression or the observation of bioluminescent microorganisms within the host (Aballay *et al*., 2000; Labrousse *et al*., 2000; Kurz *et al*., 2003; Alegado and Tan, 2008). Consequently, as synchronized populations can be obtained relatively easily, researchers can work with very large numbers of animals of an identical age, genotype and anatomy, which allows highly robust and reproducible approaches.

The insect Drosophila melanogaster

D. melanogaster is a tiny fruit fly, only 3 to 4 mm in length, and is often found in the wild around rotten fruits. The flies reproduce frequently, furnishing a new generation in less than 2 weeks; each generation includes hundreds of offspring. Their life cycle is separated into four distinct phases: embryogenesis, in which initial organogenesis occurs; the larval phase which corresponds to a 'feed to grow' phase, at which stage their mass increases by 200-fold; the pupal stage, an immobile stage where metamorphosis from larvae to adults occurs; and finally the adult stage, which constitutes the reproductive stage of the life cycle. *D. melanogaster* is easy and inexpensive to maintain, manipulate and examine in laboratories. Over the last century, numerous genetic

tools such as stable mutants and transgenic fly lines were created, and these are commonly used and updated, thus making the fruit fly an ideal model for genetic studies of specific physiological processes (Matthews *et al*., 2005).

16.2.2 Why are the worm and the fly relevant to mammals?

C. elegans *rapidly proven to be a powerful model organism*

The advantages of this invertebrate as a model system have contributed to important biological discoveries relevant to the eukaryotic kingdom. As a consequence, six scientists were recently awarded three Nobel prizes for their discoveries obtained using *C. elegans*. In 2002, Sidney Brenner, John Sulston and Robert Horvitz obtained this prize for their efforts leading to the identification of the genetic programming in *C. elegans* that is related to organ development and programmed cell death. In addition, this prize took into account the work and foresight necessary for the establishment of this animal as a biological model system in less than 40 years, as well as the wide knowledge that had been gained of its anatomy and physiology. In 2006, Andrew Fire and Craig Mello were awarded the prize for their discoveries on RNA interference (RNAi). While this phenomenon had been described since the 1980s in plants when genes were overexpressed (Ecker and Davis, 1986), the understanding of the molecular machinery involved awaited the use of the nematode. The genes identified using the nematode appeared to be highly conserved, and these studies contributed to the understanding of a process common to eukaryotes (Tabara *et al*., 1999). More recently, in 2008, Martin Chalfie, Osamu Shimon and Roger Y. Tsien were rewarded with the prize for the discovery, development and applications of the green fluorescent protein (GFP). More specifically, M. Chalfie and his team performed the first heterologous expression of this protein in an animal (*C. elegans*) and paved the way for expression patterns and GFP-reporter strategies (Chalfie *et al*., 1994).

Other results obtained with this invertebrate are also remarkable and relevant to all eukaryotes, as illustrated by the identification of the first microRNA by Victor Ambros, Rosalind Lee and Rhonda Feibaum in 1993 (Lee *et al*., 1993). However, this pioneer study awaited the year 2000 and the identification of the worm *let-7* microRNA – whose sequence is highly conserved – for the realization that the related regulation mechanism was conserved in mammals, including humans (Pasquinelli *et al*., 2000).

D. melanogaster *is prototypical of the invertebrate models*

Throughout the last century, *D. melanogaster* has been the workhorse for genetic studies in eukaryotes. These studies have provided the basis of much of scientists' understanding of the fundamental aspects of eukaryotic genomes and physiological processes (Rubin and Lewis, 2000). Consequently, several *D. melanogaster* researchers received the Nobel Prize in Physiology and Medicine. In 1933, Thomas Hunt Morgan was awarded the prize for his discoveries related to the role of the chromosome in heredity. In 1946, Hermann Muller received the Nobel honour for the discovery of the production of mutations by means of X-ray irradiation. In 1995, the Nobel Prize in Physiology and Medicine was awarded to Christiane Nusslein-Volhard, Eric Wieschaus and Edward Lewis for their pioneering work on the genetic control of embryonic development; in 2004, it was awarded to Linda Buck and Richard Axel for their discoveries on odorant receptors and the organization of the olfactory system in the mouse and *D. melanogaster*, respectively. These seminal works demonstrate the important role played by fundamental research on the *D. melanogaster* model. To date, several cloned fruit fly genes have led to the identification of mammalian cognate genes, and these discoveries have shown that the conservation between the fruit fly and mammals is much greater than ever expected, from structural proteins to higher order processes such as development, immunity, metabolism, behaviour and other physiological responses (Rubin *et al*., 2000). The conservation of biological processes from flies to mammals extends the influence of the fruit fly research to human health. Indeed, the identification of fruit fly

cognates of human genes greatly expedite the progress of human disease research (Bier, 2005).

16.3 The Fly and the Worm to Study Host–pathogen Interactions

16.3.1 Invertebrates can be used to decipher the virulence of mammalian pathogens

Pathogens with a large tropism and their universal virulence genes

From plants to humans, every organism is constantly exposed to a wide variety of microbes. Moreover, each host lives in a specific environment and has developed its own life cycle. Hence, one expectation in the competition between host and pathogen is a fine specificity of the interaction. For example, plants and insects are structurally different, one pathogen should not infect all organisms and one organism should not be affected by all pathogens. Moreover, the pathogen needs a specific environment to survive and proliferate; equally, the virulence factors of the pathogen might target certain host processes, thus again implying specificity. The host–pathogen interaction, or pathogenesis, can be defined as the complex interface between multiple factors of the infectious agent and the resistance mechanisms of the host. On the specificity issue, it might seem logical to use only higher vertebrates and mammals as model organisms to understand this interaction in humans and to identify microbial virulence factors. However, it has been shown that certain pathogens can infect a wide range of hosts, including plants and humans (Rahme *et al*., 1995). Their tropism is of great interest for large-scale studies because it permits the use of alternative models to replace mammals. Indeed, if a pathogen can infect humans as well as *D. melanogaster*, nematodes and the plant *Arabidopsis thaliana*, virulence factors that are used irrespective of the host certainly exist. Based on this, any non-mammalian organism infected by a microorganism with a wide tropism can serve as a relevant host to understand the fundamentals of the pathogenesis.

The Gram-negative bacterium *Pseudomonas aeruginosa* is prototypical of this class of microorganisms: it has a wide range of hosts, including humans. After determining the best strain of *P. aeruginosa* able to infect both plants and humans, Rahme and colleagues generated bacterial mutants for genes known to be required for infection either in animals or in plants. Strikingly, they found that all the *P. aeruginosa* mutants generated were less pathogenic for both plants (*A. thaliana*) and animals (mice) (Rahme *et al*., 1995). Following this discovery, they chose the *P. aeruginosa* strain PA14 (a clinical isolate relevant to humans) and undertook a systematic genetic screen to identify the genes required for its virulence against *A. thaliana* (Rahme *et al*., 1997). By screening randomly mutagenized *P. aeruginosa* clones, they found known as well as novel virulence determinants. Moreover, of the mutants identified as less pathogenic in plants, they showed that all had a decreased virulence in mice, thereby demonstrating that a non-vertebrate model can be used to identify components of the infection machinery relevant to mammals (Rahme *et al*., 1997).

Nematodes and insects as hosts to study microbial virulence

To extend the range of non-vertebrate hosts for the study of pathogenesis, the laboratory of Frederick Ausubel developed another experimental model with the nematode *C. elegans* and the bacterium *P. aeruginosa*. They showed that *P. aeruginosa* shortens the worm's lifespan and that virulence factors needed to kill *C. elegans* were also required for infection in plants and mice (Mahajan-Miklos *et al*., 1999; Tan *et al*., 1999a). Finally, using a *P. aeruginosa* mutant library, they screened around 2000 bacterial clones for reduced virulence against nematodes and found eight mutants, of which six were less pathogenic in *A. thaliana* and five were attenuated in mice (Tan *et al*., 1999b). These results paved the way for the use of *C. elegans* as a host model relevant to mammals for the identification of virulence factors. Following this pioneering discovery, several genetic screens involving *C. elegans* as host and pathogens with a large tropism (such as *Serratia marcescens* and *Burkholderia*

pseudomallei) have been undertaken. As previously illustrated with *P. aeruginosa*, these approaches were successful and relevant to mammals: using the nematode for a first round of screening allowed the definition of a subset of attenuated bacterial mutants for subsequent testing against mice (Gan *et al*., 2002; Kurz *et al*., 2003). Moreover, contrary to expectations, limiting the use of *C. elegans* to microbes with a broad host range, it has been shown that more specialized vertebrate pathogens can infect the worm as well, and that this invertebrate can be an alternative host for identifying pathogenic strategies required for infecting both nematodes and mammals. For example, *Salmonella typhimurium*, well known for infecting poultry and humans, and for its ability to highjack the host cellular machinery, was shown to kill nematodes (Aballay *et al*., 2000; Labrousse *et al*., 2000). A genetic screen was undertaken and demonstrated the relevance of the worm model (Tenor *et al*., 2004). Similarly, a non-exhaustive list of pathogenic agents that demonstrated the existence of virulence factors required to infect both nematodes and mammals includes both bacteria and fungi: *Enterococcus faecalis* (Garsin *et al*., 2001), *Cryptococcus neoformans* (Mylonakis *et al*., 2002), *Yersinia pestis* (Styer *et al*., 2005), *Vibrio cholerae* (Vaitkevicius *et al*., 2006) and *Candida albicans* (Pukkila-Worley *et al*., 2009).

On the other hand, *D. melanogaster* is naturally resistant to bacterial infection and presents the ability to eliminate most invading bacterial pathogens encountered in nature. To date, only three 'natural' *D. melanogaster* pathogens (*Erwinia carotovora* ssp. *carotovora* strain 15 (Ecc15), *S. marcescens* strain Db11 and *Pseudomonas entomophila* strain L48) have been identified and utilized in the laboratory to characterize their virulence strategies (Vodovar *et al*., 2004). Of note is the fact that these three pathogens belong to multihost Gram-negative bacterial genera (*Erwinia*, *Serratia* and *Pseudomonas*). *Erwinia* species also infect plants, and most *Serratia* and *Pseudomonas* strains are ubiquitously found in soil, plants and animals. Moreover, several strains of *Serratia* and *Pseudomonas* are human opportunistic pathogens which can cause disease in animals. *E. carotovora* ssp. *carotovora* Ecc15 rapidly colonizes the intestinal tract of the *D. melanogaster* larvae and adults, but is efficiently recognized and eliminated by the host immune response, thanks to the potent mucosal and systemic production of antimicrobial compounds. which result in a non-lethal infection for *D. melanogaster* (Basset *et al*., 2000; Zaidman-Remy *et al*., 2006). Genetic studies on the bacteria have shown that one virulence factor called *evf* promotes Ecc15 pathogenicity and, when transferred to other Gram-negative bacteria, confers infectivity and promotes bacterial persistence in the intestine (Basset *et al*., 2003; Acosta Muniz *et al*., 2007). *S. marcescens* Db11 is a lethal pathogen for *D. melanogaster*. Upon ingestion, it can bypass mucosal immune responses to reach the body cavity and cause a systemic lethal infection after 6 days (Flyg *et al*., 1980; Nehme *et al*., 2007). If directly introduced into the body cavity of *D. melanogaster*, it is insensitive to the host's systemic immune response and kills flies in a day (Kurz *et al*., 2003; Nehme *et al*., 2007). Using this specific route of infectivity, and by means of bacterial genetic screening, it has been possible to identify *S. marcescens* virulence factors, some of which are also important in infecting worms or mice (Kurz *et al*., 2003; Nehme *et al*., 2007). *P. entomophila* L48 is also lethal when ingested by *D. melanogaster*, or upon injection, despite its ability to induce a potent systemic immune response (Vodovar *et al*., 2005). In fact, this bacterium has developed efficient ways to antagonize the principal arm of the *D. melanogaster* immune response, i.e. the production of antimicrobial peptides, by abundantly secreting a metalloprotease AprA, which can degrade antimicrobial peptides (Liehl *et al*., 2006). Further bacterial genetic studies on *P. entomophila* infection have revealed that its virulence is multifactorial, with a clear differentiation between factors that promote pathogenicity and those that trigger the immune response. The GacS/GacA two-component system plays a key role in *P. entomophila* pathogenicity, controlling many putative virulence factors and AprA (Vodovar *et al*., 2006; Vallet-Gely *et al*., 2010a). Besides, the *pvf* genes, which are regulated independently of the Gac system, encode proteins involved in the production of a secondary metabolite that is essential in *P. entomophila* virulence through

its promotion of bacterial persistence within the gut, which leads to the activation of the fly immune responses and the infliction of gut damage (Vallet-Gely *et al*., 2010b). Taken collectively, these studies have highlighted the power of using a 'natural' pathogen and its host model to decipher bacterial virulence strategies employed by multi-host microorganisms.

Alternative strategies are also employed using *D. melanogaster* as an animal host model to study bacterial virulence strategies of human pathogens. In this case, the flies are used as a living 'test tube' because *D. melanogaster* is not a 'natural' host for these bacteria. However, the model is still relevant in the case of an apparent pathology associated with the infection, which most probably relies on a common set of virulence factors used upon infection of its natural host. In these cases, the infection procedure has bypassed the natural barriers of the host by injecting the bacteria into the body cavity. As an extreme proxy of pathology, the viability of the injected flies are analysed over time. Using this strategy, several laboratories have identified new virulence factors or confirmed the importance of known virulence strategies in the following human pathogens: *Francisella tularensis* (Vonkavaara *et al*., 2008), *Listeria monocytogenes* (Mansfield *et al*., 2003), *S. typhimurium* (Brandt *et al*., 2004), *Staphylococcus aureus* (Needham *et al*., 2004) and *P. aeruginosa* (D'Argenio *et al*., 2001). In addition, other studies have established *D. melanogaster* as a suitable model for screening the bacterial virulence factors of *V. cholerae* (Blow *et al*., 2005), *Ehrlichia chaffeensis* (Luce-Fedrow *et al*., 2009), *Porphyromonas gingivalis* (Igboin *et al*., 2011a,b) and *Burkholderia cepacia* (Castonguay-Vanier *et al*., 2010). These studies clearly establish *D. melanogaster* as an alternative non-mammalian model to decipher the virulence strategies of several human pathogens.

16.3.2 The response to insults such as chemical exposure or microbial infection is specific

Living organisms are constantly exposed to abiotic and biotic insults that range from rapid variations of their environment to pollution and predators. They have, therefore, developed numerous strategies and processes to either be constitutively protected or to react more specifically. An understanding of the stress or immune machineries induced in a given condition is a prerequisite to the development of model host testing systems. In this aspect, *D. melanogaster* and *C. elegans* are well suited owing to the extensive studies related to infections or cytotoxicity that have been made with them.

D. melanogaster *immunity is inducible, complex and its study is relevant for mammals*

For more than 15 years, *D. melanogaster* has emerged as an ideal animal model to study the host immune responses to microbial infections. Mainly through genetic approaches, work from several laboratories has put together a detailed characterization of the molecular mechanisms involved, ranging from microbial detection by pattern-recognition receptors to innate immune signalling and immune effectors involved in host defence against microbial infections (Lemaitre and Hoffmann, 2007). Like most animals, *D. melanogaster* protects itself from microbial infections through physical barriers, local immune reactions and systemic responses (Lemaitre and Hoffmann, 2007). After exposure to infectious microorganisms, the intestinal and tracheal epithelia secrete antimicrobial peptides (AMPs) and reactive oxygen species (ROS) in a highly controlled manner (Leulier and Royet, 2009). In addition, circulating and tissue-restricted phagocytic cells engulf foreign intruders and thereby complement the response of the intestinal or tracheal epithelia (Stuart and Ezekowitz, 2008). The fat body, the functional equivalent of the mammalian liver, ultimately triggers a systemic immune response which produces large amounts of humoral immune effectors that include AMPs (Ferrandon *et al*., 2007). Parallels have been drawn between the innate immune systems of vertebrates and insects (Hoffmann, 2003), and discoveries in *D. melanogaster* have been instrumental in the identification of the central role played by the Toll-like

receptors in mammalian immunity (Lemaitre, 2004), thus demonstrating the utility of this animal model for studying host–microbe interactions.

Worms do possess inducible and specific defence mechanism

The elegant studies that have been performed with *D. melanogaster* (see above) illustrated the relevance of genetically tractable invertebrates for the elucidation of innate immunity (Lemaitre and Hoffmann, 2007) and prompted studies of defence mechanisms in *C. elegans*. Contrary to the *D. melanogaster* studies, which were primarily host oriented, the different infection models involving *C. elegans* that have already been described in Section 16.3.1 were a prerequisite and offered a ready means to clearly demonstrate whether inducible host defences were present or not. Two main approaches were undertaken, transcriptomic and genetic. Using microarrays and the infection of nematodes by *S. marcescens*, Mallo *et al*. (2002) demonstrated the upregulation of a number of genes following bacterial infection, including lectin- and lysozyme-encoding genes. Moreover, mechanistic insights were obtained and elements of a TGF (transforming growth factor)-beta pathway were demonstrated as necessary for the worm's resistance to infection (Mallo *et al*., 2002). This proved that *C. elegans* can respond to infection by inducing genes whose regulation is dependent upon signalling pathways required to achieve full resistance. The genetic approach developed by Kim *et al*. (2002) was based upon the isolation of mutants with an enhanced susceptibility to pathogens. The subtlety of this assay was to identify mutants that die precociously from the infection, but that (fortunately) retained some eggs within the body, thereby allowing the establishment of a mutant population. This study resulted in the identification of elements of the conserved p38 mitogen-activated protein kinase (MAPK) pathway.

Subsequently transcriptomic approaches, forward genetic screens and candidate gene approaches, combined with the wide range of established infection models, allowed the identification of several host signalling pathways required for the direct response to infection (Couillault *et al*., 2004; Estes *et al*., 2010), as well as the mechanisms necessary for repairing immune-related damages (Garsin *et al*., 2003). The current view of nematode immunity is of a pathogen-specific response to infection involving a network of pathways with signalling modules such as p38 MAPK (Kim *et al*., 2002), extracellular signal-regulated kinase (ERK) (Nicholas and Hodgkin, 2004), c-Jun N-terminal kinase (JNK) (Kim *et al*., 2004), insulin-like receptor (ILR) (Garsin *et al*., 2003), TGF-beta receptor (Mallo *et al*., 2002; Zugasti and Ewbank, 2009) and proteins required for programmed cell death (PCD) (Aballay and Ausubel, 2001; Jia *et al*., 2009). At the same time, various microarray analyses revealed one of the main features of *C. elegans* response to infection, that is, the robust transcriptional induction of peptide-encoding effectors (Mallo *et al*., 2002; Couillault *et al*., 2004; Troemel *et al*., 2006). These genes, such as putative AMP-encoding genes, are becoming precious molecular tools for characterizing more precisely the pathways responsible for their regulation. The principle is to use the promoters of these genes fused to *gfp* (the GFP gene) to obtain biosensors for nematode immune activation. Thus, it becomes possible to identify mutants or conditions that alter the expression of these effectors (Ferrandon *et al*., 1998; Couillault *et al*., 2004). The animal's transparency permits the convenient visualization of the relative expression of these immune reporter genes. Such a strategy was originally successfully exploited for deciphering the *D. melanogaster* innate immunity (Ferrandon *et al*., 1998) and was adapted to *C. elegans* (Pujol *et al*., 2008).

16.3.3 *In vivo* platforms to assess cytotoxicity and pathogenicity

The importance of invertebrates as models for investigating specific biological processes relevant to mammals has now definitely been proven. Concerning more specifically the issues related to the toxicity of a given molecule or the pathogenicity of a microorganism,

the next step would be to systematically increase the use of invertebrates as alternatives to mammals. The basic idea supporting this principle is to consider that a chemical or a microbe harmful to a non-mammalian metazoan might be a threat for humans. The different studies described above proved this principle to be true in many cases, but it has to be kept in mind that an effect seen with the invertebrates can be specific, and that an absence of phenotype upon exposure does not necessarily imply that the molecule or microbe is innocuous. Nevertheless, in the context of assays involving potential biocontrol agents, an absence of any visible effect toward insects and nematodes can be promising (Zachow *et al*., 2009).

The nematode is successfully used for drug screenings

Nematodes are usually grown on solid media previously seeded with a bacterial culture as a food source. However, *C. elegans* can also be grown in liquid media and, therefore, becomes highly suitable for high-throughput approaches such as drug screening or toxicity assays in 96-well plates (Garsin *et al*., 2004). Such approaches are currently used to assess the cytotoxicity of pharmaceutical compounds, using worms as a multicellular alternative prior to tests with mammals. The increasing amount of testing required before commercialization of a product such as a pesticide increases the problem of bioethical and financial issues, and worms are, consequently, increasingly seen as a good *in vivo* alternative for cytotoxicity tests. Along the same lines, by using the nematode to model human illnesses such as muscular dystrophy (Giacomotto *et al*., 2009), Parkinson's disease (Braungart *et al*., 2004) or Huntington's disease (Parker *et al*., 2004), several laboratories have developed screening protocols to identify molecules able to alter the associated phenotypes. These approaches have been successful and allowed the discarding of molecules with toxic effects against a multicellular organism and the selection of a subset of products modifying the established phenotype.

An interesting illustration of what can be done with nematodes to conveniently assay toxicity and pathogenicity at a large scale comes from recent screenings aiming to identify new antimicrobials. Indeed, the characterization of several infections (such as with *E. faecalis* or *C. neoformans*) with the nematode as a host prompted several teams to test large banks of molecules to identify products able to cure the worms (Tampakakis *et al*., 2008; Moy *et al*., 2009). These screens allowed thousands of molecules to be tested, were successful in identifying new antimicrobials and permitted the ready exclusion of cytotoxic compounds as the assays were performed *in vivo*.

However, several drawbacks have to be mentioned, and these are related to the administration of the tested product to the nematode. The molecules cannot be easily and reproducibly injected into the animals, but have to be present within the media (solid or liquid), from which they would then enter the body through free diffusion. In this context, the cuticle that surrounds the animal can be a problem as a result of the concentration of the molecules that might be necessary to observe an effect. The other way for a molecule to reach nematode cells is to enter through natural openings such as the mouth. Without question, nematodes are almost constantly ingesting external media that will interact with the apical layer of intestinal cells; this is how nutrients are taken up after the grinding of bacterial cells. However, defaecation occurs every minute, with an almost complete renewal of the luminal content, thereby preventing the test products from interacting with intestinal cells for a long time. In addition, certainly due to their constant exposure to the external environment, intestinal cells are active and efficient sites of detoxification (Mahajan-Miklos *et al*., 1999), in which the mechanisms involve modifications and efflux of molecules.

Survival is one phenotype to monitor microbial virulence or toxicity

Monitoring host survival as a read-out for pathogen virulence or toxicity has been used for decades, and is highly relevant. However,

it is necessary to view this as a multifactorial result, with pathogen virulence, host immunity, stress resistance and the fitness of the two interacting organisms being tightly interdependent over the course of several days. Therefore, alternative approaches can be exploited for either saving time, addressing one aspect of the infection/toxicity, or both. Several subtle and very attractive methods include the use of fluorescent biosensors in the host during infection/toxicity assays, determination of variations in the life cycle and analysis of the balance of commensalism.

In addition to visible pathologies (i.e. lethality), the infection of *D. melanogaster* individuals can be monitored by the activation of their immune responses. Thanks to more than two decades of research, the core innate immune responses of *D. melanogaster* have been characterized in detail (Lemaitre and Hoffmann, 2007). Mainly through genetic studies, the components of two signalling cascades have revealed the Toll and immune deficiency (Imd) pathways, which lead to the potent activation of *D. melanogaster* immune responses upon infections. These signalling events share striking parallels with signalling events occurring during mammalian immune responses – the TLR (R (Toll-like receptor)/IL1-R (interleukin-1 receptor) and TNF-R (tumour necrosis factor receptor) pathways, respectively – demonstrating an ancient evolutionary origin of the mechanisms regulating host innate immune response in the animal kingdom. Activation of the Toll and Imd signalling pathways lead to the expression of hundreds of genes via the respective activation of the NF (nuclear factor)-kappaB factors Dif/Dorsal and Relish. Some of these NF-kappaB target genes encode antimicrobial peptides which are potent immune effectors that are synthesized in immunocompetent tissues, such as the tracheal epithelium, circulating 'blood' cells and the fat body (Ferrandon *et al*., 2007). The expression of such antimicrobial peptide genes is a hallmark of the *D. melanogaster* immune response activation and, as such, has been extensively used to monitor mucosal and systemic immune responses to infection (Romeo and Lemaitre, 2008). Interestingly, a battery of reporter genes, composed of the promoter of several antimicrobial peptide genes fused to a gene coding for a green or red fluorescent proteins, have been created and these allow a rapid and easy detection of the activation of immune responses by visualizing the living animals under a fluorescent stereomicroscope (Ferrandon *et al*., 1998; Tzou *et al*., 2000; Charroux and Royet, 2009). These tools have been instrumental in the success of multiple genetic screens designed to identify the signalling events controlling *D. melanogaster* immune responses and are available for monitoring microbial pathogenicity.

C. elegans is well known for its resistance to toxic compounds, including microbial products (Mahajan-Miklos *et al*., 1999). So a microbial molecule such as a pore-forming substance can be especially noxious, but the animal may still be able to survive it without any obvious phenotypic changes. However, several genes whose products are necessary to detoxify or export noxious molecules have been identified and used as fluorescent reporters for the induction of the related machinery (Daniells *et al*., 1998). As for the stress response, immune activation in nematodes is under investigation and specific fluorescent biosensors have been developed. These reporters for host immune activation can be used as read-outs for evaluating the pathogenicity of a specific microbe. Moreover, it appears that the type of effectors and the sites of expression are related to the pathogen and the mode of attack. For instance, upon infection by *P. aeruginosa*, a specific set of genes is induced mainly in the intestine (Shapira *et al*., 2006; Troemel *et al*., 2006), while another set of genes is induced in the epidermis surrounding the nematode upon exposure to spores of the fungus *Drechmeria coniospora* (Couillault *et al*., 2004; Wong *et al*., 2007). Unfortunately, transgenic animals containing two or three such specific reporters with different fluorochromes have not yet been described, but will be easy to generate and worth using as a sensor strain for microbial pathogenicity.

Thus, such reporters in insects and nematodes can not only directly inform us about the potential cytotoxicity of a single tested molecule, they can also reveal the virulence of

a microbe through the activation of the host stress response. Consequently, the evaluation of virulence or toxicity during an assay can be based on the induction by the tested samples of the animal's detoxifying machinery and/or specific immunity. This kind of approach can be initiated by the specific transcriptomic analyses of the animal response upon exposure to biotic or abiotic agents, and aims to identify the immunity and stress machinery induced (Kurz *et al*., 2007).

Nematodes and insects to underline biocontrol agents' host specificity

Among invertebrates, *C. elegans* and *D. melanogaster* are currently considered as representatives owing to their intensive use as animal models. In addition, these two animals are widely distributed around the world, easily found in the soil or on decaying products and certainly are part of numerous and various ecosystems. Therefore, when developing a biocontrol agent that should specifically target a pest, assays involving these two organisms can be extremely informative. Indeed, several aspects relevant to life in the wild can be monitored, such as the developmental period, amount of progeny or behaviour. More subtle experiments involving stress or immune fluorescent reporters can also be fruitful. Thus, a lack of obvious phenotype changes upon exposure to a microbial biocontrol agent at a relevant concentration will be encouraging, while the opposite should lead to a severe reconsideration of the specificity of that agent (Zachow *et al*., 2009).

16.3.4 The interactions between invertebrates and microbes are not limited to infections

Commensal microbiota used to determine host fitness and detrimental environmental conditions

A feature unique to *D. melanogaster* compared with *C. elegans* is the presence of commensal bacterial communities in their intestine. This characteristic is rather the rule in the animal kingdom, and *C. elegans* is an exception in this case. The exception probably relates to the artificial laboratory environment and the adaptation of *C. elegans* to this environment, its eating preference being as a 'soil bacteria feeder' as well as the fact that it carries a 'grinder' organ which destroys most if not all ingested bacteria. Natural isolates often harbour bacteria in the gut, but it is not yet clear whether this corresponds to infection or symbiosis.

Most multicellular animals carry dynamic bacterial communities – called the microbiota – in their guts. The microbiota confers enzymatic activities which help the host to optimize the digestion of ingested food and the assimilation of nutrients. In mammals, these communities are highly complex. They are also large, with more than 500 species, most of which are uncultivable in the laboratory and dynamic, being influenced by many environmental factors (Ley *et al*., 2008). In contrast, the bacterial communities of the gut of *D. melanogaster* are simple. They are composed of about 20 species with a limited set of three to five main species, which are aerotolerant and can be grown in the laboratory. The dominant *D. melanogaster* commensal species belong to the genera *Acetobacter* and *Lactobacillus* (Brummel *et al*., 2004; Corby-Harris *et al*., 2007; Cox and Gilmore, 2007; Ren *et al*., 2007; Ryu *et al*., 2008). Of note, lactobacilli are also found in the human gut and vaginal microbiota, and specific strains are commonly used on the market as probiotics (Kleerebezem and Vaughan, 2009) (see also Wassenaar and Alter, Chapter 13, this volume). This similarity again highlights the potential of using *D. melanogaster* as a model to study not only bacterial infectivity, but also beneficial commensal bacterial strains.

Recent studies have revealed that there are active immune mechanisms that control *D. melanogaster* gut microbiota and promote gut integrity (Ryu *et al*., 2008; Buchon *et al*., 2009). Like in most animals, the integrity of the intestinal epithelium of *D. melanogaster* relies on the homeostasis of the balance between intestinal epithelial cell death and their renewal from a pool of intestinal stem cells (Casali and Batlle, 2009). Interestingly, several recent reports demonstrate that pathogenic intestinal infections or dysbiosis of commensal communities trigger epithelial cell death

associated with proliferation and differentiation of intestinal stem cells in order to maintain tissue integrity (Lee *et al*., 2009). Thus, the reaction of the *D. melanogaster* gut to microorganisms is not restricted to the activation of the immune system but also extends to integrated responses to maintain gut tissue integrity (Lee *et al*., 2009). In this light, following the same logic as that of the previous analysis of signalling pathways in controlling immune responses, a 'stem cell' fluorescent reporter gene using the promoter of the *escargot* gene fused to the coding sequence of *gfp* has been instrumental in monitoring intestinal stem cell division and dissection of the signalling pathways involved in the gut regeneration process (Lee, 2009). Following these recent developments, we can now seriously envision the use of a mono-association of germ-free *D. melanogaster* (i.e. deprived of any indigenous bacterial community) with any putative probiotic strains of lactobacilli, and assay their ability to colonize their host intestine, thereby monitoring their impact on the host gut immune response (using AMP-reporter genes) or intestinal epithelial renewal (using stem cell reporter gene). In addition, and similar to recent studies performed in *C. elegans* (see below), co-infection of germ-free animals with established *D. melanogaster* pathogens and a putative probiotic strain may be developed to screen new beneficial probiotic strains that may counteract the infectivity of natural pathogens, either directly or indirectly, by enhancing host immune response or host tissue repair mechanisms. Finally, the presence and the diversity of the fly commensal microbiota can be used as a read out to monitor the impact of a given molecule or of an infection. Indeed, the existence and fitness of these bacterial communities depends both on the host and the external environment.

Co-infections and probiotic diet impacts on the host can be deciphered

A promising recent approach for studying host–pathogen dynamics using *C. elegans* is related to the co-infection of worms by two microbes, or by influence of the diet, followed by the monitoring of nematode survival. For instance, Peleg *et al*. (2008) used the standardized infection of *C. elegans* by the yeast *C. albicans* to test whether a co-infection with another pathogen, the bacterium *Acinetobacter baumannii*, might impair fungal activity within nematodes. They found that *A. baumannii* did impair filamentous growth of *C. albicans* upon co-infection of the nematodes and, additionally, increased worm survival through a secreted factor. Importantly, despite the fact that the detrimental effect of *A. baumanii* over *C. albicans* was also demonstrated *in vitro*, the *in vivo* experimental co-infection model *A. baumanii–C. albicans–C. elegans* allowed specific genetic screening for bacterial products that increased worm survival through the attenuation of fungal virulence (Peleg *et al*., 2008). Another study, from Ikeda *et al*. (2007), tested the effect of lactic acid bacteria (LAB) on resistance to bacterial infection. The species of *Lactobacillus* and *Bifidobacterium* tested for being beneficial were: *L. helveticus*, *L. plantarum*, *L. rhamnosus*, *B. infantis* and *B. longum*. Ikeda *et al*. compared the resistance to *S. enterica* of nematodes fed with the standard *E. coli* OP50 strain with that of worms fed with LABs before infection. Strikingly, animals with a LAB diet were more resistant to *S. enterica* infection without diminishing the pathogen load within the intestine. This work indicates the existence of bacterial natural products that attenuate *in vivo* bacterial virulence or stimulate nematode immunity – molecules that are waiting to be identified.

16.4 Conclusions

While approaches aimed at the rapid determination of potential pathogenicity or cytotoxicity using *C. elegans* or *D. melanogaster* as hosts are totally feasible and relevant for mammals, several limitations have to be mentioned. For instance, microbes interacting with humans adapt their metabolism and virulence to the body temperature of 37 °C, while flies and worms are not grown at this temperature. Therefore, despite the demonstrated commonalities between infections of vertebrates and invertebrates by specific microbes, some aspects of the virulence

specifically related to the growth temperature cannot be addressed using insects or nematodes. Very interesting aspects of microbial pathogenicity in mammals concern the hijacking of host immunity to favour proliferation. The absence of adaptive immunity within invertebrates severely limits the deciphering of these subtle and crucial interactions. Moreover, worms lack organs such as a liver or heart, and do not possess any circulatory system, thus impairing studies specifically related to these tissues or cells. Finally, despite the numerous advantages of a genetically tractable invertebrate, flies are not well suited for high-throughput approaches such as drug screening owing to the difficult logistics associated with these assays.

None the less, the existence of universal virulence strategies employed by pathogens and the conservation of defence mechanisms across phyla have turned the two introduced invertebrate models discussed in this chapter into attractive and relevant methods for studying microbial pathogenicity and host defence mechanisms. Up to now, flies and worms were essentially used as cheap and bioethical alternatives to mammalian hosts for microbial virulence assays. The molecular and genetic advantages of these models have not yet been fully exploited in this context, as only a few microbial infections have been assayed. Furthermore, the availability of genetic tools for the host and pathogen opens up immense perspectives towards the understanding of the mode of action of a specific chemical or virulence factor.

One can anticipate that, in the coming years, *D. melanogaster* and *C. elegans* will become powerful, comprehensive, cheap and rapid *in vivo* screening platforms for assays of the cytotoxicity of molecules and the pathogenicity of microbes prior to more subtle, specific and expensive tests with mammals. Indeed, despite the numerous advantages and proofs of principle described in this section, these arthropods cannot completely replace assays with animals like rodents, but rather have to be seen as powerful alternatives.

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17 Assessing Potential Cytotoxicity of Biocontrol Microorganisms Using Invertebrate Assays

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17.1 Introduction

There is increasing interest in the exploitation of microorganisms for the biological control of crop pests, weeds and diseases (Whipps and Davies, 2000; Butt *et al*., 2001). A number of biological pesticides (biopesticides, biological control agents or BCAs) containing fungi or bacteria as the active ingredient are already marketed in Europe, the USA and developing countries (Copping, 2009), while many other biocontrol strains or formulations are expected to be placed on the world market in the next few years. However, many microorganisms are known to produce bioactive secondary metabolites, including toxins that may be harmful to humans and animals (Cole and Cox, 1981; Cole and Schweikert, 2003; Strasser *et al*., 2011). In particular, some fungal microbiological control agents (MBCAs) have been reported to secrete biologically active compounds (Vey *et al*., 2001), which might be potentially toxic when present in formulated products, crops or the environment. Therefore, risk assessment procedures, including hazard characterization of MBCA metabolites, are required before the introduction and use of MBCAs (Blum *et al*., 2003; Strasser *et al*., 2008; Strauch *et al*., 2011). In Europe, the European Parliament and Council Regulation 1107/2009/EEC (OJEU, 2009), repealing Council Directives 79/117/EEC and 91/414/EEC, identifies the dossier requirements for authorization of the production and marketing of pesticides, including those whose active substance is a MBCA. In particular, the regulation requires the provision of information on short-term cytotoxicity, as well as ecotoxicity, of any relevant metabolites (i.e. metabolites of toxicological, ecotoxicological and/or environmental concern) formed by the BCA.

The technical, economic and ethical issues related to the use of mammals for toxicity testing have prompted the search for *in vitro* tests

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that can, at least at some stages of toxicological assessment, replace tests on laboratory animals (Lilienblum *et al.*, 2008; Andersen and Krewski, 2009). Cell lines or tissue cultures are valid predictive toxicology systems that can be used in the study of bioactive compounds. These tests are particularly useful to gain information about the mechanisms of cytotoxicity or genotoxicity of a substance, such as the destruction of cell membranes, apoptosis, cytolysis, oxidative stress, impairment of mitochondrial function, cell proliferation, quantification of DNA damage and repair, and the analysis of micronuclei. However, in many cases such detailed information is not essential in the early stages of development of a product, when what is urgently needed is a qualitative response of the type toxic/non-toxic in order to identify a potential toxicological risk and drive the decision process. In these cases, toxicity tests that are simpler and less expensive than cell line or tissue assays, but have comparable predictive value, are highly desirable (Eisenbrand *et al*., 2002; Lilienblum *et al*., 2008). Ideally, the results of these alternative cytotoxicity assays should have high correlation with those of both cell line tests and *in vivo* tests in order to allow predictive inference of toxic effect.

Recent years have seen the development of a number of toxicity tests in which the response has been measured in simple eukaryotic organisms, such as protozoa (Gerhardt *et al*., 2010) and arthropods (Calow, 1993; Lagadic and Caquet, 1998). These tests offer some advantages over other *in vitro* toxicity tests, which can be summarized in the following points:

- Test organisms can be cultured under laboratory conditions at low cost.
- High fecundity and short lifespan of test animals.
- Animals can be hatched synchronously from dormant eggs (arthropods).
- Low genetic variability among individuals.
- Tests give an indication of potential adverse effects on the environment (ecological relevance).
- Kits are often available for better standardization and higher repeatability and reproducibility of tests.

These characteristics of invertebrates account for their extensive use in toxicity tests. Many invertebrate species can be cultured easily under laboratory conditions because of their small size, high fecundity and short lifespan. This makes the maintenance of invertebrates and the simultaneous breeding of various species easier than for vertebrates. The handling of the animals is also easy and therefore the number of individuals exposed to each tested concentration or dose may be increased. This improves the statistical significance of test results without a significant increase in cost.

Many invertebrate test organisms form dormant eggs (cysts) that remain viable for a long time. Cyst-based toxicity tests eliminate the need for stock culturing of test species. Animals can be hatched synchronously, the neonates originate from genetically defined stocks and are of the same age and in the same physiological condition. In addition, some invertebrate species (e.g. *Daphnia magna*) are parthenogenetic and genetic variability is thereby reduced. As a result, standardization of the test and reproducibility of the results are significantly enhanced.

Finally, invertebrates occupy key positions in the food webs of both aquatic and terrestrial ecosystems. Some species (e.g. daphnids, paramecia) are present throughout a wide range of habitats. Therefore, they are 'biosensors' for the potential ecotoxicological risks associated with the presence of chemicals and toxicants in the environment (Lagadic and Caquet, 1998). Invertebrates are already used in tests that are required by some regulatory authorities for the environmental risk assessment of pesticides, chemicals and pollutants (OJEC, 1992; US EPA, 2002).

Without the ambition to offer a comprehensive review on the use of invertebrate systems for toxicity testing, on which topic an extensive literature and several critical reviews are already available (Calow, 1993; Lagadic and Caquet, 1998; Sauvant *et al*., 1999), in this chapter we will show four examples of how invertebrate test organisms, namely *Artemia salina*, *Daphnia magna*, *Paramecium caudatum* and *Tetrahymena pyriformis*, have been successfully used for the toxicological assessment of the metabolites of fungal BCAs.

As Appendix 1 to this chapter, we include one developed standard operating procedure for *P. caudatum* to provide guidance for a modern approach to MBCA metabolite toxicity testing.

17.2 Invertebrate model systems

17.2.1 *Artemia salina* (Crustacea: Branchiopoda)

Artemia salina is an anostracan crustacean, commonly known as the brine shrimp, which is widely distributed in tropical, subtropical and temperate climate regions. Brine shrimps are inhabitants of extreme saline water environments, although they have great powers of adaptation and are able to tolerate physiologically large changes in salinity. *A. salina* acute toxicity tests have often been used for ecotoxicological studies of xenobiotic substances (Nunes *et al*., 2006), as well as for the toxicological screening of chemicals (Barahona and Sánchez-Fortún, 1999; Cleuvers, 2003) and natural compounds (Solis *et al*., 1993; Caldwell *et al*., 2003), including mycotoxins (Harwig and Scott, 1971; Schmidt, 1989; Hartl and Humpf, 2000). Acute toxicity to *A. salina* has been reported to be correlated with cytotoxicity, as the following examples indicate. McLaughlin *et al*. (1993) found a correlation between brine shrimp kill and the cytotoxicity of plant extracts to 9-KB (human nasopharyngeal carcinoma) cells. Solis *et al*. (1993) found the *A. salina* test predictive of KB cell cytotoxicity, except for compounds requiring metabolic activation, because brine shrimps lack the necessary cytochrome P-450 enzyme. Logrieco *et al*. (1996) reported that the median cytotoxic concentration (CC_{50}) of the fungal toxin fusaproliferin to the human B-lymphocyte cell line IARC/LCL 171 and the insect cell line SF-9 were approximately the same as the median lethal concentration (LC_{50}) found in the *A. salina* assay. Moreover, Lagarto Parra *et al*. (2001) found a strong correlation ($r = 0.85$, $P < 0.05$) between the results of *in vivo* tests on mice and *in vitro* tests with *A. salina* of various plant extracts. The authors concluded that the *A. salina* bioassay was a useful tool for prediction of the oral acute toxicity of plant extracts in mammals.

17.2.2 *Daphnia magna* (Crustacea: Branchiopoda)

Daphnia magna (the water flea) is a freshwater cladoceran crustacean. Cladocerans can reproduce asexually by parthenogenesis, without male fertilization. This is one of the main arguments that led to the widespread use of *Daphnia* spp. in toxicity testing. *D. magna* has been used for a long time as a laboratory animal for testing ecotoxicity (Anderson, 1944). Other *Daphnia* species than *D. magna* may occasionally be used (Lilius *et al*., 1995), but laboratories mostly use *D. magna* as the standard. *D. magna* is specified to be used in the OECD *Guidelines for the Testing of Chemicals* (OECD, 2004, 2008). The OECD Test No. 202 is a 48 h acute toxicity test in which young *Daphnia* are exposed to varying concentrations of the test substance and the (effective) concentration estimated to immobilize 50% of the daphnids (EC_{50}) is determined. It has been reported for a number of compounds that the toxicity data of *Daphnia* tests correlate well with toxicity to vertebrates, as has also been shown for *A. salina*. For instance, correlation between toxicity to *Daphnia* and toxicity to fish has been shown for a group of 42 industrial chemicals (Walker *et al*., 1991). Also, a strong correlation between the acute toxicity of 54 different compounds to *D. magna* and rats was found by Guilhermino *et al*. (2000).

17.2.3 *Paramecium caudatum* and *Tetrahymena pyriformis* (Ciliophora: Oligohymenophorea: Urocentrida and Tetrahymenida)

Paramecium caudatum and *Tetrahymena pyriformis* are unicellular ciliate protozoa which are widespread in freshwater systems. They have been extensively used in the last decade as tools for toxicological assessments because they have almost the same metabolic systems as higher animals (Hauser, 1990). In addition,

they can be purchased from most of the prominent strain culture collections of protozoa, and can be easily grown in unsterile liquid culture at room temperature. Members of both *Tetrahymena* and *Paramecium* have been reported to be highly sensitive to a number of xenobiotic substances and have been used as sensitive eukaryotic cells to detect potential (eco)toxic substances (Komala, 1982, 1984). *T. pyriformis* is more commonly used in toxicological studies and therefore more toxicity data are currently available for this species than for *P. caudatum*.

Tetrahymena species have been used as model organisms in scientific and industrial research (Pauli *et al*., 1993; Pauli and Berger, 1997). Uma *et al*. (2008) demonstrated that *T. pyriformis* is a useful tool to assess the acute toxicity of heterocyclic compounds. Skrobek *et al*. (2006) reported that *T. pyriformis* was more sensitive to the synthetic pesticide amidosulfuron than rats. In studies on the acute toxicity of the mycotoxin patulin, comparable results in rats and in *T. pyriformis* were obtained by Escoula *et al*. (1977) and by McKinley and Carlton (1980).

P. caudatum has been successfully used to study the toxicity of the mycotoxin produced by the basidiomycete *Hebeloma edurum* (Pöder, 1982) and also that of other fungal secondary metabolites (Stemer, 2004; Gierner, 2005). Mijatov (1975) reported a good correlation between the sensitivity of *P. caudatum* and that of white mice, guinea pigs and rabbit skin treated with water extracts of maize or peanut meal colonized by *Fusarium graminearum* and *F. poae*. Hussain *et al*. (1985) validated *P. caudatum* as a biosensor by assessing the sensitivity of this organism to different pesticides. Miyoshi *et al*. (2003) demonstrated the high sensitivity of *P. caudatum* to a number of organic solvents and heavy metals, but also to mutagenic, cancerogenic and teratogenic substances.

17.3 Assessment of Acute Cytotoxicity of Fungal Metabolites

The simplification of risk assessment procedures for fungal MBCAs and their relevant metabolites without compromising high levels of safety was the main objective of the EU-funded project 'Risk Assessment of Fungal Biological Control Agents' (RAFBCA, QLKI-2001-01391) that took place from 2001 to 2004. This project was the first attempt to address scientifically the issue of risk assessment of MBCAs, which process has greatly hindered the registration of fungal biopesticides. Indeed, the risk assessment procedures requested by Directive 91/414/EEC for the registration of MBCAs and derived products had been tailored for chemicals and extended, almost unmodified, to biological agents and their metabolites. Among biocontrol stakeholders there was a general consensus that such requirements, hardly affordable for the small and medium-sized enterprises involved in biopesticide production, were a major hurdle in the registration and subsequent commercialization of MBCAs. Therefore, one of the goals of the project was to identify sensitive biosensors that could be used for assessment of the cytotoxicity of metabolites of fungal MBCAs with a significant simplification of procedures and reduction of cost.

In the RAFBCA project, *A. salina*, *D. magna*, *P. caudatum* and *T. pyriformis* were used to assess the acute toxicity of seven bioactive fungal metabolites (Tables 17.1 and 17.2) with different molecular structures and modes of action (Pernfuss *et. al*., 2003; Stemer, 2004; Gierner, 2005; Favilla *et al*., 2006; Skrobek *et al*., 2006). Those unique mycotoxins are produced by mycoparasitic (*Gliocladium* spp., *Trichoderma* spp.), entomopathogenic (*Beauveria brongniartii*, *Metarhizium anisopliae*) or phytopathogenic (*Stagonospora* spp.) fungi used for biological control of fungal plant diseases, insect pests or weeds, respectively, and had not been previously evaluated in the above invertebrate models.

For the *A. salina* assays, materials for aquarists, commercially available at low cost, were used. For the higher levels of standardization and reproducibility of *D. magna* assays, a laboratory kit (Daphtoxkit F, Creasel BVBA, Deinze, Belgium) was used with some modification of the protocol to minimize the amount of the test compounds needed (Favilla *et al*., 2006). Both crustaceans proved to be sensitive to all the fungal MBCA metabolites tested, regardless of the chemical nature of the

Table 17.1. Toxicity of fungal BCA (biological control agent) toxins to the crustacean test organisms *Artemia salina* (brine shrimp) and *Daphnia magna* (water flea).

Metabolite (toxin)/ producer BCA	Type of chemical	*A. salina*[a]	*D. magna*[a]	Toxicity data (Reference)[b]
Alamethicin(s)/ *Trichoderma viride*[c]	Polypeptide	LC_{50}, 24 h = 5.32 μM LC_{50}, 36 h = 1.96 μM	LC_{50}, 24 h = 1.19 μM LC_{50}, 36 h = 0.99 μM	Human erythrocytes, CC_{50} = 16 μM (Brückner *et al.*, 1984) Human HL-60 cell line, CC_{50} = 10 μM (Macchia *et al.*, 2003) Human U-937 cell line, CC_{50} = 3 μM (Macchia *et al.*, 2003) Lepidopteran SF-9 cell line, CC_{50} = 2 μM (Macchia *et al.*, 2003) Mice, oral administration, LD_{50} = 80 mg kg^{-1} (Taylor, 1986)
Paracelsin(s)/ *Trichoderma* spp.[c]	Polypeptide	LC_{50}, 24 h = 21.26 μM LC_{50}, 36 h = 9.66 μM	LC_{50}, 24 h = 7.70 μM LC_{50}, 36 h = 5.60 μM	PC12 cells, CC_{50} = 21.8 μM (Abu Raya *et al.*, 1993) Human erythrocytes, CC_{50} = 37 μM (Brückner *et al.*, 1984) Mice, intraperitoneally, LD_{50} = 5 mg kg^{-1} (Brückner *et al.*, 1984)
Antiamoebin(s)/ *Gliocladium catenulatum*[c]	Polypeptide	LC_{50}, 24 h = 19.79 μM LC_{50}, 36 h = 8.25 μM	LC_{50}, 24 h = 14.61 μM LC_{50}, 36 h = 7.17 μM	Human erythrocytes, CC_{50} = 125 μM (Brückner *et al.*, 1984)
Gliotoxin/ *Gliocladium virens*[c]	Epidithiodiketo-piperazine	LC_{50}, 24 h = 39.49 μM LC_{50}, 36 h = 20.44 μM	LC_{50}, 24 h = 0.85 μM LC_{50}, 36 h = 0.50 μM	Lepidopteran SF-9 cell line, CC_{50} = 4 μM (Fornelli *et al.*, 2004) Human lung carcinoma A549 cell line, IC_{50} = 0.3 μM (Kreja and Seidel, 2002) Human HL-60 cell line, CC_{50} = 100 μM (Macchia *et al.*, 2003) Human U-937 cell line, CC_{50} = 200 μM (Macchia *et al.*, 2003) Mice, intraperitoneally, LD_{50} = 25 mg kg^{-1} (Johnson *et al.*, 1943)
Destruxin A/ *Metarhizium anisopliae*[d]	Cyclodepsi-peptide	LC_{50}, 24 h = 16.92 μM LC_{50}, 36 h = 5.05 μM	LC_{50}, 24 h = 0.35 μM LC_{50}, 36 h = 0.27 μM	Mice, intraperitoneally, LD_{50} = 1 – 1.35 mg kg^{-1} (Kodaira, 1961)
Oosporein/*Beauveria brongniartii*[d]	Hydroxybenzo-quinone	24 h, not toxic at 200 μM 36 h, not toxic at 200 μM	LC_{50}, 24 h = 223.54 μM LC_{50}, 36 h = 19.10 μM	Mice and hamsters, intraperitoneally, LD_{50} = 0.5 mg kg^{-1} (Wainwright *et al.*, 1986) Day-old cockerels, oral administration, LD_{50} = 6.12 mg kg^{-1} (Cole *et al.*, 1974)
Elsinochrome A/ *Stagonospora* spp.[e]	Perylene-quinone	LC_{50}, 24 h = 20.18 μM LC_{50}, 36 h = 15.26 μM	LC_{50}, 24 h = 0.53 μM LC_{50}, 36 h = 0.40 μM	Monkey embryo R366.4 cell line, CC_{50} at 5 min = 100 μM (Ma *et al.*, 2003) Human Hce-8693 cell line, CC_{50} at 5 min = 100 μM (Ma *et al.*, 2003)

[a]From Favilla *et al.*, 2006; LC_{50} = lethal concentration for 50%.

[b]CC_{50} = cytotoxic concentration for 50%; IC_{50} = inhibitory concentration for 50%; LD_{50} = lethal dose for 50%.

[c]BCA of fungal plant diseases (Meyer and Reusser, 1967; Aluko and Hering,1970; Solfrizzo *et al.*, 1994; Jaworski and Brückner, 2000).

[d]BCA of insects (Kodaira, 1961; Strasser *et al.*, 2000).

[e]BCA of weeds (Nicolet and Tabacchi, 1999).

Table 17.2. Toxicity of fungal BCA (biological control agent) toxins to the ciliate protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*.

Metabolite (toxin)/ producer BCA	Assay material	*P. caudatum*	*T. pyriformis*	Toxicity data (Reference)[a]
Destruxin A/ *Metarhizium anisopliae*[b]	Pure compound	LC_{50}, 2 h = 9870 μM[c]	10 min, not toxic at 100 μM[d] 4 h, not toxic at 100 μM[d]	*Plutella xylostella*, LC_{50}, 24 h = 97 μM (Amiri *et al.*, 1999) *Phaedon cochleariae*, LC_{50}, 24 h = 151 μM (Amiri *et al.*, 1999) *Pseudomonas syringae*, 18 h, not toxic at 100 μM (Skrobek *et al.*, 2006) Human HL-60 cell line, 4 and 24 h, not toxic at 865 μM (Skrobek *et al.*, 2006) Lepidopteran SF-9 cell line, 4 h, not toxic at 86.5 μM LC_{50}, 24 h = 9 μM (Skrobek *et al.*, 2006)
Destruxin B/ *Metarhizium anisopliae*	Pure compound	2 h = No effect up to 6000 μM[c]		*Plutella xylostella*, LC_{50}, 24 h = 633 μM (Amiri *et al.,* 1999) *Phaedon cochleariae*, LC_{50}, 24 h = 842 μM (Amiri *et al.*, 1999) Human HL-60 cell line, 4 h, not toxic at 841 μM (Skrobek and Butt, 2005) Lepidopteran SF-9 cell line, 4 h, not toxic at 841 μM (Skrobek and Butt, 2005)
Destruxin E/ *Metarhizium anisopliae*	Pure compound	2 h = No effect up to 25,000 μM[c]		*Plutella xylostella*, LC_{50}, 24 h = 89 μM (Amiri *et al.*, 1999) *Phaedon cochleariae*, LC_{50}, 24 h = 84 μM (Amiri *et al.*, 1999) Human HL-60 cell line, 4 h, not toxic at 841 μM (Skrobek and Butt, 2005) Lepidopteran SF-9 cell line, 4 h, not toxic at 841 μM (Skrobek and Butt, 2005)
	Culture filtrate (mixture containing destruxins)		LC_{50}, 0.2 h > 500 μM[d] LC_{50}, 4 h = 367 μM[d]	
Oosporein/ *Beauveria brongniartii*[b]	Pure compound	LC_{50}, 2 h = 750 μM[e,f] (positive control, chlorpyrifos LC_{50}, 2 h = 10 μM) MIC = 100 μM[f]		***Ascomycota*** *Candida utilis*, ED_{50} (–); MIC > 327 μM (Taniguchi *et al.*, 1984) *Chaetomium cochlioides*, ED_{50} = 131 μM; MIC (–) (Brewer *et al.*, 1977) *Fusarium moniliforme*, ED_{50} = 30 μM; MIC (–) (Brewer *et al.*, 1977) *F. oxysporum*, ED_{50} > 327 μM; MIC (–) (Brewer *et al.*, 1977) *F. poae*, ED_{50} > 327 μM; MIC (–) (Brewer *et al.*, 1977) *Penicillium expansum*, ED_{50} = 327 μM; MIC (–) (Brewer *et al.*, 1977) **Bacteria** *Bacillus subtilis*, ED_{50} (–); MIC = 3270 μM (Brewer *et al.,* 1984) *Bacteroides succinogenes*, ED_{50} (–); MIC = 1630 μM (Brewer *et al.*, 1984) *Micrococcus luteus*, ED_{50} (–); MIC = 327 μM (Brewer *et al.*, 1984) *Mycobacterium smegmatis*, ED_{50} (–); MIC >1633 μM (Brewer *et al.*, 1984)

Continued

Table 17.2. Continued.

Metabolite (toxin)/ producer BCA	Assay material	*P. caudatum*	*T. pyriformis*	Toxicity data (Reference)[a]
Oosporein/ *Beauveria brongniartii*	Pure compound	LC_{50}, 2 h = 750 μM[e,f] (positive control, chlorpyrifos LC_{50}, 2 h = 10 μM) MIC = 100 μM[f]		**Paramecia** *Paramecium tetraurelia*, ED_{50} = 849 μM; MIC (–) (Stemer, 2004) **Vertebrates** Mice and hamsters, intraperitoneally, LD_{50} = 1.6 μmol kg^{-1} (Wainwright *et al.*, 1986) Day-old cockerels, oral administration, LD_{50} = 20 μmol kg^{-1} (Cole *et al.*, 1974) Human HL-60 cell line, ED_{50} = 32.7 μM to 82 μM (Semar, 1993) Human HL-60 cell line, 4 h, not toxic at 353 μM (RAFBCA report, unpublished results[g]) RBL-1 cell line (rat), ED_{50} = 32.7 μM (Semar, 1993) L1210 cell line (mouse), ED_{50} = 32.7 μM (Semar, 1993) **Insects** Lepidopteran SF-9 cell line, 4 h, not toxic at 353 μM (RAFBCA report, unpublished results)
	Culture filtrate (mixture containing oosporein)	LC_{50}, 2 h = 1100 μM[f]		
Elsinochrome A/ *Stagonospora* spp.[h]	Pure compound		LC_{50}, 10 min = 5.2 μM[d] LC_{50}, 4 h = 0.64 μM[d]	Human HL-60 cell line, 4 h, CC_{50} = 1.12 μM (Skrobek *et al.*, 2006) Lepidopteran SF-9 cell line, 4 h, CC_{50} = 0.37 μM (Skrobek *et al.*, 2006) Monkey embryo R366.4 cell line, CC_{50} at 5 min = 100 μM (Ma *et al.*, 2003) Human Hce-8693 cell line, CC_{50} at 5 min = 100 μM (Ma *et al.*, 2003)

[a]CC_{50} = cytotoxic concentration for 50%; ED_{50} = effective dose for 50%; LD_{50} = lethal dose for 50%; MIC = minimum inhibitory concentration.
[b]BCA of insects.
[c]Gierner, 2005.
[d]Skrobek *et al.*, 2006.
[e]Pernfuss *et al.*, 2004.
[f]Stemer, 2004.
[g]RAFBCA, EU-funded project 'Risk Assessment of Fungal Biological Control Agents', 2001–2004.
[h]BCA of weeds.

compound (Table 17.1). The toxic response was dose and time dependent. For both *A. salina* and *D. magna*, the acute toxicity end point was the concentration of metabolite estimated to kill or immobilize 50% of the organisms (LC_{50}) after 24 and 36 h exposure (Favilla *et al*., 2006). Determination of LC_{50} at 36 h was preferred over LC_{50} at 48 h because mortality of *Artemia* naupli (larvae) at 48 h showed high variability in both treatments and controls, possibly as a consequence of starvation. This exposure time was also adopted for *Daphnia* in order to allow for comparison of the data. *D. magna* generally showed higher sensitivity and reproducibility than *A. salina*. LC_{50} values at 36 h were in the ranges of 2–20 μM for *A. salina* and 0.3–20 μM for *D. magna*. In *D. magna* bioassays, a lower variability in mortality response and less marked differences between the 24 and 36 h responses were observed compared with *A. salina*. This was probably due to the use of more standardized materials for the *D. magna* assay. As the test animals hatching from ephippia (cysts) were of similar age, genotype and physiological conditions, test variability was greatly reduced. On the other hand, *A. salina* cysts are easily available commercially and inexpensive and hence this assay may be useful in situations where rapidity and low cost make it practical to test large number of samples for preliminary toxicity screening.

Pernfuss *et al*. (2004) showed (Table 17.2) that *P. caudatum* was more sensitive to the fungal metabolite oosporein than hamster tumour cells, baby hamster kidney cells (Wainwright *et al*., 1986), selected human promyelocytic HL-60 cells (Semar, 1993) and epithelial lepidopteran SF-9 insect cells (RAFBCA report, unpublished results). In contrast to these findings, data presented by Amiri *et al*. (1999), Gierner (2005), Skrobek and Butt (2005) and Skrobek *et al*. (2006) showed that paramecia are not as sensitive as insects (i.e. *Phaedon cochleariae*) or insect cell lines (lepidopteran SF-9) to the three destruxin (Dtx) types Dtx A, B and E.

In 'spot tests' (short time biotests, 2–4 h) oosporein was administered to paramecia in small volumes (drops of 20 μl) (Stemer, 2004). The organophosphate insecticide chlorpyrifos (Agritox®) was also tested as a positive control. Growth tests with *P. caudatum* resulted in an inhibition of growth at concentrations of oosporein between 1 μM and 5 μM. Stemer (2004) reported that *P. caudatum* was more sensitive than hamster tumour cells and selected human and insect cell lines to oosporein. However, limitations in the use of this test system were recognized because of great differences in the sensitivity to the different metabolites that were tested. The LC_{50} of oosporein to paramecia after 2 h of exposure was 750 μM. When paramecia were exposed to the culture filtrate of the MBCA (*B. brongniartii*) containing oosporein, the calculated LC_{50} was 1100 μM. Gierner (2005) exposed *P. caudatum* to Dtx A, B and E under the same standard conditions. The ciliates survived to concentrations up to 25 mM of Dtx E and approximately 6 mM of Dtx B, but were killed at just over 9 mM of Dtx A. The LC_{50} of Dtx A against *P. caudatum* was 9.87 mM (Table 17.2).

Skrobek *et al*. (2006) adapted the BACTOX test system using *T. pyriformis* to assess the toxicity of crude extracts from cultures of the fungal MBCA *M. anisopliae*, and of two major fungal metabolites, namely Dtx A (from *M. anisopliae*) and elsinochrome A (ELA, from *Stagonospora* spp.). *T. pyriformis* was highly sensitive to all of the tested extracts and compounds except Dtx A. In general, ELA, with an LC_{50} value of 5.2 μM, was the compound most toxic to *T. pyriformis*, whereas Dtx A had a LC_{50} value >100 μM after 4 h incubation. Moreover, the authors compared the protozoan test system with the bacterium *Pseudomonas syringae* and two cell line systems under the same test regime (Table 17.2). They concluded that all the above test systems were suitable for assessing the toxicity of fungal metabolites and crude extracts from fungal BCAs.

A comparison of RAFBCA results with cytotoxicity and animal test data retrieved from the literature is also presented in Tables 17.1 and 17.2. In general, even if different cell lines exhibit different sensitivity to one particular metabolite, the CC_{50} values for alamethicin (from *T. viride*) and gliotoxin (from *G. virens*) in the SF-9 model, one of the most sensitive cell lines among those tested, were in the same order of magnitude as the LC_{50} values for *A. salina* or *D. magna* (Table 17.1). Interestingly, in the

case of gliotoxin, the invertebrates seem to be even more sensitive than the two human cell lines, HL-60 and U-937, for which cytotoxicity data are available (Macchia *et al*., 2003). The low cytotoxicity of oosporein assessed with *P. caudatum* (LC_{50} = 750 µM) was consistent with the results of cytotoxicity tests carried out with human and insect cell lines (Wainwright *et al*., 1986; Abendstein and Strasser, 2000). *T. pyriformis* was very sensitive to ELA and as sensitive as the human HL-60 cell line (Skrobek *et al*., 2006). Dtx A had low toxicity to both *T. pyriformis* and the HL-60 cell line after either 4 or 24 h exposure, while it was cytotoxic to the insect SF-9 cells only after a relatively long (24 h) exposure (Skrobek *et al*., 2006).

As a whole, our experience with *A. salina*, *D. magna*, *P. caudatum* and *T. pyriformis* bioassays indicates that these invertebrate models, which are especially relevant to the assessment of the ecotoxicity of the bioactive metabolites of MBCAs, may also be of use for preliminary evaluation of the cytotoxicity of these compounds to vertebrates (Strasser *et al*., 2008; Strauch *et al*., 2011). In addition, these assays have the virtue of being inexpensive, reproducible, easy to carry out, do not require the maintenance of cell cultures and may have an interesting predictive value for the toxicity of the metabolites tested to animals. Skrobek *et al*. (2006) argued that the use of *T. pyriformis* or *D. magna* for the toxicity assessment of BCA metabolites may be not practical because of the low throughput and high cost due to the high amounts of test substance required. However, commercial test kits available for *D. magna*, or currently available protocols, can be adapted to reduce the amount of active compound needed for each assay and so, consequently, reduce the per test cost (Favilla *et al*., 2006). In addition, invertebrate test systems also proved to be suitable for testing crude extracts of fungal cultures (Altomare *et al*., 2004; Skrobek *et al*., 2006), which contain a mixture of bioactive metabolites and can be produced easily at low cost. It is conceivable that the toxicological risk associated with a particular MBCA may be better foreseen by assaying mixtures of metabolites with possible additive or synergistic effects (like those in crude culture extracts) on test organisms characterized by sensitivity to a large spectrum of different molecules, instead of assessing the toxicity of single metabolites, which are difficult to identify and expensive to purify.

Based on this approach, *A. salina*, *D. magna*, *P. caudatum* or *T. pyriformis* bioassays could be used for inexpensive and rapid screening of new candidate MBCAs or their culture extracts at an early stage of development. If appreciable toxicity is found, it might be decided not to proceed with the development of an MBCA before any toxicity testing is carried out in appropriate vertebrate models.

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Appendix 1: *Paramecium* sp. – Acute Toxicity Test

Specific scope

This standard provides a protocol for an acute toxicity test with secondary metabolites of fungal biocontrol agents using *Paramecium caudatum* (Ciliophora: Oligohymenophorea, Urocentrida), and addresses the specific needs for the evaluation of secondary metabolites derived from fungal biocontrol agents.

Introduction

According to the opinion of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) this RAFBCA Standard was based on the protocol of how chemicals were assessed regarding their potential effects and risks on terrestrial ecosystems (CSTEE, 2000).

According to CSTEE (2000), the criteria used to decide the acceptability of environmental risks are generally based on the concept of 'Toxicity Exposure Ratio' (TER). This ratio should be calculated for each of the environmental compartments at risk so as to choose critical thresholds as triggers for the need of further information. TERs may also be used for making comparisons with appropriate 'safety factors' representing the acceptable limit for the different components of the environment in terms of risk. A feasible approach is the ranking of chemicals in terms of their environmental hazard by specified criteria. In general, the proposed systems are based on the development of a score for a set of physicochemical properties and their toxicological and ecotoxicological potential of the substances considered. Many examples of risk indices have been published. The indices are fully based on the information required by Annex VI of Directive 91/414/EEC for placing plant protection products on the market (OJEC, 1991; later amended by Council Directive 2005/25/EC for plant protection products containing micro-organisms; see OJEU, 2005). Different indices have been developed for (i) the hypogean soil system, (ii) the epigean soil system, and (iii) water ecosystems. For each system, two different time–space scales are considered. The short-term indices at local scale refer to a risk posed by a pesticide immediately after a treatment. On the contrary, other indices, in a broader time–space scale context, are finalized to evaluate the pesticide impact in a medium period and in a wider area than the treated one.

Dose–response analysis

Most ecotoxicity tests are performed with several exposure levels to allow the assessment of the dose–response relationship.

Acute toxicity data are usually described with a log-logistic or log-normal curve, after which an EC_{50} or LC_{50} is determined: the calculated exposure at which a 50% effect (or lethality) is observed.

Specific approval and amendment

First approved in March 2004.

Acute toxicity test for secondary metabolites from fungal biocontrol agents

Principle of the test

Paramecium caudatum Ehr. or *P. tetraurelia* Sonneborn cells grown at standardized conditions are exposed to the test substance at a range of concentrations for a period of 2–4 h (see also OECD Guidelines For Testing Chemicals. Test No. 202: *Daphnia* sp. Acute Immobilisation Test; OECD, 2004). Immobilization is recorded every 15 min and compared with control values. The LC_{50} at 2 h is calculated.

Information on the test substance

The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of detection should be available.

Useful information includes the structural formula, purity of the substance, stability in water and light, lipophilicity (log $P_{oct/wat}$; see Fujita *et al.*, 1964; Leo, 1993) and the results of a test for ready biodegradability.

Reference substances

A reference substance (positive control) should be tested for LC_{50} as a means of assuring that the test conditions are reliable.

Description of the method

EQUIPMENT. Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Test vessels will normally be glass test tubes or beakers and cavity slides; they should be cleaned before each use using standard laboratory procedures. Test vessels should be loosely covered to reduce the loss of water due to evaporation and to avoid the entry of dust into the solutions. For the toxicity test, paramecia exposed to the solutions are incubated using a closed humidity chamber.

Determinations of whether paramecia are dead or alive are made using a dissecting microscope.

TEST ORGANISMS. As already noted, the species *P. caudatum* or *P. tetraurelia* are used in this test. The strains should be obtained from a public/quotable culture collection or be physiologically described in literature. Paramecia must be cultured under sterile and standardized conditions (Mayr, 1956).

Enterobacter aerogenes Hormaeche and Edwards (one medium-sized colony) is incubated in salad extract medium for 24 h under sterile and standardized conditions before being fed to the paramecia.

REARING PARAMECIA. Paramecia are cultured in 10 ml glass tubes with a medium volume of 6 ml at 25°C. Every 4 days a volume of 2 ml of the *Paramecium* culture is exchanged for the same volume of salad extract medium containing *E. aerogenes*. Any deficiency caused by evaporation is provided in addition. Bacteria are pre-grown in 200 ml of salad-extract medium in a 500 ml conical flask. Incubation is done at 30°C for 24 h at 200 rpm (Pöder, 1982).

DILUTION WATER, MEDIUM OR BUFFER. Any suitable water, either tap water or reconstituted water, or dechlorinated tap water, are acceptable as dilution water if the paramecia survive in it for twice the time of the test duration without exhibiting signs of stress. If test substances are not water soluble, a buffer must be chosen which fulfils the above-mentioned criteria for the survival of paramecia. An appropriate solution is 0.1% (w/v) $NaHCO_3$. Water, medium or buffer should be of constant quality during the test period. In order to check the chemical quality of the water, the quality parameters should be measured at least twice a year. If dechlorinated water is used, daily chlorine analysis is desirable.

TEST SOLUTIONS. Test solutions with the chosen concentration of the test substance are usually prepared by dilution of a stock solution. As far as possible, the use of solvents, emulsifiers or dispersants should be avoided. But, such compounds may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol and methanol. When a solvent is used, it must not have a significant effect on the survival of the paramecia nor cause a visible adverse effect. This has to be confirmed by a solvent-only control.

Procedure

CONDITIONS OF EXPOSURE. The cavities of clean slides are filled with 20 µl of the test substance solution. A 20 µl drop of *Paramecium* culture is added. At least 30 animals, preferably divided into three groups of ten animals each, should be used at each test concentration and for the controls. One test medium control series and also, if relevant, one control series with the solvent should be run in addition to the treatment series.

TEST CONCENTRATION. A range-finding test should be conducted to determine the range of concentrations for the definite test. For this purpose, the paramecia are exposed to a series of widely spaced concentrations of the test substance. A minimum of ten paramecia should be exposed to each test concentration for 4 h or less, and no replicates are necessary. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. At least five test concentrations should be used. They should be arranged in a geometric series with a dilution rate factor of two.

Justification should be provided if fewer than five concentrations are used. The highest tested concentration should preferably result in 100% mortality. The lowest concentration tested should preferably give no observable effect.

INCUBATION CONDITIONS. The temperature should be in the range of 18–22°C. To avoid desiccation, incubation is done in a moist chamber.

DURATION. The test duration is 4 h. Each cavity should be checked for dead paramecia at 15 min intervals up to 240 min. Checking is done under a dissecting microscope with a minimum magnification of ×16. In addition

to death, any abnormal behaviour or appearance of the animals should be noted.

ANALYTICAL MEASUREMENTS. The concentration of the test substance should preferably be measured at each concentration at the beginning and end of the test. It is recommended that results are calculated based on measured concentrations. If it can be demonstrated that the concentration of the test substance has been satisfactorily maintained within ±20% of the nominal initial concentration throughout the test, then the results can be based on nominal values.

Data and reporting

DATA. Data should be summarized in tabular form, stating the total number of cells and the number of dead paramecia for each observation in treatments and controls. The percentages of mortality for each observation are plotted against the tested concentration. Data are analysed by appropriate statistical methods (e.g. probit analysis; see Sakuma, 1995; Throne *et al*., 1995) to calculate the slopes of the curves and LC_{50} with 95% confidence limits ($P = 0.05$). Where the data obtained are inadequate for the use of standard methods for calculating the LC_{50}, the highest concentration at which no paramecia are killed, and the lowest concentration resulting in 100% mortality, should be used for an approximation for the LC_{50}; in that case, the LC_{50} value is estimated by the geometric mean of these two concentrations.

TEST REPORT. The test report must include the following:

- Test substance:
  - physical nature and relevant physical-chemical properties;
  - chemical identification data, including purity.
- Test species:
  - origin of the paramecia, culture conditions used (including food source, feeding amount and frequency of feeding).
- Test conditions:
  - test procedure: final volume of tested solution in cavity, number of paramecia per test, number of replicates per concentration, any treatment of cavity slides, preparation of the test substance solution, and, if used, data on solvents, pH and temperature;
  - dilution water: source, and chemical and physical characteristics.

RESULTS. The presentation of results must include the following:

- Data on the number and the percentage of dead paramecia and/or of paramecia negatively affected, in the controls and in the treatment groups, at each observation time; add a qualitative description of the effects observed; date of test and results from experiments with reference substances, if available.
- The nominal test concentrations and the result of all analyses conducted to determine the concentration of the test substances in each test; the recovery rate of the method and the limit of detection should also be reported.
- Details of all physical-chemical measurements made during the test.
- The LC_{50} at 2 h, with confidence intervals and graphs of the fitted model used for their calculation, the slopes and dose–response curves and their standard error; statistical procedures used for determination of $LC_{50.}$
- An explanation for any deviation from the standard protocol.

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18 Assessing Genotoxic Effects of Microbial Products

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18.1 Introduction

In almost every aspect and activity of our life, for good or bad, microbes and their products play an important role. They make the soil fertile; they clean up the environment; they produce vitamins and antibiotics; they improve food; they may even protect us from less desirable microbes; and they are exploited by industry for their biotechnological potential (see Sundh *et al.*, Chapter 1, this volume). Microbes, and more particularly fungi, on which emphasis will be placed in this chapter, can be found in every habitat, parasitize any organism and decay all possible materials. So, in several cases, they are associated with undesirable side effects, such as food spoilage and human, animal and plant diseases – reasons that make them generally regarded as nasty and unpopular.

Unfortunately, it is under the latter circumstances that they mainly attract people's attention and thereby create fears for toxicity, disease and even deadly events! For almost eight decades, chemicals were exclusively used in the protection of crops against pests and they have been been applied in most cultivated land throughout the world. However, because chemical pesticides have repeatedly been proven to be mutagenic and carcinogenic (various European Union (EU) guidelines; Ruiz and Marzin, 1997), the need for environmentally friendly and safe alternatives is continuously increasing. This was realized early enough by the European Commision which,

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through the Common Agricultural Policy (CAP), which aimed towards a sustainable agriculture in a global environment, and introduced criteria of quality control, taking responsibility for the countryside and keeping consumers confident about food safety (European Commission, 2008). Through recent years, both bacteria and fungi have demonstrated several advantageous properties over chemicals when used for crop protection and are considered to be a relevant and safe replacement of (at least) part of the large quantities of chemicals used (Strasser *et al*., 2000; Hunter, 2009). Thus, the application of microbial biological control agents (BCAs), known as MBCAs, which are based on microorganisms is expanding and gaining acceptance from scientists, regulators and farmers.

The best known and most successful microbial product, which has been exploited commercially for almost 50 years, is the bacterium *Bacillus thuringiensis* (Bt), with its toxins and the genes (*cry*) that produce these toxins (Flexner *et al*., 1986). Bt and its toxins, either used solely or genetically incorporated into transgenic crops, are only effective against insects within certain families and not others (for reviews see McClintock *et al*., 1995; Glare and O'Callaghan, 2000; Kumar *et al*., 2008; and references therein). In that sense, recent reports on the develoment of insect resistance to Bt in transgenic plants, even under conditions that are designed to delay this resistance, such as the refuge strategy, strongly indicate that crop protection cannot solely depend on this bacterium and its toxins (Tabashnik *et al*., 2008), and other solutions are also being sought. As several species of fungi have lately been used as MBCAs, an emphasis will be placed in this chapter on fungi and their products, as they comprise a benign alternative for the protection of crops worldwide (Butt *et al*., 2001).

A well-established fact is that many fungi produce and secrete mycotoxins (i.e. natural secondary metabolites of diverse chemical structure) which provide them an edge of survival through: (i) symbiosis and syntrophism; (ii) antagonism or competition for nutrients within an ecological niche; and (iii) pathogenesis (i.e. the ability to weaken and/or kill their host) (Vey *et al*., 2001; Mayer *et al*., 2008). In addition, there are several fungal metabolites that are highly toxic (e.g. fumonisins, ochratoxins, patulin, zearalenone) or carcinogenic (e.g. moniliformin, aflatoxins). These pose serious risks to human and animal health when foods are contaminated by the saprophytic fungi that produce them (Abramson, 1998; Strasser *et al*., 2000). Until now, several bodies of legislation and directives have been implemented that provide details of the requirements for authorization and commercial exploitation of microbial products. These directives aim to guarantee that the products or the microorganism that produces them have no immediate or delayed harmful effects on humans, non-target organisms and the environment itself. In addition, they satisfy criteria which verify that there is no impact on the existing biodiversity and the ecosystem to which they will be applied, e.g. OECD (2008); and European Council Directive 1107/ 2009 (OJEU, 2009), amending Council Directive 91/414/CEE (OJEC, 1991). So the putative exploitation and commercialization of any MBCA raises two important questions: (i) are hazardous metabolites produced by a specific strain used as an MBCA – uniquely or in combination with other microbes; and (ii) do the microbes and their metabolites pose any risk for humans and environment?

The first question is easily answered by the numerous reports on metabolites that have been isolated from pure microbial cultures of MBCAs (examples are given in Table 18.1). However, it is interesting to note that even species from different ecological habitats, such as entomopathogenic and saprophytic fungi, may produce similar compounds (Mayer *et al*., 2008).

The second question hits the heart of the problem because a major obstacle in the registration process and the subsequent commercialization and exploitation of a microbial product is the assessment of its risks and of the metabolites that it produces. So far, it is known that some of the metabolites produced by MBCAs are toxic *in vitro* to animal cell lines (Vey and Quiot, 1989; Dumas *et al*., 1996; Fornelli *et al*., 2004), whereas other metabolites have antibiotic, fungicidal, insecticidal or antiviral properties (Terry *et al*., 1992; Amiri *et al*., 1999; Kershaw *et al*., 1999; Bandani *et al*.,

Table 18.1. Fungal microbial biological control agents (MBCAs), their target hosts and metabolites, and references to work on these. Details of saprophytic fungal genera producing the same metabolites, with relevant references, are included in parentheses.

Fungal strains	Target host	Major metabolites	References
Beauveria bassiana (*Fusarium*)	Insects	Beauvericin, bassianin, bassianolide, tenellin	Hamill *et al.*, 1969; El-Basyouni *et al.*, 1968; Suzuki *et al.*, 1977; Wat *et al.*, 1977; (Moretti *et al.*, 1995)
Beauveria brongniartii (*Chaetomium*)	*Melolontha melolontha* (Coleoptera)	Oosporein	Abendstein *et al.*, 2000; (Cole *et al.*, 1974)
Gliocladium fimbriatum (*Aspergillus*)	Fungi	Viridin, gliovirin, glisoprenins, heptelidic acid, gliotoxin	Taylor, 1986; Di Pietro *et al.*, 1993; (Nieminen *et al.*, 2002)
Gliocladium catenulatum	*Pythium* sp.	Presumed as for *G. fimbriatum*	
Lecanicillium spp., formerly *Verticillium lecanii*	Insects	Destruxins, dipicolinic acid, hydroxycarboxylic acid, cyclosporin	Claydon and Grove, 1982; Patrick *et al.*, 1993
Metarhizium anisopliae (*Alternaria*)	Insects	Destruxins A, B, D, E (from the over 28 types), swainsinone, cytochalasin C	Pais *et al.*, 1981; Wang *et al.*, 2004; (Ayer and Pena-Rodriguez, 1987)
Stagonospora convolvuli	Weeds	Elsinochrome A, leptosphaerodione, cercosporine	Ahonsi *et al.*, 2005; Boss *et al.*, 2007
Trichoderma harzianum	*Pythium* sp., *Rhizoctonia* sp., *Sclerotinia* sp.	Harzianic acid, alamethicin, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoilactone	Wiest *et al.*, 2002

2000; Vey *et al.*, 2001). There are many points that still need to be addressed before a microbial product is considered safe or, alternatively, whether its metabolites are genotoxic and/or mutagenic (European Council Directive 1107/2009). These include:

- Under which conditions should the microbial products be screened?
- What would the appropriate screening protocols be?
- What is the mode of action of a metabolite produced?
- What quantity of this metabolite is needed to be genotoxic or cytotoxic?
- How are assays selected in order to evaluate metabolites?
- How should environmental risks be assessed?

Therefore, an attempt will be made in this chapter to present, in a comprehensive way, information on the available battery of tests and appropriate assays that can determine whether or not a microbial product has genotoxic and cytotoxic effects, and also to examine the various parameters that may affect the final judgement made. Particular emphasis is placed on fungal products, as these are the least studied and data on their effects and potential risk are still limited, but are urgently needed for their registration and commercialization.

18.2 How Are Genotoxicity Data Used in Safety Assessment? Detection of Putative Toxicity and Carcinogenicity of Metabolites Produced by Microbials

Genetic toxicity evaluation relies primarily on the use of *in vitro* tests for the detection of

agents that cause mutations and chromosomal aberrations of the DNA and, consequently, affect its expression. Because *in vitro* tests are used as the cornerstone of genotoxicity testing, it is necessary to correctly interpret *in vitro* findings in relation to the potential *in vivo* risk of genetic damage in a living organism. Yet, as experience shows, the interpretation of results from *in vitro* genetic toxicity testing is usually very subjective, and experts often evaluate the same set of test results differently. This clearly increases the risk for making the wrong decision, especially in cases where conclusions rely on minimal additional information beyond the genetic toxicity data. As a result, knowledge of the microbe and the metabolites it produces is of crucial importance for its risk assessment. Each case is somewhat different from another and it is always important to consider all the available information for decision making (Dearfield *et al*., 2011). Nevertheless, if *in vitro* assays indicate the lack of any mutagenic or genotoxic potential of a microbial product or of its secondary metabolites, then no further action is needed. However, additional evaluation will be required if ambiguous or, even worse, positive results are obtained from the primary *in vitro* assays (Gatehouse *et al*., 1994).

Apart from a good understanding of a MBCA itself, particular attention must be placed on whether genotoxicity tests have been performed only with pure metabolites – putatively produced by the microbes in scrutiny – because such tests may lead to wrong conclusions, as it is often a synergistic effect that renders a substance mutagenic or genotoxic (Kouvelis *et al*., 2011). As shown in Table 18.1, fungi produce several different secondary metabolites which are secreted simultanously into their environment. Their mode of action, the interactions among them and with other substances in the environment, the amounts in which they are produced and secreted, and their persistence in the field, may differ a lot in nature compared with laboratory conditions and, until now, little is known about all these parameters.

Therefore, it is very important that any confounding factors are taken into consideration and are included in the testing and interpretation of results. For example, the conditions applied (e.g. pH and osmolality other than those under normal conditions), the possible interactions with the other substances besides the one examined (such as the medium used for the assay), the involvement of a metabolic activation system (such as the S9 homogenate – see Section 18.2.2, and other cofactors), and the possible genotoxic effects of any impurities are some of the parameters that need to be considered (for reviews see Mayer *et al*., 2008; Dearfield *et al*., 2011). Crude extracts from the microorganism may provide an important alternative to pure metabolites, because, under natural conditions, mixtures of metabolites are secreted by microbes and it is often unclear to what extent synergistic effects account for toxicity (of, for example, mycotoxins). In addition, crude extracts provide the advantage of including in the assays metabolites that have not been properly characterized as they may be present in low concentrations or difficult to isolate owing to the domination of other major metabolites. Finally, the ease by which the crude extracts can be produced, even in large quantities, renders them accessible to many laboratories at low cost, and thus provides material for a reliable quick test for any toxicogenic effect on standard tester organisms (Kouvelis *et al*., 2011).

The genotoxic activity of a secondary metabolite may result from either a direct or an indirect effect on the genetic material. A direct mode of action indicates that DNA is the primary target for the substance (i.e. resulting in DNA adducts, thymidine dimers and, in some cases, strand breaks), while an indirect mode of action has primary targets other than DNA. In the latter case, the DNA is being secondarily damaged via free radicals, reactive oxygen or nitrogen species, nucleotide pool imbalance, spindle disruption, or the inhibition of DNA synthesis or topoisomerases. In both cases, the end result is still a genotoxic insult and possible genetic alteration. Distinguishing the mode of action of a metabolite is very important because the type of assay used for screening of the metabolite may otherwise provide false results. For instance, the bacterial reverse mutation assay (Ames test) detects gene mutations (Mortelmans and Zeiger, 2000), while a chromosome aberration

assay, such as the single cell gel electrophoresis assay (SCGE) is appropriate when the compound causes structural and/or numerical chromosome damage (Fairbairn *et al*., 1995). As an example, when gliotoxin, a secondary metabolite of *Aspergillus fumigatus*, was examined with the two test systems mentioned above, at high concentrations it showed genotoxic activity with the SCGE test but not with the Ames test (Nieminen *et al*., 2002).

For all the above reasons, a large number of assays have been (and are being) developed or transformed from previously existing assays suitable for chemicals in order to assess fully and without doubt the potential risk of MBCAs (Skrobek *et al*., 2006). Consequently, bioassays, biosensors, and *in vitro* and *in vivo* tests based on a wide array of organisms as test strains are continuously being developed in order to cover every possible mode of action of secondary metabolites and simultaneously exploit the different sensitivity which each assay presents (Gatehouse *et al*., 1994; Kirkland and Speit, 2008; Dearfield *et al*., 2011; Lynch *et al*., 2011).

18.2.1 Bioassays and biosensors

A bioassay is used to quantify the amount of a substance an organism can be exposed to before adverse effects are observed. In bioassays, this potential for adverse effects is estimated by exposing a biosensor to the substance. A biosensor consists of a sensitive biological element (biological material, tissue, microorganism, organelle, cell receptor, antibody, enzyme, nucleic acid, etc.). Both bioassay and biosensor must provide reliability and allow a clear determination of the effects of the agent/product under scrutiny. Common biosensors include sensitive cell lines from different organisms, such as mammals and insects (Odier *et al*., 1992; Fornelli *et al*., 2004), microbes (bacteria – Girotti *et al*., 2008; yeasts – Miloshev *et al*., 2002), plants (e.g. *Lemna minor* – Richard *et al*., 1987), and invertebrates, such as *Artemia salina* (Favilla *et al*., 2006; see also Altomare *et al*., Chapter 17, this volume), *Daphnia magna* (Favilla *et al*., 2006), *Mysodopsis bahia* (Genthner *et al*., 1994) and *Paramecium caudatum* (Sako *et al*., 1978).

18.2.2 Generally approved tests for the identification of genotoxic, mutagenic and carcinogenic substances

Regulatory agencies such as the US Food and Drug Administration (US FDA), International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Committee on Mutagenicity (COM) and Health Protection Branch of Canada have laid down recommendations for the minimal number of tests required in order to assess the possible genotoxicity, cytotoxicity and mutagenicity of a specific compound. Genotoxicity testing usually involves at least two different end points at several levels of biological complexity, and typical testing assays are performed using a living organism, either a prokaryote or eukaryote (Kroes, 1995; Dearfield *et al*., 2002). The agencies, based on experiences with chemicals, require that at least two or three assays are included: (i) a test for gene mutation in bacteria; (ii) an *in vitro* test in mammalian cells for chromosome damage; and (iii) an *in vivo* test for chromosomal damage (OJEC, 1991; Health Protection Branch (Canada) Genotoxicity Committee, 1992; Sofuni, 1993; COM, 2000; US FDA, 2006; OECD, 2007; ICH, 2011). In other words, assays should address two types of genetic damage: (i) gene mutations; and (ii) chromosome damage.

Because of the ease of handling and using prokaryotes, the low cost of experiments and the speed of toxicological prescreening, bacteria-based short-term assays such as the Ames test are widely used to identify materials that induce genetic damage leading to gene mutations (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). A number of alternative tests, such as the umu/SOS gene test (Odam *et al*., 1985), the Vitotox assay (van der Lelie *et al*., 1997) and the Toxi-Chromo test (Bitton and Koopman, 1992) have also been used by many researchers for genotoxic and cytotoxic assessment of mycotoxins and secondary metabolites (e.g. Odier *et al*., 1992; Nieminen *et al*., 2002; Fornelli *et al*., 2004; Skrobek *et al*., 2006; Kouvelis *et al*., 2011). However, regulatory authorities are still reluctant to include these tests as the sole requisite in their registration

procedures for both microbials and chemicals, as most prokaryotic genotoxicity assays seem to have some inherent limitations. For example, a natural characteristic of bacteria is that they are potentially comparatively resistant to many toxic compounds. Additionally, some genotoxic compounds become toxic only after they enter the body and are processed in the liver, therefore they can be easily overlooked in tests based on prokaryotic systems (Liu *et al*., 2008; Dearfield *et al*., 2011). This hurdle can be overcome by the addition of a crude extract of enzymes from homogenized livers of rats previously treated with Aroclor to enhance liver enzyme activity, to which enzyme cofactors are added (generally known as the S9 mix). Another shortcoming, not only restricted to bacteria-based assays, but also affecting other genotoxicity assays, is that the tests simply report whether any DNA damage has occurred but do not offer any information on the potential mode of action that led to this damage (Kroes, 1995). Conversely, the most commonly used eukaryote-based assay is the Comet assay (single-cell gel electrophoresis), a method for measuring DNA strand breaks in eukaryotic cells (Singh *et al*., 1988). Because of its sensitivity, versatility and accuracy, this test has been applied to genotoxicity testing, human biomonitoring and molecular epidemiology and ecogenotoxicology studies, as well as being used in fundamental research on DNA damage and repair (Tice *et al*., 2000; Brendler-Schwaab *et al*., 2005). However, the disadvantage of eukaryote-based assays is that they are expensive, time-consuming and often associated with complicated protocols (Rusling *et al*., 2007).

Recently, the Organisation for Economic Co-operation and Development (OECD) presented a document on the evaluation of microbials used specifically for pest control, taking into account five issues that may present barriers to the introduction of an MBCA (i.e. taxonomy of the organism at strain level, its putative genetic toxicity, exposure of operators and consumers, the possibility of residual superinfection from the MCBA in treated food crops and its efficacy evaluation; OECD, 2008). For all the above possible barriers to market introduction, the solution that the agency proposes is a case-by-case study with exploitation of the relevant literature and bridging studies wherever this is possible, as well as a thorough study of all parameters from at least two independent laboratories so that conclusions can be solid and well substantiated.

Common in vitro *genetic toxicity assays*

AMES *SALMONELLA*/MICROSOME MUTAGENICITY ASSAY (AMES TEST). Undoubtedly, the Ames *Salmonella*/microsome mutagenicity assay still remains the most accepted and widely used *in vitro* assay for the detection of genotoxicity. It is a bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage which leads to gene mutations (Mortelmans and Zeiger, 2000). The test uses a number of histidine auxotrophic *S. typhimurium* strains carrying different point mutations in genes of the histidine operon. Standard recommendations call for testing of chemicals in strains TA98 and TA100, with and without S9 mix as an external enzymatic metabolizing system. The auxotrophic mutations can act as hot spots for mutagens and revert the bacterium to its prototrophic state. The use of different auxotrophic strains increases the sensitivity of the test because some (e.g. TA1535) detect mutagenic events that others (e.g. TA100) fail to detect. The number of spontaneously induced revertant colonies per plate is relatively constant, but when a mutagen is added to the plate, the number of revertant colonies per plate is increased, therefore indicating a possible genotoxic activity (for reviews see Maron and Ames, 1983; Mortelmans and Zeiger, 2000).

So far, the Ames assay has been applied in a few studies concerning fungi and their secondary metabolites, e.g. destruxins (from *Metarhizium anisopliae*) (Genthner *et al*., 1998) and gliotoxin (Seigle-Murandi *et al*., 1990; Nieminen *et al*., 2002). No mutagenicity for either substance was detected by the use of *S. typhimurium* strains TA98 and TA100. In the former study, the destruxins used were partially purified and appeared to have no toxicity on some test organisms (e.g. mysids), but some toxicity towards others (e.g. developing

grass shrimps and mosquito fish) (Genthner *et al*., 1998). Gliotoxin was also tested with the SOS-chromotest and the bacterial repair assay (Nieminen *et al*., 2002) and showed no mutagenicity in the former but genotoxicity at high concentrations in the latter. Lately, several fungal biological control agents were studied with the whole battery of the Ames test strains and neither pure secondary metabolites nor crude extracts of the MBCAs showed any genotoxicity (Kouvelis *et al*., 2011).

UDS ASSAY. Unscheduled DNA synthesis (UDS) in primary rat hepatocytes is frequently used to assess genotoxicity and predict carcinogenicity (Williams, 1976). The test looks at effects on DNA repair damage (which is reduced by many classes of genotoxic substances) and has been validated with carcinogenic and non-carcinogenic mycotoxins (Mori *et al*., 1984). Primary cultures of rat hepatocytes serve as both the source of metabolic activation for many classes of chemicals and the target cells for measurements of a genotoxic response (Williams, 1976). The importance of the UDS test as a genotoxic assay cannot be ignored as OECD has implemented it as a test of *in vivo* screening of chemicals or physical agents (OECD, 1997b). However, caution should be taken in interpreting results because UDS tests of four different mycotoxins from *Fusarium moniliforme* (fusarin C, fumonisin B_1, moniliformin and bikaverin) indicated that they are non-genotoxic, even though these secondary metabolites have provided indications of possible genotoxicity in other assays (Norred *et al*., 1992).

IN VITRO TEST IN MAMMALIAN CELLS FOR CHROMOSOME DAMAGE: MOUSE LYMPHOMA *tk* ± ASSAY AND *hprt* ASSAY. There are two commonly used *in vitro* mammalian gene mutation assays, the mouse lymphoma assay (MLA) using the thymidine kinase (*tk*) gene as the reporter gene, and assays with various cell lines using the hypoxanthine-guanine phosphoribosyl-transferase (*hprt*) gene as the reporter. Because these two reporter genes detect different types of genetic damage, it is important to understand the properties of both genetic loci. Thymidine kinase, a non-essential enzyme, is part of a system that recycles free thymidine that is eventually incorporated into DNA. When a toxic analogue of thymidine is used, it interferes with DNA metabolism, killing the cell, but if the functional copy of the *tk* gene ceases to function as the result of a mutation, the analogue is not metabolized and is no longer toxic. The *tk* gene, probably because of its autosomal location (i.e. located on a chromosome that does not determine the sex of the organism), detects a full array of genetic damage, including point mutations, deletions, chromosomal rearrangements, mitotic recombination and non-disjunction (Applegate *et al*., 1990; Wang *et al*., 2009). The *tk* mutants generally fall into two categories based on colony size (small and large). Chemicals that are known to induce point mutations but have little or no clastogenicity (i.e. do not promote or produce disruption or breakages of chromosomes) will induce primarily large colony mutants. Chemicals that are known to be clastogens (and have little or no ability to induce point mutations) will induce primarily small colony mutants and will also be negative in the bacterial reverse mutation test. Though the proportion of small versus large colonies is helpful information, it should be acknowledged that most chemicals induce both point mutations and chromosomal changes, and thereby induce both small and large colony *tk* mutants.

The *hprt* gene is located on the non-homologous part of the X-chromosome and controls the enzyme hypoxanthine-guanine phosphoribosyl-transferase, which participates in purine salvage and also catalyses the transformation of purine analogues rendering them cytotoxic to normal cells. If the gene is mutated, the enzyme cannot phosphoribosylate the analogues and cells survive treatment because the analogue is no longer toxic. T-lymphocytes deficient in *hprt* used in the assays detect DNA pair substitutions, inversions and small deletions, but are not efficient at detecting large deletions and cannot detect mitotic recombination (Moore *et al*., 2000). Therefore, if a metabolite is positive in an *in vitro* mammalian assay such as the *tk* or the *hprt* test, but negative in a bacterial assay (e.g. Ames), it could represent a difference between prokaryotic and eukaryotic cells, but it is also

likely that the chemical/metabolite induces chromosomal damage rather than point mutations (Dearfield *et al*., 2011).

Until now, only a couple of studies have employed the *hprt* assay for testing mycotoxins and, as shown for enniatin B (a mycotoxin produced by *Fusarium* species), there was no genotoxic activity up to the level at which this secondary metabolite turned out to be cytotoxic (Behm *et al*., 2009).

Common assays with both in vitro *and* in vivo *application*

MICRONUCLEUS TEST (MN). The purpose of the micronucleus assay (MN test) is to detect modifications of chromosome structure and segregation – both effects associated with carcinogenicity – in a way that leads to the induction of micronuclei in interphase erythrocyte cells. In particular, structural chromosome changes such as breaks and rearrangements that occur in the S phase of cell division, and numerical chromosome aberrations (aneuploidy) that occur in the M phase, cause the formation of small membrane-bound DNA fragments (micronuclei) which are easily detected by microscopy (Fenech and Morley, 1985; Obe *et al*., 2002). The test is performed with different test organisms and cell types, depending on the mode of action of the chemical and the questions asked. It is traditionally performed in mice, either animals or cell lines, and the bone marrow or peripheral blood, or cells in the case of cell lines, are analysed for the presence of micronuclei. In terms of assay validation, the micronucleus assay is already recommended as a default *in vivo* test in many regulatory guidelines (e.g. OECD, 1997a; ICH, 2011). Even though this is an intensive and time-consuming assay, it has the potential to be applied in tissues other than erythrocytes and to have an automated scoring (e.g. flow cytometry or image analysis) (Hayashi *et al*., 2000; Tweats *et al*., 2007).

MN is the second most used assay, after the Ames test, for the genotoxic screening of microbial products and their secondary metabolites. Two interesting examples of the sensitivity of this assay are given from studies concerning the potential genotoxicity of two mycotoxins, beauvericin (Çelik *et al*., 2010) and apicidin with its derivatives (Yoo and Lee, 2005). The MN assay has given opposite conclusions in comparisons with other tests. For example, it gave positive results for beauvericin and weak mutagenic potency for apicidin and its derivatives, while the Ames test showed that neither of these mycotoxins were genotoxic (Fotso and Smith, 2003; Çelik *et al*., 2010). Again, these results underline the necessity of testing a substance with more than one assay, because each of the tests indicates a different mode of action of the metabolites.

'COMET ASSAY' (SINGLE CELL GEL ELECTROPHORESIS). The Comet assay is one of the most popular tests for the detection of DNA damage by electrophoresis in mammalian cells. It detects single- and double-strand breaks, oxidative-induced base damage and DNA–DNA/DNA–protein cross-linking and, therefore, is a rapid and sensitive procedure for detecting genotoxicity (Singh *et al*., 1988; Fairbairn *et al*., 1995). In its most commonly used form, the Comet assay involves lysis with detergent and high salt concentration after embedding cells in agarose so that the DNA is immobilized for subsequent electrophoresis. The Comet tail is a result of a halo of relaxed loops of the DNA pulled to one side by the electrophoretic field. The assay has been developed and modified so that it can be applied to both *in vitro* and *in vivo* systems and, potentially, to any cell type or tissue (reviewed by Collins *et al*., 2008). It has been recommended by the UK Committee on Mutagenicity (COM, 2000) as a follow-up second *in vivo* test and an alternative to the UDS assay. Recent comparative studies of Comet with UDS or transgenic rodent mutation assays have shown that it is the most predictive of the three for *in vitro*-positive rodent carcinogens that give a negative result in the conventional bone marrow MN assay (Kirkland and Speit, 2008). Recently, the effects of beauvericin on kidney epithelial and human leukocytes cells were found to be genotoxic using the Comet assay (Klaric *et al*., 2010).

In vivo *tests according to the ICH guidelines*

Initially, the ICH workgroup produced two guidelines that provided the basis for

genotoxicity testing and assessment of pharmaceuticals in different countries worldwide (Müller *et al*., 2001). More recently, new drafts have been published that combine the original two documents, with the goal of optimizing the standard genetic toxicology battery for interpretation of results and prediction of potential human risk. The latest version is dated November 2011 (ICH, 2011). Concerning *in vivo* testing, ICH proposed that: (i) both rats and mice are valid for the *in vivo* detection of genotoxins; (ii) the bone marrow micronucleus test and metaphase analysis are interchangeable for regulatory purposes; and (iii) when no specific association with a specific sex is observed, male animals are sufficient for the detection of genotoxins. In addition to the above two tests, a micronucleus test in the peripheral blood of mice is also acceptable, as is the mouse spot test according to OECD guidelines (OECD, 1986).

Nevertheless, it should be mentioned here that assessing the 'human risk' or 'potential human risk' of an MBCA solely by testing its metabolitic products based on the harmonization of genotoxicity assays may be highly erroneous, as it is well established that in many cases where the microorganism produces a metabolite in mass cultures in the laboratory, it does not produce this in the environment (Strasser *et al*., 2000).

MOUSE SPOT TEST. The mouse spot test is an *in vivo* test in mice in which developing embryos are exposed to the test chemicals. The target cells in the developing embryos are melanoblasts, and the target genes are those that control the pigmentation of the coat hair/fur. The developing embryos are heterozygous for a number of these coat colour genes. A mutation in, or loss of (by a variety of genetic events) the dominant allele of such a gene in a melanoblast results in the expression of the recessive phenotype in its descendant cells, constituting a spot of changed colour in the coat of the mouse that develops. The frequency of offspring with these spots is compared with that among offspring from embryos treated with the test substance solvent only. The mouse spot test detects presumed somatic mutations in fetal cells, and a substance that induces a biologically significant increase in the number of genetically relevant spots is genotoxic (Russell *et al*., 1981).

18.3 Limitations of Genetic Toxicity Tests

The current standard regulatory test battery generally includes an assessment of genotoxicity in bacterial and mammalian cells *in vitro* together with rodent assays for chromosomal and/or DNA damage. The limitations of these standard tests for predicting chemical carcinogenicity, and in particular the *in vitro* genotoxicity assays, were brought to the attention of the scientific community some time ago (Tennant *et al*., 1987; Zeiger, 2001). Nowadays there is a growing concern that the *in vitro* mammalian tests may be overly sensitive because there are several compounds that prove positive *in vitro* but which do not result in genotoxicity *in vivo* (e.g. rodent carcinogenicity; Kirkland *et al*., 2006). In addition, there are data showing that mammalian *in vitro* cell assays exhibit a higher rate of positive results than other assays (Snyder and Green, 2001; Kirkland *et al*., 2006). Therefore, it seems necessary that additional tests in rodents should be performed before reaching a verdict about a product's genotoxic potential (Lynch *et al*., 2011).

18.4 New Methods and Improvements in Genotoxicity Testing

The guidelines of agencies such as ICH encourage the development of new test systems, as it is obvious that testing for genotoxicity is a complex task which cannot be addressed solely by an *in vitro* or an *in vivo* assay (Kirkland *et al*., 2000; Skrobek *et al*., 2006). Many microbial products are based on either fungi or bacteria, both of which produce secondary metabolites which, in some cases, may present genotoxic activities. Thus, a wide range of technologies, at various stages of maturity, are presently in development and up-to-date results suggest that research within the discipline is robust, with quality science and highly innovative technologies

emerging to address the challenges of genotoxicity (Elespuru *et al*., 2009).

The potential of the emerging or improving assays, i.e. the Toxi-Chromo test, Mutatox and other tests based on luminescent bacteria, the *Pig*-α Gene Mutation Assay, the Flow Cytometry Micronucleus Assays *In Vitro*, the 3D Reconstructed Human Skin Models, the DNA Adductome and the Toxicogenomics approach was recently reviewed by Lynch *et al*. (2011). The most validated and promising alternatives of the existing assays are presented below.

18.4.1 Vitotox assay

Vitotox is based on a *S. typhimurium* strain (TA104 *rec*N2-4) that contains the *lux* operon of *Vibrio fisheri* under the transcriptional control of the *rec*N gene, which is part of the SOS system. Incubation of bacteria in the presence of a genotoxic compound results in the de-repression of the *rec*N promoter, and consequently, in the expression of the *lux* operon. This expression results in light production as a function of genotoxicity (van der Lelie *et al*., 1997).

The Vitotox assay has been used in a recent study for the screening of fungal MBCAs and their metabolites, either as pure substances or as part of crude extracts. It was used as complementary to the Ames test because it provides additional information on the cytotoxicity of the metabolites/crude extracts (Kouvelis *et al*., 2011). It is interesting to note that no genotoxicity was observed for metabolites or crude extracts with either test and, further, that although no cytotoxic activity was observed with pure metabolites such as beauvericin (from *Beauveria bassiana*) and cytochalasin, swainsonine and destruxin A (all from *M. anisopliae*), the Vitotox test detected toxic effects at the higher concentrations of oosporein (from *B. brongniartii*), gliotoxin (from *Gliocladium fimbriatum*) and destruxins B, D and E (from *M. anisopliae*) that were used. The toxicity of these metabolites is not surprising because there have been other reports on their toxic effects on different cell lines (Vey and Quiot, 1989; Jeff and Khachatourians, 1997; Nieminen *et al*., 2002). However, it is very important to point out here that the entomopathogenic fungi used in the above studies produce either none or only traces of the metabolites *in vivo*, and this always in significantly lower quantities (10^4–10^6 times lower) than those secreted in nutrient-rich liquid media (Wang *et al*., 2004; Skrobek *et al*., 2006). Therefore, as mentioned earlier, a crucial factor is not only the assay chosen for the genotoxic screening of the metabolite but also the conditions of growth of the MBCA.

18.4.2 Yeast DEL assay

Yeast-based assays are attracting great interest as genotoxicity assays because they can track (i) gene mutations, (ii) chromosomal changes and (iii) mitotic recombination in organisms such as *Saccharomyces cerevisiae* and *Pichia pastoris* reliably, inexpensively and rapidly. In contrast, bacteria-based tests that lack eukaryotic chromosomes are unable to detect clastogenic and aneugenic events (Chen and Kolodner, 1999; Forsburg, 2001).

In yeasts, the RAD54 protein participates in the recombinational repair of double-stranded DNA breaks together with the RAD51, RAD52, RAD55 and RAD57 proteins. RAD54 interacts with RAD51 and stimulates DNA strand exchange, promoted by RAD51 protein (Krogh and Symington, 2004). In yeast-based assays, the DNA damage-inducible promoter of the *RAD*54 gene is fused to the green fluorescent protein (GFP) gene (Walmsley *et al*., 1997), or the DNA damage-inducible promoter of the *RAD*51 gene is fused to the luciferase reporter gene (Liu *et al*., 2008). The former version of the assay has been developed into a commercially available kit, called GreenScreen (Gentronix, Manchester, UK; Cahill *et al*., 2004). The Yeast DEL Assay (aka the *in vitro* DNA deletion (DEL) recombination assay) is a promising new test with high-throughput potential. In this assay, intrachromosomal recombination events between a repeated DNA sequence lead to DNA deletions which have been shown to be inducible by a variety

of carcinogens, including those both negative and positive in the standard Ames assay (for a review see Brennan and Schiestl, 2004).

18.4.3 Microarrays

In an era where -omics technologies are blossoming, gene expression may be a sensitive indicator of toxicant exposure, disease state and cellular metabolism. It can reveal how cells and organisms adapt to changes in the external environment, provide information about the mechanism of action of toxicants and form a sort of 'genetic signature' from the pattern of gene expression changes. The development of such gene expression signatures would allow fast screening of unknown or suspected toxicants on the basis of their similarity to known toxicants (Lettieri, 2006). This can be achieved with microarrays, which are in essence a collection of microscopic spots of DNA, RNA or peptides (probes), attached to a solid substrate that assays through high-throughput screening differences in chemiluminescence due to probe–target interactions. So a biological product can be tested against hundreds of thousands of different probes in a single step.

DNA microarray technology has been applied to toxicity analyses of natural and anthropogenic substances with yeast, for which whole-genome chips are available. Yeast DNA microchips provide the possibility of monitoring gene expression levels as a function of toxin exposure and, consequently, provide a means to determine the mechanism of toxicity (Lettieri, 2006, and references therein). The tests with this assay of the mycotoxin citrinin provide an example which reflects the essential features of this system: the small volume of yeast culture required for the analysis, high reproducibility of the expression profiles and availability of the massive functional information of genes on the DNA microarray. However, problems which still have to be resolved are the high cost of the assay and the variability in gene expression levels due to different genetic and physiological states (Mizukami *et al.*, 2004; Iwahashi *et al.*, 2007).

18.4.4 Transgenic rodent model

The introduction of a specific reporter gene into the mouse genome and the selection of the rodents that carry that gene as a germ-line insertion provide a newly introduced technology that allows the transgene to be transmitted in a faithful manner to the progeny of the recipient rodent. With this methodology, *in vivo* gene mutation studies may be performed in any tissue. Its main advantage is the lack of selective pressure on mutations and therefore the accumulation of damage over time. As reporter genes, available known markers, such as *lac*Z and *gpt* (guanine phosphoribosyl-transferase), may be used in shuttle vectors (e.g. lambda phage or plasmids) and thus, gene mutations and deletions may be detected and thereby reveal the mechanisms of genotoxicity of the substance under examination. The main disadvantages of the method are the need for multiple dosing and transgenic animals. Also, this is a labour-intensive and time-consuming assay which needs trained personnel. However, the assay has a great potential of identifying the mode of action of metabolites and so it appears to be a promising alternative to the existing battery of assays (Lambert *et al.*, 2005; OECD, 2009).

A summary of all the assays that are currently in use or can potentially be exploited for the screening of metabolites produced by MBCAs, and have been analysed previously, is presented in Table 18.2.

18.5 Further Development of Genotoxicity Testing

Several guidelines issued by regulatory agencies propose genotoxicity assessment of any product (drug, chemical, pesticide, biological product) with a number of assays chosen from the battery of existing methodologies. However, the criteria for making choices and the suitability of each assay are still debated. The main question still remains: 'Are there grounds for improvement?'.

New technologies have shown that improvements can be achieved by: (i) enhancing the sensitivity of tests; (ii) constructing new

Table 18.2. Advantages and disadvantages of different assays which are used or have a potential to be used for the genotoxic screening of the metabolites of microbial biological control agents (MBCAs).

Assay[a]	Type/Target	Advantages	Disadvantages
Ames	*In vitro*/Gene mutation test	Widely exploited, recognized guideline, inexpensive, with high-throughput screening potential	Assay based only on bacteria
UDS	*In vitro*/DNA damage assay	Frequently used, with potential to be fully or partially automated	Acceptability sometimes in question, labour intensive, time-consuming, limited use for cells from tissues other than liver
Mouse lymphoma *tk* ± assay	*In vitro*/Chromosomal aberration test	Applicable to a plethora of cell types with the potential of testing individual cells, provides data on the chromosomal aberration type	Labour intensive, time-consuming, requires personnel with expertise, limitations to the number of cell types evaluated
Cell line *hprt* assay	*In vitro*/DNA damage assay	Detects several types of mutations, even small deletions	Not widely used, often with controversial results when compared with other assays
Micronucleus test	*In vitro* and *in vivo*/ Chromosomal aberration test	Fast and easy to perform assay, has the potential for testing individual cells, has potential for automation	Labour intensive and time-consuming to obtain and analyse results, does not distinguish chromosome breaks from complex rearrangements
Comet assay	*In vitro* and *in vivo*/DNA damage assay	Fast and easy to perform assay, has the potential for testing individual cells, a few cells can provide valid results, has potential for automation	Labour intensive and time-consuming to obtain and analyse results, does not detect mutagens causing strand breaks and/or alkali-based lesions
Mouse spot	*In vivo*/Germ cell assay	Sensitive assay, results easy to analyse	Seldom used, time consuming, large numbers of animals needed
Vitotox	*In vitro*/Gene mutation test	High-throughput assay, extremely sensitive	Not widely accepted yet, assay based on bacteria only
Yeast DEL assay	*In vitro*/Gene mutation and chromosomal aberration test	Inexpensive, fast, detects bost clastogenic and aneugenic events	Not widely accepted yet
Microarrays	*In vitro*/Chromosomal aberration test and gene mutation assay	High-throughput assay, highly sensitive	Still seldom used, high cost, personnel with expertise required, often variation at expression levels of the genes due to other factors than the metabolite tested
Transgenic rodent model	*In vivo*/Germ cell assay and gene mutation assay	Performed in any tissue, lack of selective pressure on mutations, reveals mechanisms of genotoxicity for the metabolite examined	Still seldom used, personnel with expertise required, high cost, needs many animals, labour intensive, time-consuming

[a]See text for details of each assay.

detector strains with improved capabilities; and (iii) aiming for high-throughput assays. Should these factors be addressed succesfully, the assays would be rapid, sensitive, easily reproducible and of low cost. Recent advances suggest that these goals can be achieved with: (i) the selection of the appropriate organism/cell line; (ii) the introduction of new reporter genes/enhancers/sensors (e.g. Lac, Lux, Gal operon, *umu*DC genes); (iii) the introduction of new promoter regulatory boxes in the reporter genes; and (iv) the improvement of the existing promoter efficiency and induction (Lettieri, 2006; Liu *et al*., 2008; Biran *et al*., 2010; Lynch *et al*., 2011).

However, for the precise genotoxic assessment of a new microbial product, the selection and optimization of the test is not the panacea. Several other questions regarding the biological properties of the microbe under evaluation must be addressed, of which the most important are:

1. *The efficacy of the organism involved in the product*. – Key action: encourage selection of microbial strains that are intrinsically poor toxin producers to humans and to other benign organisms. – Impact example: high-toxin-producing strains will not normally be approved.
2. *Knowledge of the profile of metabolites produced and secreted*. – Key action: develop tools and methodologies that will identify and detect trace amounts of toxic metabolites. – Impact example: the ability to trace sources of contaminants in food chain and thus, quantify risk factors.
3. *Knowledge of the conditions that regulate the production of secondary metabolites by the microorganism*. – Key action: develop methodologies and protocols for minimization of harmful toxins during production. – Impact example: reduce risks for the employees and the consumers.
4. *Knowledge of the properties of the metabolites and their mode of action*. – Key action: develop assays and diagnostic kits that will facilitate high-throughput screening and selection. – Impact example: easy and fast identification of the genotoxic substances.

In this way, there will be a scientific basis for the support of health and environmental policy making organizations and committees to really assess the genotoxic properties of a microbial product from the information gathered and the data generated.

18.6 Conclusions

Genotoxic assessment is of significant interest to researchers, regulatory authorities, industries and the public. Until now, several assays have been developed and used for testing the cytotoxic, mutagenic and genotoxic properties of microbial products. However, at present, the risk assessment of microbes for genotoxicity is rather imperfect and there is a need to develop appropriate guidelines and recommendations. The accumulated data strongly suggests that firm conclusions on the genotoxicity and cytotoxicity of microbial products can only be drawn when several different bioassays have been used. Currently available information shows that the majority of metabolites produced by MBCAs are not mutagenic or genotoxic. Even those metabolites that show varying results among assays are often produced by the organisms in trace amounts and only under laboratory conditions of induction, and therefore clearly do not pose any threat for humans or the environment. Although the available assays may provide the requested information, they also have limitations, and consequently a continuous demand exists for the improvement of assays so that they become more sensitive, simpler, easier to handle, faster, accurate, reliable and reproducible.

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19 Assessing the Sensitization and Irritant Properties of Microorganisms

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19.1 Introduction

Occupational skin diseases, including irritation and sensitization, are among the most important risks related to workplace exposure and use of chemical, physical and biological agents. Approximately 4 million working days are estimated to be lost every year owing to absenteeism resulting from work-related skin diseases (English, 2004). In Europe, skin diseases account for 7.1% of all occupational diseases and skin sensitization has the highest incidence rate, at 5.5%, although similar incidence rates have been reported elsewhere in the world (Ale and Maibach, 2008; EU OSHA, 2008). In the UK, 70–90% of all cases of occupational dermatoses between 2002 and 2005 were determined to be caused by a contact dermatitis irritant and/or an allergen (Chew and Maibach, 2006; Turner *et al*., 2007; Sasseville, 2008). The risk of developing an occupational skin disease is present in practically all occupations, being highest in mining and quarrying, at 31.5%, and the lowest in education, at 0.5% (EU OSHA, 2008). A long list of agents could induce contact dermatitis in humans, including chemical substances, chemical mixtures, physical agents and also proteins of natural origins (EU OSHA, 2008; Frosch and Kügler, 2011). Therefore, there is a need to identify the hazards and risks of irritants and allergenic contact dermatitis that may be associated with exposure in the workplace and in the final use of microorganisms. A series of *in vivo* animal tests which were initially designed and recognized as adequate to test the hazards of chemical irritants and sensitizers (Basketter, 2008; Hartung and Daston, 2009) were later included in the testing guidelines for evaluating products

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based on microorganisms, such as microbial biocontrol agents (MBCAs) and biocides (OJEU, 2005, 2006), but without even a formal validation of these procedures for testing microbes. Recently, new methods, such as the murine local lymph node assay (LLNA) for skin sensitization or the Reconstructed Human Epidermis Test Method (RhE) for irritation were adopted in the OECD (Organisation for Economic Co-operation and Development) Guidelines for testing chemicals after a long and complex validation process (OECD, 2010). However, the introduction of products based on live microorganisms has posed questions regarding the relevance of using chemical-based methodologies for human hazard evaluation of living organisms such as bacteria and viruses.

In this chapter, current methods for predicting the effects of microorganisms on skin, as well as available human exposure data, are critically reviewed. Tentative strategies for interpreting experimental data obtained with animal and cellular protocols for assessing the irritation and sensitization hazards of microorganisms are discussed.

19.2 Irritation and Allergic Contact Dermatitis

When we refer to irritation and sensitization, we refer to two different processes that share some common pathways, but show different progressions and have different evolution and consequences. In most cases, they are clinically similar, and show non-specific signs either in the acute and/or chronic phases. As a consequence, the final diagnosis is generally established after a specific medical treatment which solves the dermatitis (*ex adiuvantibus*), or when it is possible to clearly recognize the causative agent, i.e. by patch tests, and remove it or introduce adequate protective measures (*ex nocentibus*). However, caustic burns from corrosive chemicals such as chrome, or allergic hand dermatitis induced by *p*-phenylene diamine (PPD) in hairdressers can be easily recognized as irritative and allergenic, respectively (Chew and Maibach, 2008). Finally, both of these processes may coexist in the same subject, although a number of occupational skin manifestations may mimic contact dermatitis, thus complicating the matter (Frosch and Kügler, 2011).

19.2.1 Irritation contact dermatitis (ICD)

Skin irritation could be defined as a direct insult to the skin surface which occurs quickly after exposure to chemical or physical agents (Rustemeyer *et al*., 2011). The result is a clinically evident lesion that varies from a simple transient effect in the contact area that quickly recovers, up to a severe irritation which could result in large blisters (*bullae*), erosion or ulceration, and leave scars. The skin reaction is usually proportional to the nature of the causative agent or to the exposure condition in an apparent dose–response pattern. The complete spectra of all forms and lesions observed related to irritancy, as well as the endogenous and exogenous factors involved, are too extensive to be reported here, but more details are given by Weltfriend *et al*. (2006). Irritation contact dermatitis (ICD) was considered for years to be a simple *monomorphous* process localized on the skin surface. Recently though, ICD has been recognized as a complex biological syndrome based on skin irritation induced by diverse external stimuli, which leads to an inflammatory response without the production of specific antibodies and/or cell clones (Chew and Maibach, 2008).

Two theoretical models have been proposed to better understand what happens and how skin reacts to irritancy. The first one is based on the assumption that irritancy occurs when the toxic action of the insult exceeds a specific threshold of clinical evidence. After the insult, the skin attempts to repair itself and eventually the irritancy disappears. This model includes skin responses either after a single and strong insult or a series of small ones, both exceeding the threshold. According to this model, specific irritation patterns have specific and different thresholds (Malten, 1981).

While the first model is focused on insult intensity and frequency, the second model is more related to how the skin strata involved determine the type and degree of irritation (Table 19.1). When a chemical is applied on to

Table 19.1. Intensity of irritation due to skin strata involvement.

Skin strata affected	Observable lesion	Sensorial reaction
Skin surface	No sign (subclinical irritation)	Softness, dryness, tightness
Stratum corneum	Dryness (weak irritation)	Dryness, itches, stings
Derma	Erythema (irritation)	Dryness, stings, pain

the skin surface and acts as an irritant, it induces cellular damage in the keratinocytes in the stratum corneum. The damaged stratum corneum increases the permeability for the chemical in the lower strata and results in a more severe irritation. The first level of irritation limited to the surface is responsible for sensory impairment and dryness, while erythema is a consequence of dermal involvement (Simion, 2006).

When the insult is continuous and repeated, the ICD becomes chronic, representing the most frequent form, especially in wet environments and in the presence of weak irritants. Severe chronic ICD leads to hyperkeratosis and eczema which persist for a long time even after removal of the causative agent (Rustemeyer *et al*., 2011).

19.2.2 Allergic contact dermatitis (ACD)

While irritation is determined by damage to the epidermal skin barrier, allergic contact dermatitis (ACD) is a multi-step process leading to a specific immunological response. ACD could be defined as a delayed cell-mediated immunological response which arises following more than one contact with an allergenic agent. At the skin level, ACD is characterized by delayed erythema, oedema and vesicles, all signs of an immune system-mediated reaction. In short, ACD starts with an initial contact with a small (molecular weight usually less than 400) *hapten* that binds to the skin protein and forms a complex. The dermal immune system must be reached by the allergenic complex in order to evoke a response, and when this happens, new exposure after days or weeks provokes dermal effects that are often more severe than the initial one. The first contact between the allergenic complex and the immune system is called the *induction* phase (Marzulli and Maibach, 2008).

In the induction phase, the allergenic complex must reach the inner layers of the stratum corneum and come into contact with the Langerhans cells. These are dendritic cells that are able to initiate a primary T-lymphocyte response in the corresponding lymph node into which they migrate after binding to the allergic complex (Gober and Gaspari, 2008). The stimuli of the Langerhans cells promote specific T-lymphocytes sensitized against the allergenic complex, ending this phase.

After induction, whenever there is a new contact with the allergenic complex, sensitized T-lymphocytes occurring at the site of exposure provoke a cell-mediated inflammatory response at the skin level (type IV allergy after the Gell and Coombs classification), worsening for each exposure occasion, defined as the *elicitation* phase (Marzulli and Maibach, 2008).

In the induction phase, the hapten must penetrate the stratum corneum. Whether it does so is determined by several factors including: the chemico-physical properties of the hapten itself (dimension and lipophilicity); skin surface area, integrity and thickness; exposure time; and, finally, the action of other factors that enhance penetration, such as solvents or an occlusion patch. Again, in contrast with acute ICD, the skin response during ACD elicitation is not proportional to the amount of exposure, but is more correlated with exposure frequency because the skin response is determined by the quality and the quantity of the immune system cells recruited and with cytokine release. These aspects have been well investigated both *in vivo* and *in vitro*, with results that contribute to a better understanding of the molecular background of skin sensitization and also of some aspects of ICD (Marzulli and Maibach, 2008).

19.2.3 ICD versus ACD

A comparison of ICD and ACD is quite useful as it highlights some aspects that can

contribute to our understanding of the principles and the end points of the *in vivo* and *in vitro* test models for evaluating both effects and of how the models perform, or rather *may* perform, with microorganisms. ICD is mostly a result of damage to the skin barrier which leads to an unspecific innate immune response, whereas ACD is a more complex response that leads to a specific immunological cell-mediated response (Rustemeyer *et al*., 2011). On top of the differing bases of these different responses, there is the presence of danger signals for the immune system. The subsequent series of events are similar in both processes as histology, immunohistochemistry and electron microscopy of ICD and ACD has failed to demonstrate differences, and cytokine secretions are similar and non-specific (McFadden and Basketter, 2000) (Table 19.2).

19.3 Testing Methodologies

In order to prevent occupational skin diseases, a series of criteria are needed to enable a scientifically based and practical identification of a potential irritant or sensitizing agent. Additionally, the criteria must be able to quantify the potency of the irritancy/sensitization hazard. This information is the basis of risk assessment for workers and end users (Basketter, *et al*. 1999; Maurer, 2007).

For both irritancy and sensitization there were, until recently, only a limited number of *in vivo* tests on animals available. These methods have been widely used for a long time and are part of the OECD Guidelines as well as of other international guidelines for the testing of chemicals. They are based on the observation of clinical signs on animal skin after topical and, in some cases, systemic treatment at a given dose. Animals are scored for clinical signs at time intervals, and the results are classified on a value scale from *no reaction* to *severe reaction* according to the examiner's experience. Apart from clinical signs, no other types of observation are routinely performed. These tests still offer advantages, such as:

1. Standardized protocols that are recognized worldwide.
2. Relevant experience with chemicals in different fields of application.
3. Availability of a consistent bibliography of data on animals and comparison with human data.
4. Availability of a long list of testing laboratories with trained personnel.

Despite these favourable aspects, there are also well-known disadvantages, such as:

1. Uncertainty in translating the results obtained in animals to humans.
2. Expression of the result obtained in terms of hazard and not potency.
3. Animal-based methodologies.

Recently, a series of new tests were submitted for international validation of standardization of the methods. They were developed for answering scientific questions and practical needs, such as:

1. The need to quantify rather than qualify the effect observed to improve result objectivity.
2. The need to express the results in terms of potency in order to better categorize different agents.
3. The need to replace, reduce or refine the use of animals in skin toxicology, a sector under strong criticism by animal welfare organizations.

19.3.1 Methods for skin irritation

Dermal irritation studies are tests that predict the irritant and/or corrosive effects of chemicals that may accidentally or intentionally contact the skin (Rauckman and Soifer, 2006). In 2002, OECD revised the protocol and introduced 'A Sequential Testing Strategy for Dermal Irritation and Corrosion', which included the following points (OECD, 2002, 2010):

- Evaluation of existing human and animal data. If the tested material shows unequivocal positive or negative results in human and/or animal, it does not need to be tested again.
- Analysis of structure–activity relationships (SAR). The results of testing of

Table 19.2. Comparison between irritation contact dermatitis (ICD) and allergic contact dermatitis (ACD).

End points	Irritation contact dermatitis	Allergic contact dermatitis
Causative agent	Chemical and physical agents	Chemicals or molecular fragments able to bind to skin proteins and form an allergenic complex
Chemico-physical properties	Corrosion: agents with pH ≤ 2.0 and ≥11.5 Presence of structural alerts	Low molecular weight substances (≤ 400–500) Presence of structural alerts
Contactant concentration	Usually high even in chronic forms	Usually low
Pathogenesis	Direct cytotoxic effect	T-cell mediated immune reaction (type IV)
Affected individuals	Potentially all	A minority of individuals
Distinctive initial event	Skin barrier impairment	Hapten presence and allergic complex formation
Area affected	Topically at the contact site	Induction: at the contact site Elicitation: initially at the contact site, but extending later to other areas nearby or systemic
Skin strata involved	Stratum corneum in weak and mild irritation In corrosion and chronic form severe irritation up to derma	Whole skin up to derma and corresponding lymph node after migration of Langerhans cells
Delay of skin reaction	In acute form immediate response at the contact site. In chronic form a steady state of diffuse inflammation.	No skin reaction during the induction phase Days or weeks after the first contact elicitation reaction after exposure, usually within 24–48 h
Distinctive clinical signs	Dryness, itching, pain, ulcer if corrosive Dryness, hyperkeratosis and eczema	Vesicles, itching, hyperkeratosis
Cellular types involved	Keratinocytes, Langerhans cells, fibroblasts, inflammatory cells	Keratinocytes, Langerhans cells, fibroblasts, inflammatory cells, specific T-lymphocytes
Cytokines released	Cytokines: IL-1α[a], IL-1b[a], IL-8[a], TNF-α[a], GM-CSF[a] Chemokines: CCL20, CCL27 Growth factors: EGF[b], KGF[b]	Cytokines: IL-1α[a], TNF-α[a], GM-CSF[a], IL-1b[a, c], IL-18[a, c] Chemokines: CCL19[c], CCL21[c]
Clinical evolution	For weak and mild irritation usually benign with complete recovery For corrosive agents irreversible damage with scar formation For chronic form risk of irreversible changes in affected skin	Irreversible as elicitation occurs at every new contact Possible cross activation with other substances, or elicitation risk when the same substance is inhaled or ingested

[a]Danger signals; [b]In chronic forms; [c]Langerhans cell specific.
CC, chemokine; EGF, epidermal growth factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin; KGF, keratinocyte growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or thiazolyl blue; TNF, tumour necrosis factor.
Data from: McFadden and Basketter, 2000; Ale and Maibach, 2008; Sasseville, 2008; Gober and Gaspari, 2008; Rustemeyer *et al.*, 2011.

structurally related substances should be considered, if available, and may be considered only for corrosive and irritating material.

- Physico-chemical properties and chemical reactivity. Substances exhibiting pH extremes such as ≤ 2.0 and ≥ 11.5 may have strong local effects. In these cases, the material may be classified as corrosive without further testing.
- Dermal toxicity data. Substances which are highly toxic by the dermal route

cannot be tested for irritancy because they can induce death or severe suffering in animals. Negative findings are accepted only if the dermal toxicity study was performed on rabbits, at comparable doses and up to 2000 mg kg^{-1} body weight without signs of irritancy or corrosion.

- Results from *in vitro* or *ex vivo* tests for corrosion. If the tested material shows positive results in *in vitro* or *ex vivo* corrosivity tests, it does not need to be tested again.
- *In vivo* tests in rabbits. Starting with an initial test using one animal, it is possible to stop further testing if the substance is corrosive, otherwise the irritant or negative response should be confirmed using up to two additional animals for an exposure period of 4 h, in a sequential manner, or by exposing them simultaneously.

One *in vivo* and one *in vitro* testing model for dermal irritation are adopted by OECD, namely Test Guideline Nos 404 and 439. A comparison of the advantages and disadvantages of these two methods is presented in Table 19.3 and the guidelines will be described in more detail in the following sections.

In vivo *animal models*

The OECD Test No. 404 defines the protocol for Acute Dermal Irritation/Corrosion. This method was initially adopted in 1981, revised in 1992 and reformulated in 2002 with the inclusion of several aspects related to animal welfare and *in vitro* alternatives. The most common animal used is the New Zealand White rabbit because it has no dermal pigment and a large dorsal surface that is practical for observation. Rabbits have been shown to be rather sensitive to the irritant/corrosive effects of a wide range of chemicals. The test material is applied on the dorsal skin surface and a nearby untreated area is considered as the control. The effects on the rabbit skin are scored at timed intervals and the duration of the study is sufficient to see reversibility or irreversibility of the effects. A dose of 0.5 ml of liquid or 0.5 g of solid or paste is applied to the test site, with a semi-occlusive patch for an exposure time of 4 h. After the exposure patch is removed and the skin washed, scoring is performed at 60 min, and then at 24, 48 and 72 h. Exposure is terminated after 14 days if there are signs of irritation to determine possible recovery, or immediately at any time if the animal shows continuing signs of severe pain or distress. After one animal treatment, additional animals are treated until an unequivocal response is obtained in the majority of animals tested.

Dermal irritation grading is based on a scoring system originally developed by Draize (OECD, 2010). Scores for erythema and oedema are combined at each scoring time up to a maximum score of 8 and counted as the primary irritation index. Materials producing a primary irritation index of 0.00 are classified as non-irritant; scores from 0.01 to 2.00 rate a material as a slight or weak irritant, from 2.01 to 5.00 as a moderate or mild irritant, and above 5.01 as a severe irritant (Rauckman and Soifer, 2006).

In vitro *models*

For many years, no *in vitro* methods were available for skin irritation testing. While corrosivity as an expression of cell lethality was easily simulated *in vitro*, testing for irritancy remained a difficult task. Pre-validation studies, such as those sponsored by the European Centre for Validation of Alternative Methods between 1998 and 2006, failed to reach final objectives because the proposed methods were not suitable for safety assessment and regulatory purposes (Basketter and Jones, 2008).

Recently, OECD adopted the Reconstructed Human Epidermis (RhE) Test No. 439 as predictive for skin irritation under certain conditions (OECD, 2010). The RhE is based on non-transformed human-derived epidermal keratinocytes that are cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers representing the main lipid classes analogous to those found *in vivo*. The RhE model can be prepared

Table 19.3. Comparison between *in vivo* test on rabbit (OECD Test Guideline No. 404) and RhE *in vitro* (OECD Test Guideline No. 439) test methods for skin irritation.

End points	OECD Test No. 404	OECD Test No. 439
Test method	*In vivo* skin irritation	*In vitro* skin irritation
Test system	New Zealand rabbit	Reconstructed Human Epidermis (RhE): EpiSkin™, EpiDerm™ SIT (EPI-200), SkinEthic™ RHE
Testing material	Active substances and formulations	Active substances
	Liquid or solid	Liquid or solid Not suitable for formulations
End point	Clinical signs of irritation scored at determined time intervals	Skin cells viability (MTT[a] assay) Alternatives[b]: skin barrier efficacy, cell morphology
Recovery evaluation	Possible up to 14 days of observation	Limited to 48 h and only at keratinocyte level
Results obtained	Primary Irritation Index	EC_{50} (effective concentration)
Result expression of a hazard	Yes	Yes
Result expression of a potency	No	Potentially yes
Ability to identify weak or mild irritant	Yes	Limited to weak irritant (very limited skin damage with short time complete recovery) Not suitable with mild irritant (evident skin damage and inflammatory response with longer recovery)

[a]MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or thiazolyl blue.
[b]To be applied when tested chemical interferes with the MTT test.

in house or obtained commercially. At the moment, three commercial RhE models are available: EpiSkin™, EpiDerm™ SIT (EPI-200) and SkinEthic™ RHE.

Whichever RhE model is adopted, a series of conditions have to be respected, including:

1. Cell viability, measured by enzymatic conversion of the vital dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or thiazolyl blue).
2. Histological examination of the cell morphology should be performed to demonstrate human epidermis-like structure (including a multilayered stratum corneum).
3. Integrity of the skin barrier function, which should be sufficient to resist the rapid penetration of cytotoxic marker chemicals (measured by the skin cell viability MMT test).
4. Reproducibility and quality control.

The RhE test is performed by applying the test chemical liquid or solid to uniformly cover the epidermis surface, in three replicates. Exposure times vary from 15 to 60 min and incubation temperature between 20 and 37°C. Positive (sodium dodecyl sulfate – SDS) and negative (phosphate-buffered saline – PBS) controls should be included in order to demonstrate model responses within a historical acceptance range.

Following treatment, the viability assessment has to be performed after rinsing the exposed RhE and a recovery incubation time of 48 h, so that substantial cytotoxic effects become more evident. Again, the MTT assay is used for assessing cell viability. Results are interpreted by comparing the optical density of the treated RhE versus the negative control. Chemicals are considered as irritants if the cell viability is less than 50% (OECD, 2010). As stated by OECD, RhE-based methods may be used to determine the skin irritancy of chemicals as a stand-alone replacement test for *in vivo* skin irritation testing, or as a partial replacement test, within a tiered testing strategy.

With Commission Regulation (EC) No 761/2009 (OJEU, 2009), Europe adopted the RhE irritancy test as Method B.46. However,

the regulation clearly indicates that the RhE method is suitable only for classifying substances as *skin irritants category 2* according to the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS). Furthermore, it is not intended for testing gases and aerosols, and mixtures have not been assessed yet in a specific validation study (OJEU, 2009).

19.3.2 Methods for skin sensitization

The prediction of the allergic potential of chemicals and formulations is a common practice in regulatory and experimental toxicology. Curiously, predictive tests were defined at a time when the mechanism of sensitization was not completely known. The available testing methodologies with guinea pigs are an evolution of the first method developed by Draize in 1944 (ECETOC, 1990), which was adopted by the US Federal Drug Administration (US FDA) in 1959 (ECETOC, 1990). A series of improvements were necessary to better reproduce human exposure conditions and to increase sensitivity of the test, and seven methods had been developed by 1980. After a close examination by OECD in 1992, it was recognized that only two methods were frequently used in the USA and Europe: the Bühler test and the guinea pig maximization test (GPMT) of Magnusson and Kligman (1970), respectively. These were adopted and recommended by OECD (Test Guideline No. 406), limiting the others only to special cases (ECETOC, 1990; Maurer, 2007).

Subsequently, a new method based on mice, the murine local lymph node assay (LLNA) was internationally validated and adopted by OECD in 2002 under a separate guideline (Test No. 429). In 2010, two LLNA variants were published (Test Guidelines Nos 442A and 442B) (OECD, 2010).

In contrast to irritation, sensitization is a complex process involving the skin, derma and immune system, and actually no *in vitro* alternative is available. The LLNA has recognized this by reducing and refining the number of animals tested, as a result of pressure by animal welfare agencies and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; Kimber *et al*., 2003).

Guinea pig methods

Basically, the guinea pig methods consist of two experimental parts: the induction and the challenge or provocation phase. During the induction phase, animals are treated repeatedly for 2–3 weeks to simulate the first contact. After a resting period, the animals are treated again with a non-irritating amount of the tested material at another site. A challenge that induces an allergenic reaction topically is scored according to a four-grade scale from erythema to oedema. The more animals that show challenge effects, the stronger is the tested material, so the final judgement is based on the total number of animals affected rather than single individual responses (Magnusson and Kligman, 1970).

Both the Bühler test and the GPMT follow the above general scheme. However, treatment type and timing, together with the evaluation period, are the most relevant differences (Table 19.4). The scope of adjuvant use and injections in GPMT are to enhance the clinical reaction after challenge induction. Also, pretreatment with sodium lauryl sulfate (SLS) is to facilitate the dermal penetration of the tested chemical through the skin barrier impairment induced by the irritant, in order to increase the possibility of a sensitization. While the GPMT is conducted using two types of exposure (skin and percutaneous) during induction, in the Bühler test, animals are treated repeatedly only by the dermal route, but at a concentration which can be up to 10-fold higher than the corresponding tested material in real use. It is generally recognized that the Bühler test underestimates and the GPMT overestimates the sensitization potential of chemicals. Finally, it should be emphasized that the Bühler test and the GPMT may identify the *hazard*, but not the *potency* of sensitizers, as they cannot correctly classify weak allergens that are unable to trigger a clinical response in animals (Basketter and Kimber, 2010).

Table 19.4. Comparison of the GPMT (guinea pig maximization test) and Bühler test protocols for skin sensitization; table is based on protocol Test Guideline No. 406 (OECD, 2010).

End point	GPMT	Bühler test
Animal/strain/sex	Guinea pig Hartley or Pirbright White strains	Guinea pig Hartley or Pirbright White strains
	Male or nulliparous female	Male or nulliparous female
Animals/group	Minimum 10 test and 5 control animals 20 test and 10 control animals optimal	Minimum 20 test animals and 10 controls
Positive controls	Reliability check every 6 months with adequate positive results: hexyl cinnamic aldehyde (CAS No. 101-86-0); mercaptobenzothiazole (CAS No. 149-30-4); benzocaine (CAS No. 94-09-7)	Reliability check every 6 months with adequate positive results: hexyl cinnamic aldehyde (CAS No. 101-86-0); mercaptobenzothiazole (CAS No. 149-30-4); benzocaine (CAS No. 94-09-7)
Induction phase		
Treatment type	Week 1: a series of injections: Freund adjuvant or physiological saline tested material alone tested material with Freund adjuvant Week 2: occlusive patch for 48 h (if the testing material is not irritating, 24 h pretreatment with sodium lauryl sulfate)	Induction for 6 h on days 0, 6, 8, 13 and 15
Treatment site	Injections 1 and 2 in anterior dorsal area, injection 3 in posterior dorsal area Patch: shaved skin of median dorsal area	One shaved flank area (4–6 cm^2)
Tested material concentration	Week 1: maximal tolerable concentration Week 2: minimal irritant concentration	For each induction exposure the highest concentration able to induce irritation
Challenge phase		
Treatment type	Occlusive patch for 24 h	Occlusive patch for 6 h
Treatment site	One flank of the animal and if necessary negative control with vehicle only on other flank of the same animal	Untreated flank posterior area and if necessary negative control with vehicle only on anterior area of the same animal
Tested material concentration	Maximal non-irritant concentration	Maximal non-irritant concentration
Observation	21 h after patch removal, skin is cleaned and/or shaved: first observation 3 h later (48 h from the challenge start); second observation 24 h later (72 h from start)	21 h after patch removal, skin is cleaned and/or shaved: first observation 3 h later (30 h from the challenge start); second observation 24 h later (54 h from start)
Scoring	For each animal in the treatment site: 0 = no visible change 1 = discrete or patchy erythema 2 = moderate and confluent erythema 3 = intense erythema and swelling	For each animal in the treatment site: 0 = no visible change 1 = discrete or patchy erythema 2 = moderate and confluent erythema 3 = intense erythema and swelling
Evaluation	Sensitizer: more than 30% of the guinea pigs with clinical signs Non-sensitizer: less than 30% of guinea pigs with clinical signs	Sensitizer: more than 15% of the guinea pigs with clinical signs Non-sensitizer: less than 15% of guinea pigs with clinical signs
Re-challenge	If necessary, with same or new animals	If necessary, with same or new animals

Mouse methods

While guinea pigs were chosen for their similarity to humans in clinical signs during allergic responses, mice received attention owing to the good understanding of their immune system. The mouse-based LLNA method has advantages and disadvantages, but undoubtedly represents a step forward in the prediction and evaluation of the sensitization hazards and potentials of chemicals. Rather than looking at the clinical changes induced after challenge on animal skin, the LLNA explores and quantifies the ability of the tested material to promote cell proliferation during the induction phase. In the original LLNA protocol, cell proliferation is detected by the incorporation of a radioactive compound, such as tritiated ^{3}H-methyl thymidine or, alternatively, ^{125}I-iododeoxyuridine and fluorodeoxyuridine, which during replication are incorporated into the DNA of the lymphocytes. The use of radioactive compounds is substituted in the new, recently approved protocols by the detection of ATP content via bioluminescence (Test Guideline No. 442a) or non-radiolabelled 5-bromo-2-deoxyuridine, detected by ELISA (Test Guideline No. 442b). In the LLNA, proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitization. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle-treated control group to obtain the Stimulation Index (SI). At the threshold, it is assumed that a threefold increase in activity occurred compared with concurrent vehicle controls, the so-called EC3 value. An SI ≤ EC3 indicates that the tested material is a sensitizer. A summary of the main end points related to the LLNA is presented in Table 19.5; see OECD testing guidelines (OECD, 2010) for the detailed protocols.

Furthermore, the LLNA has the potential to reduce the number of animals required and offers a substantial refinement for allergic contact sensitization testing. The LLNA is limited to the induction phase of sensitization and, unlike guinea pig tests, does not require the elicitation of challenge-induced dermal hypersensitivity reactions, thereby resulting in less animal pain and distress.

19.4 Microorganisms and Irritation/ Sensitization

The presence of microorganisms in the workplace poses the question regarding their ability to induce skin irritation and/or sensitization. In order to determine this, it is necessary to first underline under which conditions these effects could be manifested. Enhanced exposure levels to microorganisms in the workplace can be expected in three situations:

1. In workplace activities that involve purposeful handling of products containing biological agents, for example, in biotechnology or biopesticide production.
2. Where workplace materials are contaminated by biological agents.
3. Where sources of bioaerosols are incidental to the main work activity, e.g. contamination of humidifiers and air conditioners.

First, opportunistic microorganisms could induce skin diseases under conditions that alter the efficiency of the skin barrier, such as wetting or exposure to detergents or surfactants. Secondly, in biotechnology, enzymes of bacterial and/or fungal origin can be suspected to cause irritation to the skin, eyes and the respiratory tract. Finally, there are cases in which the microorganism is the active ingredient in a formulated product for professional and non-professional end users, for example biopesticides and biocides. Examples of known workplace conditions related to these situations are reported in Table 19.6.

In the first case, represented by, e.g. metalworking fluid dermatoses, environmental bacteria are responsible for ICD in connection with small traumas and a wet environment. The role of the microorganisms in the pathogenesis of dermatoses has often been ignored, owing to the lack of specificity of skin bacterial activity (Ueno *et al.*, 2002; Taibjee *et al.*, 2003). In most of the cases reported, it is difficult to identify single microorganisms which may be responsible and it is reasonable to accept the possibility that microorganisms are

Table 19.5. Description of the LLNA (murine local lymph node assay) test (OECD, 2010).

End point	LLNA (OECD Test Guideline (TG) Nos 404, 442a and 442b)
Animals/strain	Mice: CBA/Ca or CBA/J, female nulliparous
Animals/group	4 animals/group
Positive controls	Hexyl cinnamic aldehyde (CAS 101-86-0) in acetone:olive oil (4:1, v/v) 5% mercaptobenzothiazole (CAS 149-30-4) in N,N-dimethylformamide
Negative control	Vehicle: olive oil (4:1, v/v), N,N-dimethylformamide, methyl ethyl ketone, propylene glycol and dimethyl sulfoxide The tested material must be adherent to mouse ear so water solvent is not recommended
Tested material concentration	Pretest for defining dose interval if no data available with 2 animals/dose and only irritation evaluation on topically tested ears At least 3 concentrations starting with the highest non-irritant dose in a sequential series (i.e. 100%, 75%, 50% …)
Treatment site	Topically on ear *dorsum* in an area of *c.* 1 cm^2: one ear 25 µl of tested material, other ear vehicle alone or positive control
Treatment timing	Days 1,2 and 3 treatment Days 4 and 5 rest Day 6 tracing substance injection via tail vein 5 h later, the mice are killed and lymph nodes extracted for cell suspension
Tracing system	TG 404: tritiated (^{3}H)-methyl thymidine or ^{125}I-iododeoxyuridine and 10^{-5}M fluorodeoxyuridine by β-scintillation counting as disintegrations per minute (dpm) TG 442a: ATP content via bioluminescence TG 442b: 5-bromo-2-deoxyuridine via ELISA
Scoring	Stimulation Index (SI) as comparison between tested material and control on single animal and/or pooled animals SI ≥ 3 sensitizer or SI≤ 3 non-sensitizer Considering that proliferation is a dose–effect relationship, an EC3 value (SI = 3) could also be calculated as the *threshold of sensitization*

a non-cause, due to the high levels of environmental contaminants in poor workplace conditions and incorrect procedures such as too frequent hand washing.

For enzymes and proteins produced by biotechnologically engineered microorganisms, the resulting skin diseases are immunological contact urticaria (ICU) and protein contact dermatitis (PCD). Several agents, all of natural origins, are able to induce ICU and/or PCD. Both diseases are more similar to an IgE-mediated (reaginic) allergy rather than cell-mediated sensitization, but in PCD, both clinically type I (reaginic) and type IV (cell-mediated) responses can be involved (Amaro and Goossens, 2008; Goossens and Amaro, 2011). In industrial biotechnology, workers may be exposed to the process microorganisms or their components at various levels, particularly at the downstream processing stage, including centrifugation, product concentration and waste handling (Crook and Swan, 2001). However, containment is a requirement in those instances, not only to ensure product purity but also for workplace and environmental safety. Thus, the adoption of strong preventive measures and improvements in workplace conditions have substantially reduced the risk of allergy and sensitization. In some cases, such as detergent enzymes, the risk of ICU in the general population is minimal, while occupationally it is relatively higher without the adoption of personal protective equipment (PPE) (Basketter *et al.*, 2008).

The situation for biopesticides and biocides is more complex, because the microorganism

Table 19.6. Workplace activities related to a potential for skin irritation and skin sensitization in humans resulting from exposure to microorganisms.

Activity	Potential hazards[a]	Suspected agents	Potential group affected
Contamination in workplace by microorganisms			
Contact with metal-working fluid	ICD	Chemicals, traumas, wet hands, bacteria	Workers[b]
Proteins or enzymes in food/feed ingredients and non-food products produced by microorganisms			
Bakery production	ACD, ICD, PCD	Organic dust, contamination with microorganisms, enzymes	Workers [c,d]
Detergent production and use	ICU, PCD	Raw and encapsulated enzymes	Workers, general population[d,e,f]
Farming	ACD, ICD, PCD	Chemicals, animal proteins, enzymes	Workers[g]
Animal feed production	PCD	Proteins, enzymes	Workers[d,h,i]
Microorganisms as active ingredients in final formulations ready to use			
MBCAs and biocide production and use	ACD	Active ingredients, toxins	Workers, professional users, bystanders and general population

[a]ACD, allergic contact dermatitis; ICD, irritation contact dermatitis; ICU, immunological contact urticaria; PCD, protein contact dermatitis.
References: [b]Awosika-Olumo *et al*., 2003; [c]Vanhanen *et al*., 1996; [d]Goossens and Amaro, 2011; [e]Kelling, *et al*., 1998; [f]Basketter *et al*., 2008; [g]Burdzik, 2009; [h]Johnsen *et al*., 1997; [i]Vanhanen *et al*., 2001.

is the active ingredient of the formulation that is ready to be used by professional and non-professional users. The risk is potentially not limited only to the user, but also concerns the general population when a biopesticide such as *Bacillus thuringiensis* is used as larvicide against Lepidoptera or mosquitoes by aerial treatment on a large scale.

19.4.1 Data on the exposed human population

Spontaneous infectious eczematoid dermatitis (aka infective dermatitis, microbial eczema) is an eczematous skin reaction induced by a hypersensitivity reaction to microorganisms (bacteria, fungi, or viruses) or their products. The eczematoid reaction is a cell-mediated immune reaction to microorganisms acting as haptens and is aggravated by scratching and rubbing. This pathology differs from infection atopic dermatitis or contact dermatitis, because it is not workplace related so that removal of the cause does not resolve the symptoms (Ostler *et al*., 2004).

Generally speaking, the sensitization potential with microbial products appears to be better documented for certain microbes than for others, so for many, the dearth of studies makes it difficult to estimate the exposure of the general population and to understand possible consequences. As an example, Gram-negative bacteria with their bacterial lipopolysaccharide (LPS) endotoxin have been proposed as a major candidate for the pulmonary inflammation reaction due to their presence in organic dusts. Rylander (2002) reviewed endotoxins in the environment and a possible relationship of these to disease among exposed persons. The author concluded that there is a relationship between exposure and disease, yet at certain exposure levels and/or at certain periods of life, exposure may even be beneficial and reduce the risk for disease.

In a 2 year study, Green *et al*. (1990) conducted a surveillance programme in *Bacillus thuringiensis* var. *kurstaki* (Btk) spray areas (inhabited by 80,000 people in 1985, and a smaller area with 40,000 people in 1986) in Lane County, Oregon. A non-sprayed community 100 km

away served as a control population. Human culture specimens were obtained for routine clinical purposes during, and for 1 month after, the spray period. Specimens that were positive for any *Bacillus* species were subcultured for *B. thuringiensis* and the presence of toxin crystals (parasporal inclusions) was investigated. Fifty-five cultures out of 105 were positive for Btk, but there was no evidence of human pathogenicity. There was no increase in the number of telephone complaints nor was there a change in the pattern of complaints.

Another study was conducted in an area with a population of 1,400,000 in Lower Mainland, British Columbia, Canada (Nobel *et al*., 1992). After a spray campaign with Btk, the study examined 26,000 telephone calls, 1140 family practice patients and 3300 hospital admissions, and closely monitored 120 workers with occupational exposure. There were no differences in emergency room visits between spray days and non-spray days, nor was there a significant change in the pattern of telephone calls. There was no evidence that Btk spraying was associated with illness or infection and, in fact, there was a lower incidence of diarrhoea in the spray zone than in the control area. However, workers showed symptoms of headache; nose, throat and eye irritation; dry skin and chapped lips, all signs of irritation and/or sensitization. The range of worker exposure was between 750 and 5.5×10^6 cfu (colony forming units) m^{-3}.

Similar conclusions of no health effects were drawn from an evaluation of the use of *B.t. israelensis* commissioned by the New Zealand Ministry of Health (Glare and O'Callaghan, 1998). However, an independent study carried out during eradication spray programmes using Foray 48B (Btk) to control infestation by the painted apple moth that was conducted in West Auckland, New Zealand, found adverse health symptoms in 315 of the resident population of 150,000 people exposed to the Btk spray operation, although these made up only 0.21% of the exposed population (Blackmore, 2003). Another study on 292 subjects, also in West Auckland, found that there was an increase in health problems in 168 subjects; these ranged from cough to dizziness and sleep difficulties. Skin irritation and eczema were not statistically relevant (Petrie *et al*., 2003).

A longitudinal, follow-up investigation of 48 workers who were involved in picking Bt (Javelin®) sprayed crops (celery, parsley, cabbage, kale, spinach, strawberries) was conducted by Bernstein *et al*. (1999). These authors presented three exposure groups, 'Low', 'Medium' and 'High'. There was no evidence of occupationally related respiratory symptoms. Four types of Btk spore and two vegetative (subsp. *kurstaki* and *aizawai*) antigen extracts were prepared, of which some elicited positive skin tests, chiefly with exposed workers, at an incidence of 35%. Yet 25% of the 'Low' exposure group also presented an atopic response. Positive skin tests were seen with water- and mercaptoethanol-sodium dodecyl sulfate extracts of Javelin, yet their incidence was not higher for Javelin extracted pro-Delta-endotoxin or proteinase K spore extracts. Specific IgE and IgG antibodies to vegetative cells were present in all groups, witnessing an immunological response.

Doekes *et al*. (2004) tested human sera from the BIOGART project, a longitudinal respiratory health study on more than 300 Danish greenhouse workers. This study had a 2 and 3 year follow-up. While many sera had detectable IgE to Bt (23–29%), all positive reactions showed a relatively weak IgE anti-Bt positive serum response. The authors concluded that even though the IgE binding components may be genuine Bt components, they could also be shared with some or many commonly found bacterial species, and in that case, similar IgE levels may be found in the general population.

A review analysis of the effect on the human population exposed to Bt spraying was redacted by Otvos *et al*. (2005). The authors concluded that Bt is one of the safest biopesticides and noted that no scientifically documented case of human infection has been reported since its introduction in the 1960s, as well as no human health problems as a result of spraying. However, the majority of the studies examined were focused on human risk of infection rather than respiratory and/or skin distress, which were considered transitory and less relevant symptoms.

In conclusion, studies on exposure and health effects on residents in areas treated with Bt show divergent results (Madsen, 2011). While some authors conclude that symptom complaints increased significantly, others conclude that there were no significant changes in physical health for residents in the affected area and that almost all of the studies were scientifically questionable. However, the conclusions drawn by studies on exposed workers, which showed humoral changes and skin or respiratory distress, but without clinical relevance, are more pertinent.

19.4.2 Strategies for assessing irritation or sensitization by microorganisms

As mentioned previously, after direct infection, sensitization and irritation are the most relevant adverse effects for workplace exposure to microorganisms. There is need for a correct identification of the potential risk in order to evaluate the need for preventive measures such as gloves or, in some cases, also the limitations of their use. Madsen (2011) estimated the amount of microbial pesticides to which workers are exposed and concluded that exposure occurs in occupational settings but that residents in treated areas and neighbourhoods are also exposed. According to the author, exposure can also occur days or weeks after treatment, posing the need to correctly predict the irritation and sensitization potential of the final product.

For chemicals, the use of predictive tests on animals is scientifically recognized as appropriate and, for many years, molecules and products were classified for human risk according to experimental animal data. If we look at microorganisms and products based on microorganisms such as MBCAs, the question of predicting sensitization and irritation potential is more complex.

Microorganisms are not chemicals and, in most cases, the living microorganism is unable to penetrate the skin barrier. However, sensitization and/or irritation could arise from the presence of other substances in microbial products, such as toxins, enzymes, proteins, fragments, contaminants and formulants. For biological material produced by the active ingredient microbe, such as toxins and/or enzymes, the risk of irritation is related to the possibility of direct toxic effects on skin keratinocytes. In the same way, the sensitization risk by MBCAs could be similar to that of PCD, with a mixed pattern of IgE and IgG production and cellular response as weakly indicated by studies on population and workers exposed to Bt. Formulants could play a relevant role but, in practice, sensitizers and irritants are avoided in final products.

If we look at the available methodologies for testing end-use products based on microorganisms, such as enzymes or MBCAs, there are several questions that must be answered. For irritation, the possibility of using *in vitro* methods is only related to the possibility of classifying the tested material as *irritant* or *corrosive*, i.e. as positive. In other words, these tests are useless if we have a negative result, i.e. *non-irritant*; they are also useless if we need to distinguish between a mild and/or a true irritant. The only way to solve the problem is to accompany the *in vitro* tests with a physicochemical examination and/or a structural alert investigation (by SAR analysis). This approach, with difficulty, was proposed for chemicals but has not been tried with microbials, and could be better defined only after years of experience which, at the moment, is of course lacking. A tentative strategy is the possibility of applying RhE tests on MBCAs and, if the results are negative according to other aspects, e.g. human data, waiving animal testing.

For sensitization, the question is even more complex, because methodological improvements such as LLNA may not be suitable for microorganisms. LLNA is a test based on Langerhans cell recruitment, lymph node migration and lymphocyte cloning. As Langerhans cells are immune competent and react *naturally* against bacteria and exogenous intruders, it can be suspected that in the case of, e.g. MBCAs, the presence of the microbe or its fragments could elicit a response from Langerhans cells, thus overestimating the sensitization potential or shifting the sensitization response into a *true* aspecific immune response. The only animal test method for sensitization that could be suitable for MBCAs is the Bühler test, which is more

representative of real exposure conditions in humans and is already validated for formulations. Bühler is considered so far a more sensitive test, while companies prefer the GMPT, but the use of an adjuvant and percutaneous exposure should be avoided. In the case of a positive result with Bühler, further tests of the technical preparation without the active ingredient could help to identify the component responsible for sensitization.

In conclusion, none of the testing procedures for irritation and sensitization have been validated for microorganisms, and it can be questioned whether a special test for microbial products is really needed. If we look to bacteria, spores are unable to pass the skin barrier so they cannot induce irritation or sensitization. Viruses and fungi are unable to induce toxic effects per se, but only through infection and/or toxin production. If viruses are not human pathogens or they are unable to infect skin cells, and fungi are unable to produce toxins dangerous for human skin cells, there is no need to test these organisms. In this case, a positive result is obtained by a combination of biological activity (i.e. the ability to infect and/or damage skin cells) and physical properties (the ability to pass through the skin). Based on currently available data, the need for testing microorganisms alone is scientifically questionable.

19.5 Conclusion

The use of microorganisms under working conditions can be responsible for non-infective adverse effects, mainly irritation and sensitization. In recent years, the knowledge and perception of this risk has increased and the number of workers affected by skin dermatoses induced by microorganisms or by enzymes in the workplace has been reduced significantly, thanks to better working conditions and protective measures.

Unfortunately, this progress has not been accompanied by any in-depth investigations on the conditions and the mechanisms that are the basis of sensitization and irritation induced by living organisms. In the meantime, the use of microorganisms has conquered new sectors and diffused into all human activities, leading to exposure of not only workers, but also the general population, to possible toxic effects that are not limited to infection. At present, bacteria, viruses and fungi have to be considered for some non-infectious toxicological end points, such as irritation and sensitization.

Despite the lack of a specific analysis, the response by the regulatory agencies has been to transfer knowledge and experience from the chemical sector to the microorganisms used for biological pest control (Bailey *et al.*, 2010). The background to this approach is easily understandable because testing methods and evaluation schemes perform well with chemicals and, after all, microbial products, like enzymes or toxins, are chemicals. However, it is scientifically difficult to understand the need for classifying living organisms for their non-infective properties, once they have been determined to be non-pathogenic for humans and recognized as safe by several government agencies and also in Europe, by the European Food Safety Authority (EFSA, 2007; Ehlers, 2011). Nevertheless, there is a need to develop new approaches for estimating the health risks of microbial products, considering their diffuse spread and the potential threat that they could present for humans and the environment.

These aspects, although apparently minor, risk limiting the development and relevance of microbial products which, in some cases, are the only real alternatives to chemicals. At the moment, the only practical solution is to involve academia, industry and regulatory agencies in developing societal strategies that could lead to a revised position of microbial safety in which infectious and non-infectious aspects are holistically considered. Finally, it should be stressed that an improvement in production control and quality of microbial products could also play a relevant role.

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20 International Conventions and Agreements – Consequences for International Trade and Utilization of Biological Matter, Including Microorganisms

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20.1 Introduction

Developments in biotechnology, molecular genetics, intellectual property regimes and systems for regulating access to genetic resources and related information over the last two decades are rapidly changing the conditions for public research on microorganisms, plants and other types of biological matter/genetic resources. Moreover, these developments also influence the possibilities for exploiting these biological resources in beneficial products, e.g. in food production or environmental applications. While no multilateral or international regulatory regimes specifically covering microorganisms have so far been agreed upon, the emerging 'proprietary science landscape' is comprehensive and includes several international agreements that can strongly influence trade with and the utilization of biological matter (Table 20.1).

The CBD (Convention on Biological Diversity) is a legally binding agreement on biodiversity under the United Nations (UN). The WTO TRIPS (World Trade Organization Trade-Related Aspects of Intellectual Property Rights) is a trade agreement introducing a global mini-standard on what (matter) can be protected under intellectual property rights. The FAO ITPGRFA (Food and Agriculture Organization International Treaty on Plant Genetic Resources for Food and Agriculture) is an international treaty on multilateral access to – agreed upon – plant genetic resources for food and agriculture. UPOV (International Union for the Protection of New Varieties of

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Table 20.1. Overview of the international treaties and conventions that impacts international exchange and utilization of biological matter (adapted from Thornström, 2011).

Convention on Biological Diversity (CBD) 1992/3	World Trade Organization Trade-Related Aspects of Intellectual Property Rights (WTO TRIPS) 1994	Food and Agriculture Organization International Treaty on Plant Genetic Resources for Agriculture (FAO ITPGRFA) 2001/4	International Union for the Protection of New Varieties of Plants (UPOV) 1978/91	World Intellectual Property Organization Intergovernmental Committee on Genetic Resources, Traditional Knowledge and Folklore (WIPO IGCGRTKF) 2000	Cartagena Biosafety Protocol 2000	International Plant Protection Convention (IPPC) 1997
National sovereignty SNL Art. 15 PIC/MAT Art. 8j Protection of TK ABS – Bonn guidelines Arts 16–18 Transfer of technology and technical and scientific cooperation Capacity building	Mini-standard for IP (time limited) Art. 27.3(b) *Sui generis* – protection Microorganisms Microbiological processes Plant varieties Patent Copyright Trademark Trade secret Industrial designs Integrated circuits Databases • Novelty/Non-prior article • Innovative step • Industrial application Morality Ordre public Arts 66–67 LDCs and technical cooperation Capacity building	Multilateral access to *c.* 55 crop genera Standard MTA Art. 9.3 Farmer's rights SNL Farming system knowledge Landraces Farmer's varieties SNL	PVP (time limited) • New • Distinct • Uniform • Stable Common knowledge Public domain Extant varieties Farmer's privilege SNL Breeder's exemption SNL	Protection of TK CO/DO in IP application International ABS regime Standardized PIC/MAT regime	Transboundary movement of living modified organisms (LMOs) Art. 10 Right to delay import subject to further scientific biosafety check Art. 17 Unintentional transboundary movements and emergency measures	Arts V,VI, VII regarding phytosanitary requirements in shipping of plant material

ABS = Access and Benefit Sharing; CO/DO = Certificate/Disclosure of origin; IP = Intellectual Property; LDCs = Least Developed Countries; MAT = Mutually Agreed Terms; MTA = Material Transfer Agreement; PIC = Prior Informed Consent; PVP = Plant Variety Protection; SNL = Subject to National Legislation; TK = Traditional Knowledge.

Plants) is an international convention granting specified rights to plant breeders and farmers as regards use of (there is a time limitation of 20 years) intellectually protected seed varieties. WIPO IGCGRTKF (World Intellectual Property Organization Intergovernmental Committee on Genetic Resources, Traditional Knowledge and Folklore) is an ongoing negotiation to harmonize obligations mainly between the CBD and WTO TRIPS (especially on access to and use of biological matter and traditional/indigenous knowledge). The Cartagena Biosafety Protocol deals with the regulation of transboundary movement of living modified organisms (LMOs). The IPPC (International Plant Protection Convention) deals with phytosanitary requirements in the shipping of plant material across national borders.

The conditions set by these agreements comprise an *evolving new global legal regime related to all biological matter*. This regime will have a strong impact on the exchange and use of all biological/genetic resources, including microorganisms. Lengthy work has started in both the CBD and FAO to define and develop legal instruments for multilateral exchange of biological matter that comply with regulations, for example under TRIPS.

In this chapter I will refer mainly to three treaties (several others also matter) that have considerable bearing on the exchange and exploitation (for research as well as for the trade and marketing of new products) of microorganisms in public and private sectors:

- The United Nations Convention on Biodiversity (CBD)
- The WTO TRIPS agreement and its annex on trade-related intellectual property rights
- The FAO ITPGRFA on plant genetic resources for food and agriculture.

The two latter treaties have (different) provisions for intergovernmental enforcement and sanctions, while the first (CBD) leaves this subject to national legislation. In short, the CBD means nationalization of genetic resources (previously seen as part of humankind's common heritage), TRIPS sets minimum standards for what must be protected as intellectual property (IP) and the FAO treaty stipulates multilateral access and benefit sharing (ABS) rules for around 50 crop genera of high country interdependence and for global food security. In addition, starting in 2007, several sessions in FAO have been devoted to policy issues surrounding agricultural microbial genetic resources (AMiGRs), to which I will return later in this chapter.

In short, the new regulatory regimes on access and ownership impose an enclosure of the biological and genetic commons. In this zero sum game, the public domain is continuously reduced as more and more of the commons are proprietized. Thus, this transfers organisms from being free public goods to becoming private, corporate or state property. Biological common rights are thus replaced with regulated/discriminating access. The emerging new legal regimes have deep impacts on the freedom to operate (FTO) for public science (Kowalski *et al*., 2002; Safrin, 2004). In fact, for public (and in fact also private) sector research, this can be summarized in the equation: IP × ABS = FTO. In other words, if we marry IP with ABS, how do we create a viable offspring that grants acceptable FTO for science and scientists?

In this chapter, the focus is on treaties that have strong impact on exchange and practical implementation of biological matter/genetic resources, particularly treaties that may have impacts on the utilization of microorganisms. Animal genetic resources are dealt with in FAO through a Global Plan of Action, which is not treated here. Human genetic resources are subject to other legal provisions, mainly conventions and protocols under the World Health Organization (WHO), and are not treated either. Additionally, in this chapter I do not treat defence strategies against trade and the antagonistic use of microorganisms or advanced biotechnology: bioterrorism (Suffert *et al*., 2009).

Microorganisms are included in several of the approximately 20 categories of biological matter specified in, for example, the CBD, but also in TRIPS and the ITPGRFA, and in this chapter I present those categories. A few examples are discussed (partly from the world of plants), in which the treaties

have led to direct effects on capabilities to conduct research and exploit new research findings. Finally, a few prospective ways are discussed that may lead forward towards higher FTO, and thereby could facilitate the transition of new research results into biological products that can lead to improvements of food security and environmental quality.

20.2 What is Proprietary Biological Matter?

Taking into consideration provisions under the CBD, FAO ITPGRFA, TRIPS and UPOV in the late 1990s, at least the following categories of biological matter and related information were proprietary and subject to national legislation (CGIAR, 2001):

- plant seeds or other propagative plant parts collected after 1994
- plant and animal cell lines
- plasmids
- other recombinant vectors
- gene promoters
- gene markers
- transformed bacteria/fungi
- isolated plant DNA
- plant cDNAs (complementary DNAs)
- isolated animal DNA
- bacteria and fungi (other than transformed)
- isolated/purified proteins (other than those obtained by purchase of laboratory reagents)
- equipment for specialized laboratory purposes
- information regarding laboratory methods
- genomic sequence database(s)
- other nucleotide sequence database(s) such as PCR primer databases, cDNA sequences, etc.
- ethnobiological information (traditional knowledge, indigenous knowledge)
- farming systems information.

As modern research moves further into the very basic architectures of biological matter (genomics/DNA), new and often even smaller components become targets for possible proprietary claims.

20.3 A Slowly Shrinking Public Science Domain?

National legislation regarding access to genetic resources under CBD is presently in progress or in operation in some 30 countries, mainly in the southern hemisphere. In those countries, access to genetic resources is now subject to prior informed consent (PIC) under mutually agreed terms (MAT). In South America, the Andean Community, in its decision AP391/1996, agreed to apply a common regime to genetic resources with very strict and far-reaching ABS regulations (Correa, 2001). Another example of a very restricting implementation of the CBD is Indonesia (State Ministry of Research and Technology, 2011), where a foreign scientist must move through a comprehensive set of steps in order to obtain a research permit (Fig. 20.1). These may also include a copy of the marriage certificate if a foreign scientist wants to bring his/her spouse along.

As stated above, TRIPS sets a minimum standard for IP. The current trend for IP in biotechnology is that patents are granted on ever smaller pieces of biological matter/organisms. These patents are especially broad in scope in the USA (Oldham, 2004), and restrict use by third parties even if only for research purposes. The *research exemption* (i.e. that the biological material may be used for further research without permission of the IP holder) is in principle not valid in the USA. Additionally, the so-called *farmer's privilege*, which allows reuse of seed that has been patented, is not valid either. Until quite recently, under 'research exemption' there was more or less a global reality that scientists/researchers could access most biological matter and related information free of charge or for a symbolic fee – at least if its use was for research only – the so called *bona fide use* formula. This, of course, also included AMiGRs. Further, based on UPOV, farmers were allowed (subject to national legislation) to reuse protected seed without approval of

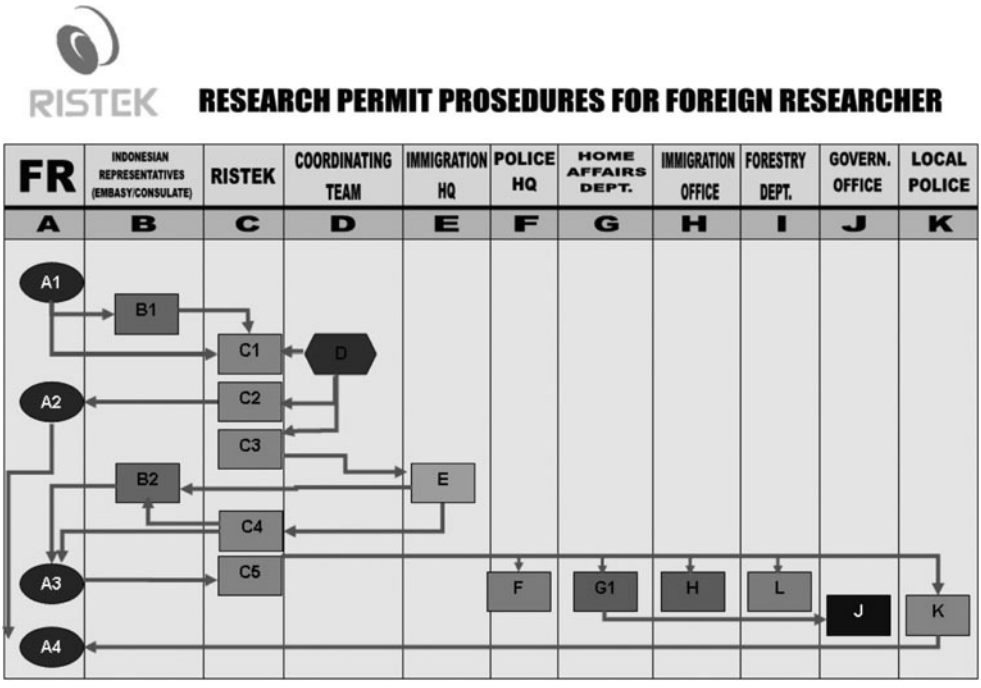

Fig. 20.1. Graphical presentation of the procedures involved from the point at which a foreign researcher (FR) applies to RISTEK (State Ministry of Research and Technology) for a research permit in Indonesia (A1) to the point at which the FR can begin his/her work on the research project (A4) (State Ministry of Research and Technology, 2011).

the owner of the variety. Implementation of CBD and TRIPS provisions in the national and international context have created complicated sector transgressing regulatory processes in which scientists and farmers, among others, have to operate. These global processes have arrested the international transboundary exchange of genetic material and related information (Fowler and Hodgkin, 2004). Figure 20.2 illustrates the continued daily multilateral interdependence of germplasm for plant breeding. The figure shows only a very minor segment of the pedigree of one single plant variety: the Sonalika wheat. Both wide and long, the pedigree reveals that, for this very minor portion of the pedigree alone, breeders used landraces and breeding lines from eight different countries. In all, *four continents* 'contributed' to this small section of a much longer pedigree. Continued national access to other nations' germplasm for plant breeding is thus a global necessity, and something that the FAO ITPGRFA is expected to lay the foundations for. Further, today there are more than 30 different legal, social, biological, ritual, etc. definitions of what comprises a seed 'variety'.

Another illustrative example, this time directly related to microorganisms, is when Dr Craig Venter's 'Ocean sampling expedition' visited the Galapagos Islands in February 2004 and collected some 50 soil and water samples for microbial characterization. Having visited the Islands in December 2003 on behalf of my university, I was asked in January 2004 by staff of the Darwin Station/National Park to assist them in negotiating a 'Memorandum of understanding for a microbial biodiversity collaboration' with Dr Venter's Institute for Bio-Energy Alternatives (IBEA). Because Ecuador is a member of the Andean Community and because of the Community's decision AP391/1996 on a 'Common regime on genetic resources', I foresaw a soap opera ensuing, as the Darwin Station/National

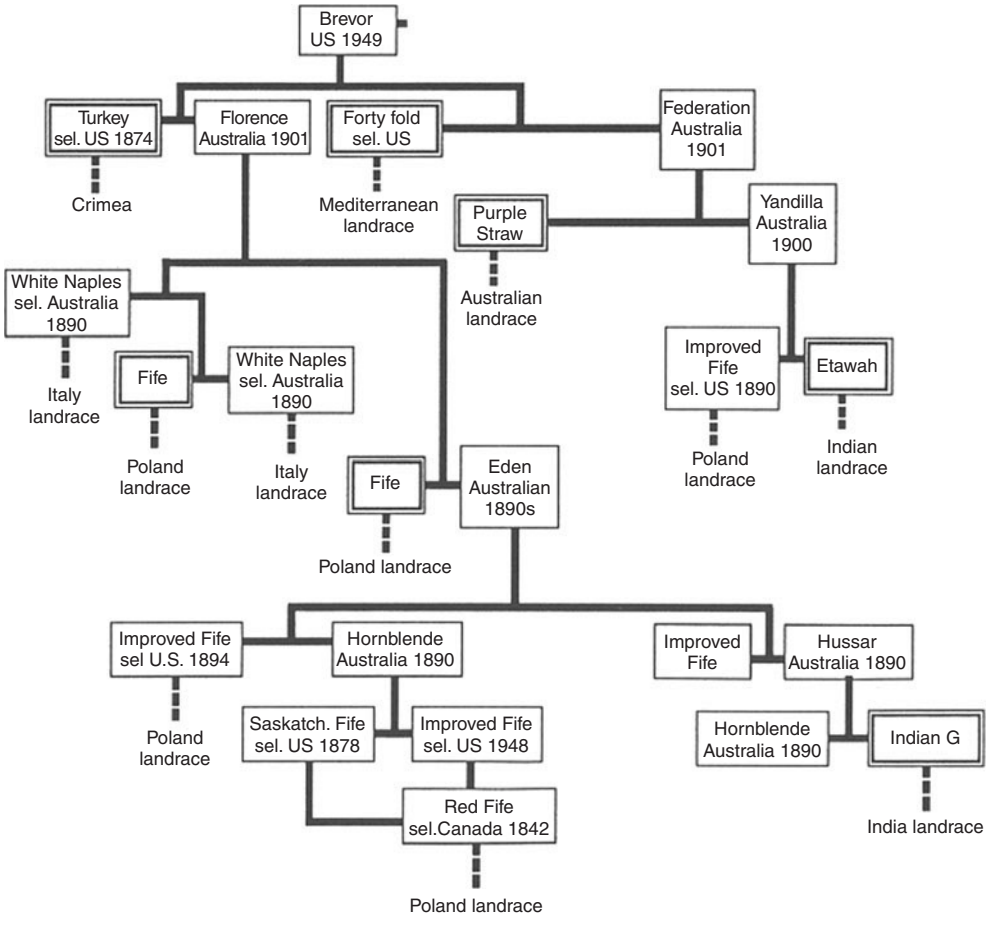

Fig. 20.2. A minor segment of the pedigree of the wheat variety Sonalika (sel., selected) (from Smale, 1996).

Park staff had no clue about the Andean Community regime, or any established procedures as regards access to biological materials by foreign research vessels. It all ended in late 2005, when the Ecuadorian government prohibited IBEA from publishing scientific results derived from microbes collected in the Galapagos, unless the Institute could guarantee that no subsequent products amenable to IP rights would occur. IBEA offered a number of different and reasonable compensations. During 2006–2007, I was deeply involved as informal ombudsman between IBEA and the Ecuadorian Ministry of the Environment to try to find an acceptable compromise. But the case had become heavily politicized – and discussions were no longer fruitful. In 2007, the Venter Institute went public with the results from the 'Ocean sampling expedition' at a press conference in Washington, DC. The Galapagos findings were excluded! In January 2008, informal consultations took place between Ecuadorian officials and the IBEA, but no progress was made. By late 2009, the case seemed dead. I am, however, convinced that as the gunpowder dries, there will be a solution to this very strange ABS-related event.

After the implementation of the CBD in December 1993, the collection of biological/genetic material on sovereign nations' territory by the Consultative Group on International Agricultural Research (CGIAR) and its 15 research institutes (see www.cgiar.org for details) has been reduced, although this varies by crop and

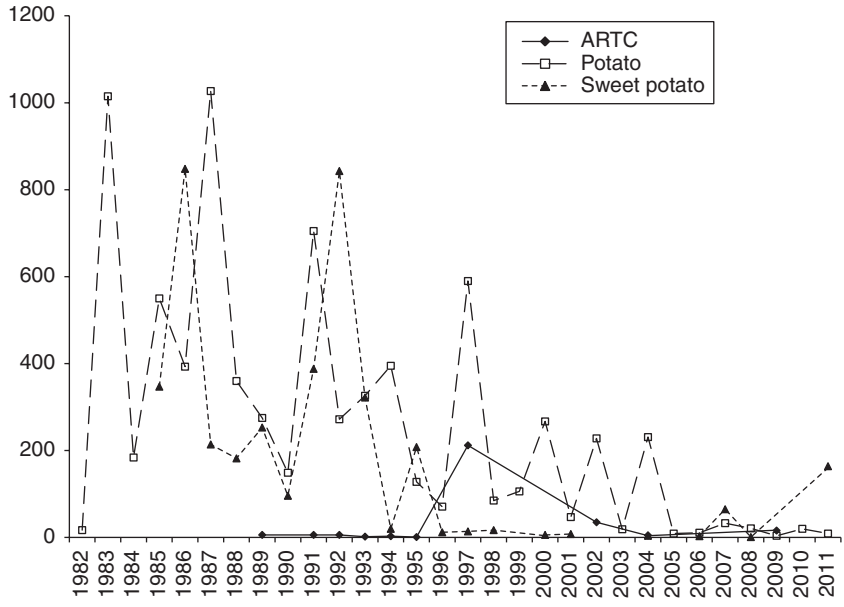

Fig. 20.3. An example of the development over time of the deposition of different plant genetic resource accessions of ARTC (Andean root and tuber crops) into the gene bank of CIP (International Potato Centre) (CIP Gene Bank, Lima, Peru, 2012).

region. One example is acquisitions of potato and sweet potato varieties by the International Potato Centre (CIP) in Lima, Peru (Fig. 20.3), which, like Ecuador, is a member of the Andean Community. The inflow of potato accessions generally dropped between 1990 and 1996. A temporary increase in 1997 was followed by an irregular but relatively low inflow until 2005 – and thereafter very few new accessions. The inflow of sweet potato was quite high from 1985 to 1993, and then there was a heavy drop towards the middle of the 1990s, followed by very low numbers until 2010, and a slight increase in recent years. For Andean Root and Tuber Crops (ARTC), a low inflow from the end of the 1980s to 1992 decreased to almost zero in the following years, but there were higher number of accessions in 1997 and 2002. Overall, inflow after 2004–2005 is amazingly low. It is probable that some of the decrease in accessions is linked to the entering into force of the CBD (1993) and the FAO ITPGRFA (2004), respectively.

This example illustrates that international (multi-local) variety testing within the CGIAR has been strangled as countries (mainly in the South) implement CBD legislation, join UPOV (as part of the WTO TRIPS review) and become aware that biological material offered to other countries for variety testing can be subject to variety protection. Taxonomists, breeders and ethnobotanists who want access to other countries' genetic material and ethnobotanical information are increasingly facing the fact that access is not granted, or is circumscribed with restrictions, and that publications are no longer freely shared with reference to national access legislation. The severe result is that this situation creates legal uncertainties that greatly hamper the international exchange of scientific information and biological material. Most threatened is the concept of *international public goods* (IPG), presently taken more or less for granted, not least in the public sector. Of course, AMiGRs will not escape from being perceived in a similar geopolitical context either, whether in processes within TRIPS, the CBD or the FAO CGRFA (Commission on Genetic Resources for Food and Agriculture).

More than a decade ago, the Monsanto seed company released its transgenic Bt (*Bacillus thuringiensis*)-soya which contains some 30 intellectual property rights (IPR) (patented

gene traits, gene sequences, processes, etc.). The recently developed (and halted because of biosafety regulations) so-called *Golden*Rice™ – a genetically modified rice variety that produce beta-carotene – was a Rockefeller Foundation initiated project and provides further illustration of the necessity for IP transfers in this sort of development project (Fig. 20.4). The project involved, among others, CGIAR's IRRI (International Rice Research Institute) and contains around 70 IPR (Kryder *et al*., 2000).

The example of *Golden*Rice is very important to understand in the context of the FAO ITPGRFA and its Standard Material Transfer Agreement, Article 6.2, which states (concerning any 'Product') that the treaty includes 'the Material or any of its genetic parts or components'. This is because 'parts or components' may include microbes and/or potentially proprietary traits/technologies contained in biological matter.

What is also new here is that while UPOV complies with the *research exemption* and the *farmer's privilege*, this is less and less accepted for genetic materials (i.e. LMOs, including genetically modified (GM) microorganisms, or GMMs) that are wholly patented, such as transgenic seed, or contain patented parts. This is especially the case in the USA. The WTO TRIPS process creates serious concern that demands by the corporate sector for segmented markets, and other restrictions of use by third parties of proprietary genetic material/information, will negatively affect continued implementation of the research exemption and farmer's privilege and thus, in the long run, also public and international research collaboration and national food security. 'Starvation' threatens the public domain, but scientists have so far only felt the first signals, often interpreting them as occasional and not as part of an

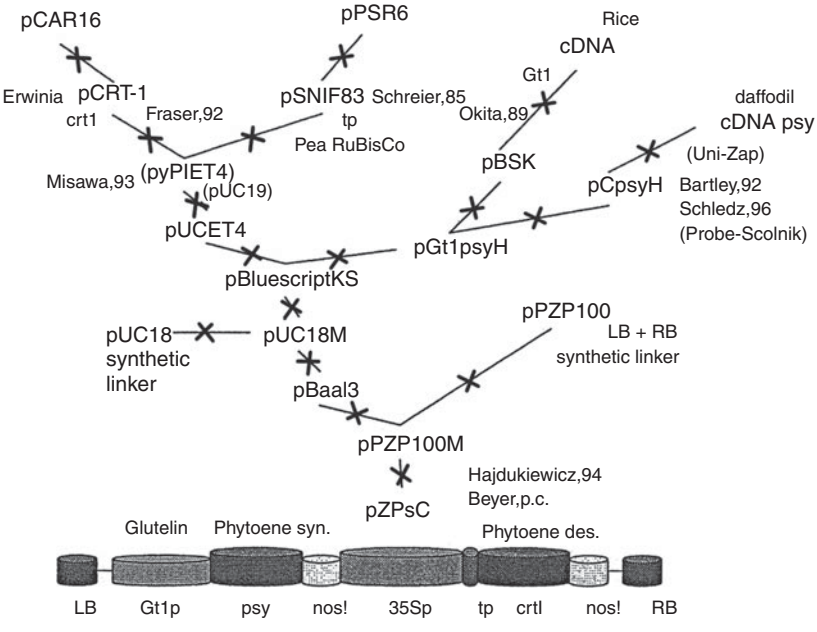

Fig. 20.4. Flow chart for tangible intellectual property (IP) transfers involved in the development of and attempts to implement *Golden*Rice™. The IP pedigree shows for example: patented methods for the isolation/cloning of DNA, patented synthesis/pathways for the production of beta-carotene and patented methods for the regeneration of transgenic plants from transformed cells. Each line with a cross implies a possible proprietary link – thus demanding access – and a licence agreement for use by a non-proprietor (Kryder *et al*., 2000).

emerging permanent new global legal regime for, e.g. GM crops and GMMs.

The TRIPs Agreement states that microorganisms and microbiological processes (involving modified or wild-type isolates) can be patented. 'Microbiological processes' should be understood as 'those used to modify or use unicellular organisms with dimensions beneath the limits of vision'. An important requirement when issuing a patent is that a 'person skilled in the art' should be capable of reproducing the invention with the support of the patent description. For microorganisms and microbiological processes, as well as gene sequences, this requirement is often difficult to fulfil. Consequently, it has been a requirement in the USA and other countries since 1949 that the applicant for a patent must deposit any such microorganism in a culture collection. In 1977, the Budapest Convention established that depositions of this kind are mandatory, forming a guarantee that the invention can be replicated. This was not accepted by Germany until 1987, when depositions of rabies virus were accepted as a sufficient description of a product patent regarding microorganisms per se. However, TRIPs does not require this form of deposition in a culture collection.

Pending a forthcoming international or multilateral regime, for example for agricultural microbial genetic resources (which of course cannot directly copy the regimen for multilateral exchange of seed materials under the FAO ITPGRFA), it is still very important to keep in mind the historical background of extreme interdependency for seed production. The AMiGR – depending on genera – may be multi-local rather than available on only one distinguishable national territory. Moreover, 'simply' to determine the geographical distribution of a specific microbial species – and even more a particular strain/isolate – is a substantial scientific undertaking, as at a species level many microbes are very widely spread. Therefore, it may be the work involved in their isolation and characterization, rather than actually obtaining the microbe in a specific region or country, that can be said to have the main IP value (see Howieson and Fox, Chapter 10, this volume). So then: how to categorize microbes under a multilateral regime – when their biology, e.g. with respect to reproduction, is very different from that of plants and seed 'varieties'?

20.4 Moving into a Global 'Legal Can of Worms'?

In a recent study (CGIAR, 2010, p. 21), the following observation was made on microbial and insect genetic resources:

> more than 53,000 accessions of living microorganisms or cell cultures and an astounding collection of insects and other arthropods numbering about 420,000 are collectively held in the CGIAR Centers. The … survey also contacted 28 bioresource centers worldwide, of which 26 responded showing more than seven million accessions of living and nonliving materials.

But what is the *legal status* of these 7 million accessions in the context of the CBD, FAO ITPGRFA, TRIPS and UPOV? The CGIAR study gives the following answer (CGIAR, 2010, p. 21):

> most collections of non-crop genetic resources are used by researchers to develop their activities and sometimes specimens are exchanged with partners, without due regard to IP or the access and benefit sharing regulations. Can these accessions be regarded as Global Public Goods? Or are they only genetic resources for current research? It seems that a serious discussion is needed as basis for further investment on conservation, capacity building, and infrastructure or to establish connections with local institutions and international repositories for the maintenance of the specimens that are indispensable for the research projects.

The CBD is one of the fastest approved international legally binding agreements. Negotiations started late 1987 and the final text was endorsed in Rio de Janeiro in 1992 and entered into force in December 1993. The CBD places every living cell and its derivatives on the planet (with the exception of humans) under national sovereignty in which access by other parties to the Convention is subject to the PIC/MAT procedures as adopted in different national

legislations. The negotiations for an exception covering plant genetic resources for food and agriculture started as early as 1983 and resulted in the FAO ITPGRFA 2001; this was implemented in 2004. In the history of international treaties, the link between the CBD and FAO ITPGRFA is unique, because negotiations for exceptions from the emerging CBD convention started without knowing the exact content in the most likely forthcoming convention. Policy making is an extremely complex game, especially when advanced genetics, evolution/genetic drift and legal matters are to be usefully – and fairly politically correctly – combined. To expect that the discussions around genetic resources for food and agriculture led by national ministries of agriculture (starting on larger scale in the 1970s) would have influenced the discussions on implementing benefit sharing under CBD would be naive, because the ministries of agriculture did not coordinate national positions with their own ministries of the environment. This is still happening: during the latter part of that period (i.e. from the late 1990s) a similar negotiation process was ongoing, led by national ministries of the environment to establish yet another internationally binding agreement, which was adopted in October 2010 by parties to the CBD – the Nagoya-Cali Protocol on Access and Benefit Sharing.

The Genetic Resources Scoping Study (CGIAR, 2010, p. 70) also makes the following observation regarding the text of the Nagoya-Cali protocol:

> While a certain degree of creative ambiguity is a hallmark of international accords, the text of the Nagoya protocol has left experts puzzled about what exactly has been agreed on for many critical issues, including the substantive and temporal scope of the agreement and the application of the definitions – derivatives and utilization, giving rise to a range of partially conflicting interpretation.

Notably, it is, e.g. unclear whether the Nagoya-Cali protocol covers microorganisms – and if so, how?

Over the years, issues related to infringement on patented crop seeds/genetically modified organisms (GMOs) – although very few countries currently allow patents on crop seeds, monopolies, denial of farmer's privileges, etc. have created a lot of opposition from G77 countries and NGOs (non-governmental organizations) in international fora such as the CBD, FAO and TRIPS. The main objections relate to a perceived risk of increased expansion of monopolies by the private sector on materials and knowledge that have been considered to be global commons.

20.5 Moving Out of the Can to Exploit Science Constructively for the Benefit of Humankind?

During the Cali negotiations for the CBD ABS protocol, it was agreed that AMiGRs would need a specialized ABS agreement/solution (SGRP, 2010):

> One possible approach would be to develop a virtual common pool of AMiGR[s], which would be available for use in agricultural research and production and subject to pre-agreed terms and conditions. It would be 'virtual' in the sense that the strains would not be pooled in a single location, but would help create and be part of a network of holders of microbial collections around the world that agree to participate in the creation of the pool.

Moreover, the CGIAR Genetic Resources Scoping Study identified the policy needs according to the following (CGIAR, 2010, p. 30):

(a) identifying patterns of exchange and use of agricultural microbial genetic resources;
(b) possibilities for addressing policy-related challenges to globally coordinated efforts to conserve and make available (with considerably lower transaction costs) microbial genetic resources for use in agricultural production and research;
(c) the impact of IPRs and seed laws on plant genetic diversity 'in the field'; and
(d) analyses of the effectiveness of the Treaty overall (and its various relevant components) and diagnoses of challenges to its successful full implementation.

By proposing specialized ABS agreement/solution for AMiGRs, we have still only

opened a minor window in Pandora's box. How much of the other different biological matter of the around 20 different categories shall we classify for the development of separate regulatory regimes? Because of their huge differences with respect to life form and biological properties, each of these could require a different ABS regime.

20.6 Still in the Can … for CGIAR

Genetic policy is to: 'analyze the relationships between the politically desirable, the technically and biologically possible and the socio-economically defensible' (Bengtsson and Thornström, 1998). This observation, originally made 15 years ago, alludes to the 2010 CGIAR observation regarding the Nagoya-Cali protocol mentioned earlier, which: 'left experts puzzled about what exactly has been agreed'. Just to provide an illustration of a few of the reasons for this puzzling situation, we can go back to the issues of (different) life forms and legal forms of control and appropriation as summarized by Howieson (2007):

> Because of a combination of factors concerning microbes used in agriculture – for example, their deployment in open environments; their extremely fast rates of reproduction and variation; their small size and portability; and historical patterns of use and distribution – it is difficult, and often impossible, to subject them to legal forms of control or appropriation.

So, what are possible ways out of this need for translating the politically desirable (all the way through ideological and almost eschatological partial fogs) into socio-economically defensible solutions/agreements, whose implementation the world community is prepared to fund?

20.7 … to Enter into Public–Private Partnerships (PPPs)

Another bottleneck for the exchange and implementation of biological material is the Rio+10 credo (the CBD – 10 years later) adopted in Johannesburg in 2002, regarding the need for increased Public–Private Partnerships (PPPs). To conform with the Johannesburg agreement, the CGIAR is expected to produce international public goods, while at the same time increasing its collaboration with the private sector; and this, especially since the 1980s, in an increasingly proprietary science and technology world created by the same governments that support the CGIAR. The expansion of IPR – especially patents – into biological matters challenges issues such as stability, annual/perennial, evolution, genetic drift, etc.

So how can members of OECD (Organisation for Economic Co-operation and Development) in favour of IPG with PPPs solve the equation? Or quite simply put: how can we produce international public goods while still respecting private property clauses? So far, most remain silent when we ask them for an informed forward-looking and useful opinion. One of the great challenges to the public sector is in the PPPs and confidentiality agreements set up by the private sector to protect intellectual innovations under IP laws. From the industry perspective, the reason is simple and clear: why should the private sector invest in PPPs if they see no financial gains pending?

During the CGIAR Mid-term-Meeting in Cairo in May 1997, the Chair of its Private Sector Committee, Mr Sam Dryden, then Chief Executive Officer from Emergent Genetics, reminded the CGIAR donors in the plenary that the rules in advanced agri/biological research were changing (because of the CBD, FAO, TRIPS, etc.), creating new legal boundaries, and thus having an impact on IPG and CGIAR. He asked the CGIAR to tell the industry representatives under which rules the CGIAR wanted to collaborate with the private sector? Few CGIAR donors understood the question or the implications for the system in relation to Mr Dryden's almost historical message. … Interestingly, this was in 1997, some 5 years ahead of the Johannesburg treaty on PPPs.

20.8 With Some Additional Work to Do …

Over the last 10 years, the CGIAR has been a prominent but somewhat silent (for a quite

uninterested surrounding world of donors) scene for an intellectual battlefield, trying to satisfy its donors requesting IPG produced in an increasingly proprietary biological world created by the very same donors! This fact is alarming or, at worst, more or less close to intellectual schizophrenia. In the Genetic Resources Policy Committee (GRPC) of the CGIAR (details available at: http://www.cgiar.org/who/structure/committees/grpc_index.html), we struggled between 1999 and 2010 to try to develop policies that would allow CGIAR centres to be attractive enough for partnerships with the private sector. In a recent draft document from the current CGIAR Consortium Board/Chief Executive Officer, an attempt was made – based on our 10 year work in the now dismantled GRPC – to formulate recommendations on how demands from the private sector for exclusivity can be combined with demands for IPG by the CGIAR. Just to complicate things further, we had as a starting point to realize that the 11 gene banks of the CGIAR are not owned by the CGIAR centres, but are legally and policy wise under the auspices of the Governing Body of the FAO ITPGRFA, with which each CGIAR-centre signed agreements in October 2006.

So in short: the trick for us in the GRPC was to make the IPG-producing CGIAR centres, which did not own their own gene banks, attractive to the private sector. For example, in such a situation, how could we arrange for public access to proprietary advanced biotechnologies and cutting edge, legally protected, genomic tools/information? After many years work in the GRPC, a final text was delivered in May 2010. In February 2011, a draft revised *Annex 1 Briefing paper on IP* was circulated on a restricted basis among CGIAR Director Generals and Board chairs. On the subject of PPPs in the CGIAR Consortium, the draft stated:

> –The Consortium and its Members may, in accordance with applicable law, impose or allow restrictions to the global availability of the intellectual assets they produce only if such restrictions further their mission by significantly enhancing the scale or scope of impact on target beneficiaries in developing countries, as provided hereunder.
>
> –The Consortium and its Members may strategically grant exclusivity for commercialization of their intellectual assets when such exclusivity is indispensable or invaluable for the further improvement or effective utilization of such intellectual assets, provided (a) it is as limited as possible in time, territory and/or field of use and (b) that these intellectual assets remain available for research and development in developing countries.

This text was later further developed into IP and IA (intellectual assets) management guidelines and policy. These were turned down by the CGIAR Consortium's Fund Council at a meeting in Washington, DC in July 2011. As this is being written (October 2011) revised texts are being worked on to be discussed by the Fund Council at its meeting in Rome in November 2011. Whether the finally adopted texts will be in harmony with the provisions set forth in the FAO ITPGRFA, and the agreement with this treaty by the CGIAR centres, will be a matter for further analysis. The expression 'remain available for research and development' (second point above) may – unless further clarified – compromise confidentiality in the eyes of the private sector or, as recently indicated from the Board chair of one of the CGIAR centres: 'We cannot possibly accept that we will have to provide unrestricted access to all agreements' and 'At the same time, we are very aware of our obligations as a publicly-funded research organization, that has a mandate to produce public goods. Balancing those two issues requires a very sophisticated IP policy'.

Yes, it does require a very sophisticated IP policy; and it may take quite some more time to elaborate that sophisticated IP policy in a way that satisfies the IPG ambitions of the CGIAR with other and contrary demands by the same stakeholders: intensified collaboration with the private sector.

20.9 Biological Matter and the Law

In this chapter, I have tried to take a broad look at public agricultural research and the exploitation of biological resources in the context of the different international regulatory regimes that have emerged. To 'analyze the relationships between the politically desirable, the technically and biologically possible and

the socio-economically defensible' (Bengtsson and Thornström, 1998) is still a challenge, not only for the CGIAR Consortium, but also for most public R&D institutions dealing with biological matter and related information. If reasonable PPPs are to be established and delivered, researchers in the public sector must stop pushing things under the carpet, and instead be realistic about the politically correct vis-à-vis accepting realities in our ever-deepening understanding of biology, genetic drift and evolution.

Thus – given current geopolitical realities – there is a need for the training of scientists in the public sector, but also of scientists in NGOs and in the private sector, on the complex legal issues that surround the exchange and use of biological matter. One example is the advanced training programme GRIP (Genetic Resources and Intellectual Property Rights) that I lead (from 2003 to 2013), which is supported by SIDA (the Swedish International Development Cooperation Agency) (details available at: https://www.slu.se/grip).

We are reluctant to accept the rapidly emerging 'proprietary science landscape' – actually created by ourselves – which constrains rather than encourages the securing of and clever use of biodiversity. To similar effect, TRIPS at its present stage of development may discourage intellectual innovation by introducing 'patent carpets' and biological monopolies that nobody may find useful to humankind in the long run – and the quite alarming challenges that we have from climate, biodiversity, ecosystems and global food security. Microorganisms are somewhere there at the base of those challenges. We must rationalize their sustainable use, as well as that of other biological matter, whether in the CGIAR, in the private sector or at public sector universities and the like in the North and in the South.

Appendix: Abbreviations

ABS	Access and Benefit Sharing
AMiGR	Agricultural Microbial Genetic Resource
ARTC	Andean Root and Tuber Crops
CBD	Convention on Biological Diversity
CGIAR	Consultative Group on International Agricultural Research
CGRFA	Commission on Genetic Resources for Food and Agriculture
CIP	International Potato Centre
CO/DO	Certificate/Disclosure of origin
FAO	Food and Agriculture Organization (of the United Nations)
FAO ITPGRFA	International Treaty on Plant Genetic Resources for Food and Agriculture
FTO	Freedom to Operate
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
GRIPS	Genetic Resources and Intellectual Property Rights (SIDA programme)
GRPC	Genetic Policy Resources Committee (of the CGIAR)
IA	Intellectual Assets
IBEA	Institute for Bio-Energy Alternatives
IGCGRTKF	Intergovernmental Committee on Genetic Resources, Traditional Knowledge and Folklore (of the WIPO)
IP	Intellectual Property
IPG	International Public Goods
IPPC	International Plant Protection Convention
IPR	Intellectual Property Rights
IRRI	International Rice Research Institute
LDC	Least Developed Country
LMO	Living Modified Organism

MAT	Mutually Agreed Terms
MTA	Material Transfer Agreement
NGO	Non-Governmental Organization
OECD	Organisation for Economic Co-operation and Development
PIC	Prior Informed Consent
PPP	Public–Private Partnerships
PVP	Plant Variety Protection
SGRP	System-wide Genetic Resources Programme (of CGIAR)
SIDA	Swedish International Development Cooperation Agency
SNL	Subject to National Legislation
TK	Traditional knowledge
TRIPs	Trade-Related Intellectual Property Rights
UPOV	International Union for the Protection of New Varieties of Plants
WIPO	World Intellectual Property Organization
WTO	World Trade Organization

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21 OECD Guidelines and Harmonization for Microbial Control Agents

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21.1 Introduction

International efforts are ongoing to harmonize the way that microbial pest control agents are regulated in different regions of the world. The Organisation for Economic Co-operation and Development (OECD) has established the BioPesticide Steering Group (BPSG) to address this subject, and several issue papers and guidelines have been produced. This chapter gives an overview of the current main discussion internationally on microbial pest control agents, and of how the work of the BPSG has influenced current legislation and registration processes.

The use of microbial biological control products (MBCPs) is an increasingly important approach for achieving a more sustainable agriculture, an agriculture that is less polluting and less dependent on synthetic chemical pesticides. MBCPs are allegedly less persistent and hazardous than their synthetic counterparts. Various such products have been used throughout the world without demonstrable negative effects to human health, wildlife and the environment; however, some microorganisms used in agriculture or horticulture may be infective or contain toxicants. For example, under particular conditions, strains of microorganisms may produce toxins which have adverse effects on human health or the environment. A proper safety evaluation of MBCPs before they are placed on the market is, therefore, essential in order to guarantee their safe use (Mensink and Scheepmaker, 2007).

The cost associated with the research and development of a MBCP, the dossier preparation by industry, the evaluation, possible acceptance and approval by the regulatory authorities, and finally the marketing of MBCPs, can be significant. Harmonization across countries would facilitate the preparation and evaluation of necessary dossiers and monographs in situations where a product could be placed on several markets. Various initiatives have been undertaken to harmonize the regulatory requirements for safety evaluations of MBCPs so that a dossier submitted to one member country and the monograph developed from that dossier can be used by other OECD countries (OECD, 2004d).

Historically the first microbial pesticide was registered in 1948 in the USA. This was *Paenibacillus popilliae* (as *Bacillus popilliae)* for the control of Japanese beetles. The first baculovirus for use as a biological insecticide was registered in 1975, also by the USA Environmental Protection Agency (US EPA). The first viral insecticide was approved in 1987 in Switzerland, while the first virus for use in greenhouse crops was approved in 1993 in the Netherlands (Ravensberg, 2010).

In the framework of this chapter, the definition for microorganisms according to Regulation (EC) No 1107/2009 (OJEU, 2009a) is used: 'any microbiological entity including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material'. In this respect, microorganisms, microbes, microbials, microbial biological control agents (microbial BCAs, or MBCAs) and microbial pest control agents are considered synonyms.

21.2 About OECD

OECD was established in 1961 and is an intergovernmental organization in which representatives of the member countries in North and South America, Europe, the Asia and Pacific region, as well as the European Commission, meet to coordinate and harmonize policies, discuss issues of mutual concern, and work together to respond to international problems. Presently, the OECD has 34 member countries and the headquarters are located in Paris, France. More than 70 developing and transition economies are engaged in working relationships with the OECD. Among currently enhanced engagement countries are Brazil, China and India. The number of member countries, as well as associated economies and international organizations such as the World Health Organization (WHO), United Nations Environment Programme (UNEP), Food and Agriculture Organization (FAO) and the European Commission (EC), working together in an international framework, stresses the importance of an organization like OECD.

Most of OECD's work is carried out by more than 200 specialized committees and working groups composed of member country

delegates. For more than 40 years, OECD has been one of the world's largest and most reliable sources of comparable statistics and economic and social data. As well as collecting data, OECD monitors trends, analyses and forecasts economic developments and researches social changes or evolving patterns in trade, environment, agriculture, technology, taxation and more. OECD is also one of the world's largest publishers in the fields of economics and public policy. Its publications are a prime vehicle for disseminating OECD's intellectual output, both on paper and online.

How do pesticides, and in particular microbes, fit into all this? One of the fields in which OECD is actively involved is the sustainability of agriculture. OECD's work on agricultural pesticides (i.e. chemical and biological pesticides) aims to help member countries improve the efficiency of pest control, share the work of pesticide registration and reregistration, minimize non-tariff trade barriers and reduce risks to human health and the environment resulting from their use. In support of these goals, the Pesticides Programme has undertaken work to: (i) identify and overcome obstacles to work sharing; (ii) harmonize data requirements and test guidelines; and (iii) harmonize hazard/risk assessment approaches.

21.2.1 Working Group on Pesticides

Initially, the OECD Working Group on Pesticides (WGP) focused mainly on conventional chemical pesticides. Due to increased interest in a more sustainable agriculture and the fact that all available OECD guidance documents were written from a chemical perspective – and in that respect were not very user friendly for the registration of biopesticides – the WGP decided to establish a separate Steering Group dealing with the authorization of biopesticides and related issues: the BioPesticides Steering Group (BPSG). In this respect, 'microbes' are covered under the BPSG.

The WGP is currently assisted by three steering groups: the Registration Steering Group (RSG), the Risk Reduction Steering Group (RRSG) and the BPSG. The Working Group and Steering Groups are chaired by representatives from OECD member countries and assisted by the OECD Secretariat. Documents produced by the WPG are circulated to the Joint Meeting of the Chemicals Committee for declassification, followed by publication. The RSG covers issues such as work sharing, global and/or joint reviews and the electronic exchange of pesticide data, and regularly carries out surveys on a variety of subjects. The RRSG addresses issues such as risk indicators and obsolete pesticides, and organizes seminars on risk reduction strategies on a regular basis.

21.2.2 BioPesticides Steering Group

The BPSG was established by the WGP in 1999 to help member countries harmonize assessments of biological pesticides and improve the efficiency of control procedures. Biological pesticides include: microbials, pheromones and other semiochemicals, plant extracts (botanicals) and invertebrates. The BPSG was chaired by Canada from its inception and by the Netherlands from mid 2005 and onwards. The first tasks of the BPSG consisted of: (i) reviewing regulatory data requirements for three categories of biopesticides (microbials, pheromones and invertebrates); and (ii) developing formats for dossiers and monographs for microbials on the one hand, and for pheromones and other semiochemicals on the other hand.

OECD's work initially focused on ways to harmonize the format/structure of reviews that are exchanged, with the primary goal of facilitating the sharing of national review reports. The OECD dossier and monograph guidance provide general layouts and standardized formats for industry applications (dossiers) and government reviews (monographs or – as they are called in the European Union (EU) – draft assessment reports, or DARs).

Participating members of the OECD BPSG from the start were Belgium, Canada, Denmark, Germany, the Netherlands, Sweden, Switzerland, the UK, the USA, the European Commission and the International Biocontrol Manufacturer's Association (IBMA). Currently,

Australia, Austria, China, France, Italy and Japan also participate on a regular basis. The BPSG meets once a year. The IBMA plays an important role as a spokesman for the industry and participates in many activities with authorities and other organizations.

21.3 OECD Guidance on Dossier and Monograph Format

In the OECD Guidance Documents, the format and contents for a dossier are described in 'documents' A–O and for a monograph (DAR) in 'volumes' 1–4. OECD Guidance Documents were available for conventional chemicals; however, these were not suitable for facilitation of dossier submission and the evaluation of microorganisms. Therefore, as one of its first tasks, the BPSG completed equivalent Guidance Documents tailored for microorganisms in 2004, which were revised in 2006 (OECD, 2004b,c).

A dossier contains information organized in the following documents:

- Doc A (purpose and context)
- Doc B (collective dossier)
- Doc C (label)
- Doc D1 (supported uses)
- Doc D2 (registered uses)
- Doc D3 (import tolerances) – *deleted for microbials*
- Doc E1, E2 (MRL information) – *deleted for microbials*
- Doc F (statement of intention to submit a dossier)
- Doc G (regulatory position of formulants)
- Doc H (safety data sheets for formulants)
- Doc I (other available toxicological information on formulants)
- Doc J (confidential information)
- Doc K (individual test and study reports)
- Doc L (Tier I quality checks for individual tests and studies and reference lists)
- Doc M (Tier II summaries and assessments of individual tests and studies and groups of tests and studies)
- Doc N (Tier III overall summary and assessment, conclusions and proposed decision)
- Doc O (completed forms for the checking of dossiers for completeness).

The documents in the dossier are related to the structure of the monograph in a specific way (Table 21.1).

Summaries as provided in the OECD format dossiers (documents M and N) make up a significant proportion of the applicants' efforts for dossier preparation; however, these are considered to be very useful by the scientific evaluators for the preparation of monographs or DARs, especially from a time-saving perspective. Initiatives are developed for a more streamlined procedure to convert these summary documents into monographs and avoid a redundancy of work.

Table 21.1. Structure of a draft assessment report and its relationship to the different documents of the dossier.

DAR/Monograph			Dossier
Volume 1	Level 1	Purpose and context	Doc A, F
		Collective dossier	Doc B
		Identity and use	Doc C, D1, D2
	Level 2	Overall conclusions	Doc N
	Appendix 1 and 2	Standard and special terms	–
	Appendix 3	List of end points	Doc D1, N
	Level 3	Proposed decision	Doc N
	Level 4	Further data to be submitted	Doc M
Volume 2	Annex A	Lists of tests and studies	Doc I, J, L
Volume 3	Annex B	Summary and assessment	Doc D1, K, L, M
Volume 4	Annex C	Confidential information	Doc B, G, H, I, J

The format is also related to the OECD numbering system. Every OECD data point has its own number, although this does not necessarily correspond to the numbering systems used by different member countries. To overcome this obstacle, a cross-walk table was developed with a comparison of the different number codes used in the EU, USA, Canada, Japan and Australia (Appendix 6, Parts 4 and 5 in the OECD *Guidance for Industry Data Submissions for Microbial Pest Control Products and their Microbial Pest Control Agents*; OECD, 2004c). As all new dossiers and monographs for active substances in the EU must be in the OECD format as of 1 January 2005, and in order to better align the numbering for microbials, new numbering for the OECD Microbial Dossier and Monograph Guidance Document was adopted by OECD in 2006.

Both the USA and Canada also accept a dossier in the OECD format, which already facilitates the international exchange of information. However, other OECD member countries are strongly encouraged to use this harmonized numbering system to enable even easier – electronic – transfer of data and finally to produce monographs in OECD format.

In this respect, the development of templates for documenting test results should also be mentioned. Proposals were developed by Canada in 2005. These templates were tested by BPSG members and initially considered to be too detailed. It was concluded that there is a need to gain more experience before deciding on the issue of 'OECD templates' for documenting test results for microbials. Canada continues work on revision of the templates.

21.4 Registration Procedures

Before manufacturers can sell any product which claims pesticidal activity in an OECD country, the respective agency in that country has to evaluate it thoroughly to ensure that it meets safety standards to protect human health and the environment. A safety evaluation should take into account the characterization and identification of the microorganism and other components of the product, the efficacy of the product and its release in the environment during and after application. Subsequently, the exposure and effects (toxicity, infectivity) on humans and non-target groups such as birds, bees or fish are assessed. However, scientific and technical guidance on the safety evaluation of microbials is scarce.

The regulatory frameworks for the USA, Canada and the EU are briefly presented below in order to provide the reader with a better understanding of the role of the OECD BPSG in the harmonization of the registration process and requirements. The registration process evaluates the risks of the *active substance* and of the *products* in relation to the intended use of the products.

21.4.1 The USA

The US EPA has a separate division dedicated to regulating biopesticides. The EPA works together with the various states, some of which also require registration of pesticides and are involved in enforcement activities (Braverman *et al*., 2010). Registration of microorganisms is authorized by the Biopesticides and Pollution Prevention Division of the EPA. The data requirements for biopesticides were recently updated (US EPA, 2007). The definition was changed from 'microbial agent' to 'microorganism'. Other changes include the requirement for more mutagenicity studies with metabolites, for immunotoxicity studies and for an additional avian study. The requirement for an intracerebral study was removed.

21.4.2 Canada

Health Canada's Pest Management Regulatory Agency (PMRA) is responsible for the federal regulation of pest control products under the Pest Control Products Act (PCPA). The PMRA also develops pest management policies and guidelines, promotes sustainable pest management, enforces compliance with the PCPA, and distributes pest management information to the general public and key stakeholders. Besides acceptable risk, all biopesticides granted approval for use

in Canada must demonstrate their efficacy, and thereby their contribution to sustainable pest management. In Canada, data requirements are outlined in Regulatory Directive DIR 2001-02 (PMRA, 2001).

21.4.3 European Union

The EU registration is divided into two parts. The first deals with the *approval* of the active substance at EU level (formerly referred to as *listing into Annex I to Directive 91/414/EEC;* OJEC, 1991). The designated Rapporteur Member State (RMS) evaluates the dossier on behalf of the EU and prepares the Draft Assessment Report (DAR). Further evaluation and peer review is done by the member states and the European Food Safety Authority (EFSA). Following this evaluation, the European Commission (EC) – in consultation with the member states – decides on approval or non-approval of the active ingredient (OJEU, 2009a). After approval of the active substance, the second part of the registration is evaluation of the formulated plant protection product at the zonal level, followed by authorization at the national level of member states. Uniform Principles (OJEU, 2005) ensure that the evaluation and decisions with regard to authorization of MBCPs are conducted in a harmonized way by member states.

Major differences between the EU and North America are the separate procedures needed in the EU for the active substance and the plant protection product (MBCP); there is only one procedure in the USA and Canada. Also, in the USA and Canada, the registration process is handled by one authority in each country while in the EU, 27 member states, the EC and EFSA are involved.

Legal framework to reduce the risk of conventional pesticides

It is noteworthy that new legislation concerning plant protection products was adopted in the EU in 2009. This concerns the following four pieces of legislation:

- Regulation (EC) No 1107/2009 for the placing of plant protection products on the market (OJEU, 2009a)
- Directive 2009/128/EC establishing a framework for Community action to achieve the sustainable use of pesticides (OJEU, 2009b)
- Directive 2009/127/EC with regard to machinery for pesticide application (OJEU, 2009c)
- Regulation (EC) No 1185/2009 concerning statistics on pesticides (OJEU, 2009d).

The new Regulation (EC) No 1107/2009 was published 24 November 2009 and applies in all member states from 14 June 2011. This new regulation replaces Directive 91/414/EEC. It introduces a number of new areas, including safeners, synergists, co-formulants, adjuvants, basic substances and low-risk substances. The EU will also be divided into three zones (northern, central and southern) and will have zonal RMSs. The regulation also includes other features such as cut-off criteria, comparative risk assessment, consideration of estuarine and coastal waters, and efficacy and consideration of vulnerable groups. Due to the number of deadlines included in this Regulation, it can be expected that it will speed up the decision-making process.

The sustainable use Directive 2009/128/EC aims at reducing the risk linked to the use of pesticides, improving the quality and effectiveness of pesticide application equipment, ensuring better training and education of users, and developing integrated pest management (IPM) schemes. In this respect, member states shall adopt National Action Plans to set up their quantitative objectives, targets, measures and timetables to achieve the goals as set out in the sustainable use Directive. The machinery Directive, 2009/127/EC, which aims at the reduction of the adverse effects of pesticides, and the Regulation on statistics, (EC) No 1185/2009, according to which up-to-date data on sales and use of pesticides at Community level have to be provided, are supportive to Regulation (EC) 1107/2009 and Directive 2009/128/EC. It is believed that this new legislation will facilitate registration and promote the use of biological products.

21.5 Data Requirements for Microorganisms

Data requirements for MBCAs in the USA, Canada and EU are largely similar in practice (Hauschild *et al.*, 2011; and Hauschild, Chapter 6, this volume). A substantial difference is that the PMRA in Canada requires a complete dossier on efficacy and phytotoxicity data, whereas the US EPA does not. In the EU, an overview of efficacy data is required for the decision on the approval of the active substance. This aspect will be dealt with in more detail at national level at the stage of product authorization.

The history of the development of data requirements for entomopathogens started in the 1960s in the USA, and a little later in Europe, first for baculoviruses, then for bacteria, with fungi following later. Many data requirements are still not appropriate for living organisms and most countries use their own sets of data requirements or use the ones developed for chemicals with some modifications. In the EU, it was only in 2001, when Directive 2001/36/EC (OJEC, 2001) came into force, that a standardized set of data requirements became available for all EU countries. Even later, Directive 2005/25/EC (OJEU, 2005) was published containing the Uniform Principles for microorganisms.

Review of regulatory data requirements by the OECD BPSG for three categories of biopesticides (pheromones, microbials and invertebrates) was achieved in 2004 and resulted in several OECD publications in the Series of Pesticides (OECD, 2001; 2003; 2004a). The microbials document (OECD, 2003) needs to be revised in the light of current developments.

It can be concluded that data requirements are still not well defined, and are often adapted from the requirements for chemicals, which are generally not appropriate for microorganisms. Also, a number of data requirements are unclear or ambiguous, and guidance documents are needed to improve this situation. These requirements relate predominantly to issues on taxonomy, metabolites, stability and genotoxicity.

The EU anticipates finalizing the updating and amending of the data requirements for chemicals in 2011. In the near future, the EU will also start the process for updating the data requirements for microorganisms. It is anticipated that a number of concerns, e.g. the fact that current data requirements are not appropriate for microorganisms, can be overcome.

21.6 REBECA: Regulation of Biological Control Agents

Microbials, botanicals and semiochemicals are regulated under legislation originally developed for chemical pesticides. Therefore, in 2006, the project Regulation of Biological Control Agents (REBECA) was initiated in Europe to develop and propose more efficient regulatory procedures, to reduce costs and, at the same time, to maintain the level of safety to producers and users of these compounds and to consumers of agricultural products. As part of this project, current legislation requirements and regulatory processes for BCAs were reviewed and compared between the EU and other countries such as the USA, Canada and Australia, where BCAs have easier access to the market. Potential risks were evaluated and a cost–benefit analysis of regulation was performed (Ehlers, 2011; Strauch *et al.*, 2011). The REBECA project brought together stakeholders from industry, science, regulatory authorities, policy and environment to disseminate knowledge and experience in the regulation and safety of BCAs and to identify those fields that need further research to assist in the regulatory process.

21.6.1 Baculoviruses

An example of a topic discussed within REBECA was how to deal with baculoviruses, which can be regarded as harmless as far as effects on human health and the environment are concerned. However, product components other than the baculovirus itself resulting from the *in vivo* production process could be problematic (e.g. microbial contaminants).

It was proposed that a separate document with generally applicable contamination limits should be developed. This recommendation has been taken up by the OECD BPSG and has resulted in a paper based on an already available Canadian proposal that covers plant protection products containing baculoviruses and other microorganisms (*see also* 21.10.1 OECD, 2011b).

Another aspect to be considered here is that the evaluation of microorganisms generally needs to be carried out at the strain level. Based on available taxonomic data, the MBCA will be evaluated and authorized at the strain level. For the EU, this is laid down in a SANCO (the EC Directorate-General for Health and Consumers) document (European Commission, 2005). In the meantime, the EC has adopted a more streamlined procedure for baculoviruses. Based on the recommendations from REBECA and conclusions from the OECD Consensus Document on baculoviruses (OECD, 2002), the EU has decided to approve baculoviruses (not genetically modified) at species level, and this is described in a special guidance document (European Commission, 2008).

The OECD BPSG has continued to work on a number of recommendations from the REBECA project for the improvement of procedures as well as data requirements, and is developing guidance for several data requirement issues.

21.7 Working Document on the Evaluation of Microbials for Pest Control

After completion of the harmonization of the format/structure of dossier and monograph guidance for microbes, the BPSG concentrated its efforts on scientific and technical issues that remain as barriers to harmonization and work sharing. Five areas were identified: (i) taxonomy; (ii) genetic toxicity; (iii) operator and consumer exposure; (iv) residues in treated food crops; and (v) efficacy evaluation. This resulted in the preparation of a Working Document (OECD, 2008) which describes the views of different OECD countries on how they assess these scientific issues in the safety evaluation of MBCPs. It is intended to be used as guidance in the safety assessment of microbials, and in the preparation and evaluation of the dossiers and monographs for MBCPs. The Working Document does not provide mandatory rules but is essentially a set of examples/case studies aimed at helping: (i) the industry to submit relevant data for risk assessment; and (ii) the regulatory authorities to be better prepared to review the submitted dossiers and monographs. The conclusions, recommendations and follow-up of the document are summarized below.

21.7.1 Taxonomic identification of microorganisms in MBCPs

When submitting a dossier on an MBCP, the MBCA in the product needs to be identified at the highest possible level of detail. Where possible, the microorganism should be specified at strain level, and the strain should be deposited in an internationally recognized culture collection. When not possible (e.g. for viruses with mosaic genomes), the identification should be performed at the species level and include any additional information on distinguishing features. Specification at strain level and deposition in culture collections are prerequisites in order to obtain authorization for the MBCP in, e.g. the EU, USA and Canada. Where the notifier argues that the strain is sufficiently identical for extrapolation of data generated for one strain to the other, the RMS should request the notifier to submit detailed taxonomic descriptions of the microorganisms (using the best available technology). Based on these data, the RMS will decide whether or not extrapolation of data between strains is possible.

21.7.2 Genetic toxicity assessment: needs and recommended approaches

It can be concluded that the existing test guidelines for chemical pesticides may not be directly applicable for testing microbial

products, and specific guidelines for each test system and type of microbial pesticide need to be developed (see Typas and Kouvelis, Chapter 18, this volume). OECD guidelines need to be developed with references to the available test methodology and appropriate protocols. The BPSG will carefully monitor any progress in this respect.

21.7.3 Occupational, bystander and consumer exposure and risk assessments

Conventional chemical pesticide models used to *quantitatively* assess occupational and bystander exposures are not easily applied to MBCAs. Therefore, the use of the available models for a *qualitative* approach to the evaluation of occupational and bystander exposure to microbial pesticides is recommended. Three elements, (i) the risk from exposure, (ii) the anticipated level of exposure, and (iii) the routes of exposure, should be considered using information and data on the proposed use pattern, product characterization, toxicity and infectivity testing, and any available information on the environmental fate of the organism. For most MBCPs, the risk from exposure can be mitigated by appropriate handling of the MBCP in relation with appropriate labelling.

21.7.4 Microbial metabolite residues in treated food crops

One of the differences of MBCAs from conventional chemical pesticides is the potential for some microorganisms to produce metabolites that could be harmful to humans if consumed. Currently, the extent of investigations on metabolites of MBCAs depends on what is already known in the published scientific literature or becomes apparent during product development. There is no widely accepted guidance offered by the regulatory authorities on how to deal with potentially toxic metabolic by-products, and applicants/notifiers can encounter new and unpredictable regulatory requirements that could ultimately delay or even prevent registration/authorization. If the plant protection action is known to be due to the residual effect of a toxin/metabolite, and consumers will be exposed to this toxin/metabolite, the microbial product should be subject to the same data requirements as a chemical pesticide. In the case that the applicant wishes to pursue registration/authorization for a food use, the regulatory authorities should require appropriate data to establish a maximum residue limit or tolerance.

21.7.5 Efficacy evaluation

There are two major types of pesticidal modes of action associated with microorganisms: (i) direct toxicological or infective interaction with a pest; and (ii) pest population regulation through processes such as competition for ecological niche (e.g. nutrients, habitat), induction of plant resistance, endophytic growth or root colonization. The efficacy data required to support a microbial product are specific to its mode of action and the proposed label claims. For microbial products that exhibit a direct, measurable toxic or lethal effect on the pests, scientifically sound laboratory and field studies designed to quantify the susceptibility, dose response, time to mortality and residual control effect on the proposed target pest are required. In the case of population regulation, the performance data must help to define any specific environmental conditions needed to maintain growth of the microorganisms; these may need to be defined on the product label. Dose–response behaviour should also be determined and the optimum dose recommended must be justified. The minimum dose required to achieve effective control (or other defined benefit) should be established.

21.8 Workshop on the Regulation of Biopesticides

A workshop was organized in April 2008 by the OECD BPSG on the 'Regulation of Biopesticides: Registration and Communication Issues', hosted by the US EPA (OECD, 2009). The goal was to improve the

registration process for biopesticides worldwide and to resolve the current barriers to joint reviews. The objectives were: (i) to collect input to resolve science issues and harmonize approaches associated with registering biopesticides (evaluation and risk assessment); (ii) to improve communication and information exchange between regulators, scientists and/or industry: and (iii) to take forward some of the conclusions and recommendations of REBECA.

The focus of the workshop was that communication between and among regulators, scientists, industry, consumer organizations, grower organizations and non-governmental organizations (NGOs) should be further encouraged. Communication requires that all parties speak a common language. For example, if we talk about biopesticides or BCAs, we have to know whether this includes genetically modified organisms (GMOs), whether growth regulators are covered by this definition, and whether products of natural origin fall within the scope of this definition. It is not necessary that everyone uses the same definition, but it should be clear which definition is being used and what types of products fall within the scope of the used definition.

Input to the workshop was collected on science issues that should be resolved, and approaches to the registration of biopesticides that could be harmonized. These recommendations have been taken forward by the BPSG and further issue papers related to microorganisms will be developed on: (i) microbial contamination; (ii) pre-submission meetings; (iii) storage stability; (iv) evaluation of environmental safety of MBCPs; and (v) fungal metabolites.

21.9 Seminars

The BPSG decided to organize a series of seminars as an outcome of the US EPA workshop in 2008. The reports of these seminars will be published in the OECD Series on Pesticides. The 2009 and 2010 seminars were both linked to the annual meeting of the BPSG. The first of these seminars, *Identity and Characterisation of Micro-Organisms,* was held in 2009 (OECD, 2010). In particular, the following issues were addressed: (i) what methods should be used for identification of micro-organisms; (ii) at what taxonomic level is verification required; (iii) what identification data package should be submitted to conclude whether strains are similar; (iv) standard operational procedures, including quality control measures regarding the manufacturing process; and (v) maximum acceptable amount of contaminants. Due to the diversity and variety of organisms among MBCAs, a major conclusion was that a case-by-case approach is necessary.

The second seminar was titled *The Fate in the Environment of Microbial Control Agents and their Effect on Non-Target Organisms*. This topic was selected because of its significance for the registration of biopesticides. The seminar was jointly organized by the OECD BPSG and the COST 873 initiative. COST is an intergovernmental European framework for international cooperation between nationally funded research activities; COST Action 873 is a large network of leading European and Mediterranean specialists in 22 countries and covers all bacterial diseases of all species of stone fruits and nuts in the European sphere.

The following issues were discussed: (i) the importance of natural and released inoculum levels of microbials; (ii) the stability of microbial strains when released; (iii) their potential persistence and mobility in the soil; (iv) the epidemiology of microbial control agents in the environment; (v) a system to control released microbial control agents in the environment; and (vi) environmental safety evaluation and risk assessment of microbial control agents. It was concluded that, in many cases, the environmental evaluation will need to be handled on a case-by-case basis. It was also highlighted that one of the conclusions was again related to the need for good communication networks and the possible need for a system of electronic information exchange. The report from this second seminar has also been published in the OECD Series on Pesticides (OECD, 2011a).

21.10 Second Working Document on the Evaluation of Microbials for Pest Control

One of the recommendations from the OECD Workshop on the Regulation of Biopesticides was to develop issue papers on a number of topics. In the following section, a preview is presented on some of the issue papers that are currently in preparation.

21.10.1 Microbial contaminant limits for microbial pest control products

Microbial pest control products are produced and manufactured using various methods depending on their unique characteristics. Most biological agents are produced in some type of submerged culture or solid-state substrate unless they are obligate parasites that require cell cultures, whole animals or other living forms as hosts. Few organic solvents or other harsh chemicals are required during formulation and manufacturing. However, all MBCAs have the potential of containing unwanted microorganisms in addition to the microbial control agent. Depending on the culture and formulation conditions, contaminating microorganisms could include pathogens, their associated toxins and other metabolic by-products of health concern. As a result, a contaminated MBCP could pose a risk if it is applied over areas populated by humans, habitats frequented by susceptible non-target organisms or other sensitive areas (e.g. drinking water supplies) as well as a risk to food crops up to, or near, the time of harvest.

The discussion in this issue paper is limited mainly to microbial contaminants of human and animal concern, including primary human pathogens, and does not consider the presence of other microorganisms of concern.

Its purpose is to highlight the current international microbial contaminant criteria on food and in drinking water and to promote a dialogue among OECD member countries on acceptable levels of microbial contamination in MBCPs (OECD, 2011b).

21.10.2 Pre-submission meetings

In various OECD member countries, meetings between the applicant and the registration authorities before submission of the final dossier have been highly beneficial in ensuring that the submitted dossier is of a high standard and appropriately addresses the requirements and concerns of the regulators, especially if it concerns applications for microbial pest control products. The main objective of pre-submission meetings is to agree on the information requirements. Although the data requirements are laid down in legislative documents, applicants may need guidance on how to interpret them, because interpretation partly depends on the identity and biological properties of the MBCA, the nature of the product and the intended use pattern. In order to simplify the registration procedure, it is, therefore, recommended that before submission of the dossier, the applicant discusses the data requirements with the authority and whether studies, published literature and/or waivers can be accepted. It is foreseen that a pre-submission meeting will facilitate the dossier submission and subsequently the review process, and finally speed up the decision-making process. However, it must be emphasized that different procedures will be followed in the USA/Canada on the one hand and in the EU on the other. In the former, the US EPA and PMRA can establish the information requirements in dialogue with the applicant. This is also the case for joint submissions in the USA/Canada. In the EU, an individual member state can only give advice on the information requirements, because the other member states (currently the EU consists of 27 member states) and the EFSA must have the opportunity to express their opinion.

21.10.3 Storage stability

There are no guidelines specific to microbials as far as storage stability is concerned; the test conditions used for assessing the storage stability of chemicals are applied to microorganisms. Such an approach is not always relevant,

for example, an accelerated storage stability test cannot be conducted with live microorganisms. A draft paper on the storage stability requirements of MBCPs will be amended to provide sections on the different type of products (bacteria, viruses and fungi).

21.10.4 Evaluation of environmental safety of MBCPs

The following issues should be covered concerning the environmental safety evaluation of microbial biocontrol agents: (i) a statement on the recovery for non-target soil microorganisms based on a literature search; (ii) no inclusion of safety factors for pathogenic MBCAs in cases where dose–response relations are not observed; and (iii) terminology should be used which – compared with chemicals – is more appropriate for microorganisms (*longevity* instead of *persistence* and *estimated/predicted environmental density* instead of *predicted environmental concentration*).

A paper on the fate of an inoculum in the environment in relation to background levels of the population and its occurrence and persistence in soil has been recently published by Scheepmaker and Butt (2010). In this paper, an overview is presented on the background levels of some indigenous fungi as well as persistence data of some applied fungal BCAs in order to provide some guidance to risk assessors and registration authorities on how to evaluate data on natural background levels of indigenously occurring species.

21.10.5 Fungal metabolites

The general understanding is that data are not required for a specific, identified toxin/metabolite in cases where the toxin/metabolite is only produced in the infected/target organism and leaves no (viable) residues. However, in specific cases, data requirements for certain metabolites can be justified.

One topic of the issue paper on this subject will be to investigate the possibility of using specific exposure scenarios for humans and non-target organisms on a case-by-case basis, and also conducting approximate calculations for such exposure scenarios. Most fungal metabolites, when produced in relation to their use as a microbiological plant protection product, would probably degrade quite quickly and would therefore not cause any residue problems. The longevity of the microorganism has to be taken into account.

21.11 Vision for the Future

Overall, the aim of the OECD initiatives is that a dossier submitted to one member country and a monograph developed from that dossier can be used by other OECD countries (OECD, 2004d). Building on progress achieved, the OECD has adopted the vision that, by the end of 2014:

- The regulatory system for agricultural pesticides has been harmonized to the extent that country data reviews (monographs) for pesticides prepared in the OECD format on a national or regional basis (e.g. EU or NAFTA – North American Free Trade Agreement) can be used for independent risk assessments and regulatory decisions made in other regions or countries.
- The preparation of data submissions (dossiers) for pesticide active substances and for end-use products is coordinated globally by industry (to the extent possible).
- Work-sharing arrangements between regulatory authorities in OECD countries take place as a matter of routine.
- The generation of a single monograph for each active substance, serving the needs of the regulatory authorities in all OECD countries, is commonplace, notwithstanding the need for separate independent risk assessments and regulatory decisions in each jurisdiction.

21.12 Conclusion

It can be concluded that, regarding biopesticides in general and microorganisms in particular, the BPSG has achieved a lot of progress

towards the OECD *vision* through the development of guidance and working documents, the 2008 Workshop on the Regulation of Biopesticides and the subsequent seminars in 2009 and 2010. It is the intention of the OECD BPSG to continue contributing to this goal by facilitating the evaluation and registration of microorganisms.

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22 Understanding Public Risk Perception for the Use of Beneficial Microorganisms

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22.1 Introduction: Divergent Understandings of Risk and the Public

Realizing the potential benefits that microorganisms could provide society hinges, in part, on scientists understanding how society at large perceives risk. Scientific understanding of microbiology has expanded remarkably, but so too has public suspicion of scientists and of (public and private) institutions that use science. Billions of dollars have been spent to create, assess and communicate technical information about the risks of technologies, yet social science surveys have consistently shown that the public has become more, not less, concerned about the risks of modern life (Slovic, 1987, 2001). Those who develop new scientific applications and novel technologies build up significant specialized scientific knowledge and a familiarity with them. Members of the public do not generally have this same knowledge, nor a favourable disposition towards using science to manage risk. Scientific risk communication across this gap in understanding is a major challenge to deriving benefit from the application of new science in modern society, from microorganisms to nanotechnology.

Effective public communication across gaps in understanding requires all parties to understand themselves as simultaneously senders and receivers of messages. This communication should foster shared understandings of scientific knowledge, the relationship of risks to benefits, and social values such as democratic decision making. Communicating across gaps in risk perception and understanding depends heavily upon scientific experts listening to feedback from a cluster of diverse public audiences in order to understand the assumptions that shape the interpretation of messages, while simultaneously improving the quality of communication. In short, scientists and their institutions must listen to the public to understand public risk perception, and devise communication strategies to overcome this gap in risk perception and understanding. The academic disciplines of social psychology and science communication can help scientists and practitioners

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understand how to communicate across this risk perception divide.

Those trained in scientific expertise are predisposed toward discounting lay risk perception as subjective and emotional, in contrast to scientific risk analysis, which experts consider to be rational and value free. Critical social scientists, however, perceive the stages of risk analysis (i.e. risk assessment, management and communication) to be influenced by social values and assumptions, to varying degrees. If scientists (and institutions that use science) misunderstand how the public perceives their knowledge, technologies and institutions, well-intended policies are likely to be ineffective (Slovic, 1987). Popular risk concerns have, in some cases, overridden expert recommendations for risk management. For example, public concerns about hazardous waste management resulted in directing the bulk of the budget of the US Environmental Protection Agency (US EPA) to that issue, when in fact hazards from indoor air pollution are considered by scientists to be more serious (Slovic, 1999).

Most lay audiences bring the following beliefs to their intuitive risk judgements about novel microorganisms, which shape their risk perception: microbes are invisible, largely unknowable and probably dangerous (because many lay audiences presume all microbes necessarily cause human disease). Microorganisms are particularly challenging for engaging public risk perception because they are generally invisible to ordinary people. Two biological control scientists summarize these challenges in the following way: 'Despite the lack of documented serious conflicts, there is an air of pathophobia that has brought to a virtual standstill the application of the classical approach in the use of plant pathogens for weed control' (Freeman and Charudattan, 1985). The term 'phobia' is apt in this context, for a chief obstacle to the beneficial use of microorganisms may indeed be unfounded fears on the part of the public or public agencies. Risk fears can serve as obstacles to appropriate regulation as well as to public support (Waage, 1995; Evans, 2000; Sheppard *et al*., 2003; Delfosse, 2005). Transforming public phobia into appropriate public support depends not only on carefully crafted scientific communication but also on social deliberative processes grounded in democratic values. Both are critical to realizing the beneficial potential of microorganisms.

No opinion surveys of public perception of the risks and benefits of microorganisms as a broad taxonomic category exist. However, many salient lessons relevant to the use of microorganisms can be drawn from social science research investigating public views of other biological or novel technologies. From a social science perspective, these other technologies function as proxies for understanding public risk perception of microorganisms. Pioneering work in the 'social psychology of risk' addressed human perception of nuclear power and toxic chemicals (Slovic, 2001). Those who developed communication strategies for crop biotechnologies did not consult social psychologists or risk perception experts, and they committed many fundamental mistakes in science communication (Wynne, 2001). These errors imposed significant costs to industry, government credibility and society at large.

To head off the polarization that accompanied the introduction of transgenic biotechnologies, funders of nanotechnology have enhanced their support for social science work (Barben *et al*., 2008). This recent research has further characterized how members of the public perceive risks of novel technologies (Kahan *et al*., 2007). From the perspective of critical social science, public perception of nanotechnology risks is functionally equivalent to public perception of microorganism risks. There are many significant biological and ecological differences between nanotechnology, genetically modified microbes, and 'wild-type' – not genetically modified – microbes. However, few members of the lay public are able to distinguish meaningfully between these in their composition and potential risk. For example, McNeil *et al*. (2010) surveyed Canadians about their perception of biocontrol pest strategies; the findings suggested that those surveyed could not distinguish between a 'beneficial microbe' and biocontrol agent, and a food contaminant. The field of science communication has investigated strategies for facilitating more

constructive public engagement with novel technologies (Burri, 2009), and these contain lessons relevant to effective and appropriate risk communication.

There is no singular, homogenous public audience, any more than there is one worldview held by all scientists in all places at all times. Thus, we must speak of many scientific perspectives and a diversity of lay audiences, both in the plural, to remind us of the many perspectives, and the error of conceiving of the public in a homogenous, anonymous way. The word 'public' necessarily bundles together people holding a wide range of scientific expertise, value predispositions and social power. For example, some opinion leaders in business, universities and non-governmental organizations, and government regulatory officials, may be considered a form of 'public' in the sense that they may be outside a specialist research community, but they are essential to mediating understandings of risk to a more general, less scientifically informed general public. Hence, understanding the diversity of views held by public audiences is essential to understanding public risk perception (Wynne, 1992; Bucchi and Neresini, 2008).

Critical social science research addresses how scientific experts and lay publics perceive, analyse, communicate and evaluate risk, and can propose examples of social processes to overcome gaps in understandings of risk. Examples of this work can be found in the fields of social psychology of risk (Beck, 1992; Slovic, 2001) and science communication (Gregory and Miller, 1998). These fields of social science incorporate natural science data into how human beings develop, use, perceive and communicate knowledge and risk within society. Therefore, this chapter does not specifically evaluate safety assessment and regulation of beneficial microorganisms, but rather how scientists and diverse public audiences understand, communicate and deliberate risks and benefits. The implications will apply broadly to any use of microorganisms for societal benefit, whether for food or feed preservation, or for agricultural, environmental or health purposes.

This chapter begins by describing the ways in which diverse members of the public perceive risk, drawing heavily on social psychology of risk literature. It then examines the ways in which scientific institutions understand the public, and describes some errors in public risk communication. The chapter concludes by outlining new, more constructive approaches to fostering public engagement with novel technologies that could help realize their potential for public benefit. Greater efforts to conduct 'upstream engagement' with nanotechnology through anticipatory public dialogues (Macnaghten *et al.*, 2005; Burri, 2009) are developing models for deliberating and negotiating risk perception, evaluation and judgement. These can inform scientists' understandings of public risk perception and improve the effectiveness of risk communication efforts.

22.2 How Does the Public 'Understand' Risk?

The Risk Society (Beck, 1992) was one of the most influential books in European social science in the late 20th century. Beck outlined the fundamental shift across industrial societies over the past five decades: from a primary concern about resource scarcity to the management and distribution of risk. He argues that the scientific and technological forces that created industrial development are themselves now evaluated by the public with their lay understanding of risk. Beck argued that debates about risk will be central in society for the indefinite future, but that diverse conceptualizations of what constitutes 'risk' are determined chiefly by social, not scientific, factors. The astonishing growth of scientific expertise (among some sectors) to create our technological society has necessarily led to divergent understandings of risk. In Beck's risk society, lay versus expert understandings of risk substantively frame public judgement on the application of science and technology. Thus, most controversies about appropriate regulation are actually predictable expressions of broader social concern about risk. Politically charged disputes over regulatory

safety criteria become an expected, even routine, in the application of science and technology in the risk society (Slovic, 1999).

The standard expert conceptualization of risk is the statistical probability of an adverse event that can be objectively quantified by a risk assessment process (National Research Council, 1996). It is usually expressed in probabilistic terms, such as 'risk = hazard × exposure' (Delfosse, 2005). This approach fulfils the criteria of consistency and quantification. Critical social scientists reject conceptualizations of risk as pre-existing in nature, awaiting human discovery and measurement (Slovic, 1999). Rather, risk is an abstract concept invented by human beings to help society manage uncertainties. Risk is a mental model constructed by humans. Harms, hazards and danger are real, but risk is a conceptual framework for evaluating and managing these. Critical social scientists have demonstrated both complexity of the concept of 'risk', and the inadequacies of conventional risk communication to the public in terms that are narrowly quantitative and probabilistic (Slovic, 2001).

The scientific method, as an abstraction, may be considered value free; however, the application of science in society through risk analysis necessarily incorporates social or cultural values (Douglas and Wildavsky, 1982). These values may be explicit or tacit. Risk analysis has scientific components, but inevitably it has elements that are subjective and value laden, meaning that the cultural values are assumed and incorporated into the process. Value judgements are embedded in the risk model in the decisions made using it, e.g. which theory is to guide the construction of models, what context is to be considered, what elements are to be considered, what possible consequences are to be considered, and what time frame is to be considered. Social values shape the assumptions made about all of these factors, which are woven into scientific risk assessment processes. How these assumptions are communicated to the public is also value laden, and they reflect the experts' perception of the public's understanding of risk.

Recent research in cognitive psychology and neuroscience has demonstrated that human beings conceptualize risk in two fundamentally different ways (Slovic *et al.*, 2004). The 'analytical' system uses formal logic, probabilistic reasoning and scientific deliberation. The 'experiential' system is an intuitive, largely automatic response to perceived danger, and often inaccessible to subjective awareness. The former is slow, but the latter is much more rapid. The experiential system has resulted from human evolutionary processes that selected against those who failed to perceive environmental risks (e.g. larger predators, foul water), and may be considered the default approach to human risk perception (Slovic *et al.*, 2004). This second 'system' of risk perception is instinctual to human beings, and scientific training develops the skills and disposition to deploy the analytical system in its place. Social science research has consistently found that the public has a broader conceptualization of risk than experts, consistent with their perspective of the world. This public perception of risk is both qualitative and complex (Slovic, 1999).

Non-scientists perceive risk through the lens of their own life experience and the decisions about uncertainties that they negotiate in daily life (Wynne, 1992). Members of the public evaluate technological risks in light of the following types of social factors: dread, catastrophic potential, equity in outcome, degree of certainty, reversibility and the potential to personally choose the risk (or not). Examples of how these criteria might be manifest in public risk perception include: death from cancer is dreaded, but death from automobile accidents is less so; exposure to environmental tobacco smoke is perceived as riskier than cigarette smoking; hazardous industrial waste is perceived as more risky because it is not chosen, as opposed to toxic household products which are purchased (Slovic, 1999).

The public develops opinions about the risks of new technology based on factors that are not included in expert risk models. For example, research into public perception of the risks of nanotechnology has demonstrated that the public holds greater concerns about personal privacy issues and equity of benefits than do scientists (Priest *et al.*, 2010). Although some scientists may perceive this as irrational or unfair, this is in fact how non-experts

develop their opinions. Research has consistently shown that trust is the chief criterion that most lay publics use to evaluate novel technology. A scientist may ask: does the proposed introduction of the technology represent acceptable risk? But lay publics ask: is the scientific claim trustworthy? They answer by evaluating the trustworthiness of scientists and sponsoring institutions, and their perceived motivations. There is both wisdom and error in public perceptions of risk (Slovic, 1987). Understanding that lay members of the public develop opinions about acceptable risk based on their level of trust in scientists and their institutions is fundamental to understanding public risk perception and, thus, indirectly, is essential to successfully introducing a novel microorganism.

Early social psychology explained how social factors such as gender, race, class, political views and individual psychology shape public risk perception (Slovic, 1999). Recent work has demonstrated the importance of world views – and the social values embedded in them – in shaping risk perception of nanotechnology (Kahan *et al*., 2007). An individualistic world view can be defined as one that prizes the autonomy of individuals and markets to operate freely from perceived collective interference. When more information about nanotechnology is provided to those with an individualistic world view, they are more likely to see it as beneficial. An egalitarian world view can be defined as one that is highly concerned with the equitable distribution of benefits (and risks) across a society. When the same risk information is provided to those with an egalitarian cultural outlook as to those with an individualistic world view, they are more likely to perceive nanotechnology as having more risks than benefits (Kahan *et al*., 2007). A key implication of this finding is that providing more information about a novel technology prompts different responses, from support to fear. More information reinforces the favourable views of those with a general risk-accepting approach to life, but for those who are more likely to be risk averse, more information can augment their concerns (Kahan *et al*., 2007). By understanding the diversity of world views held by the public, one can craft more appropriate and effective risk communication strategies.

The tone or affect of a scientific risk message for a public audience generally plays a greater role in shaping public response than scientific data. For example, if a risk communication bears tacit meanings of the inevitability of a government action, members of the public may react negatively to the perceived exercise of government power, not to the scientific assessment of the risk. This finding poses a fundamental challenge to public risk communication, and points to the need for strategies that are sensitive to broadly held social values. It also points to the critical importance of understanding science communication processes from the perspective of lay audiences, lest miscommunication and confusion occur. The rise of the digital media environment poses genuine challenges to public agencies gathering public comment on the proposed use of microorganisms. Novel communication strategies are required – more than simply posting information on a web page.

In sum, social science has demonstrated that lay public understandings of risk are more complex and instinctual – and potentially volatile – than the statistical probability of an undesirable event. Members of the public use an 'intuitive system' of perceiving and evaluating risk that differs from that of experts and scientists. Lay public risk perception is strongly shaped by social factors such as class, gender, affect and world view. These factors strongly influence differential predispositions towards the risks of novel technologies. It is inevitable that these social factors and cultural values will shape public perceptions of risk, but it is not inevitable that the debate becomes polarized or negative, or undermines the introduction of new technologies (Kahan and Rejeski, 2009).

22.3 How Do Scientists Understand the Public?

Expert risk communication to the public carries the potential of a perverse outcome. Expert efforts to communicate 'acceptable' risk to lay publics can backfire. Providing

more scientific information about risk may increase risk fears, at least among some publics, and undermine the intended communication effort (Douglas and Wildavsky, 1982). Effective risk communication between scientists and the public depends upon the public learning about science, but also upon scientists adopting a realistic approach to the public and its risk perception. A recent survey of American scientists found that they perceive the public to have an understanding of science that is deficient: 'While the public holds scientists in high regard, many scientists offer unfavorable, if not critical, assessments of the public's knowledge and expectations. Fully 85% see the public's lack of scientific knowledge as a major problem for science, and nearly half (49%) fault the public for having unrealistic expectations about the speed of scientific achievements' (The Pew Research Center, 2009).

Research into science communication has taken up the question of how scientists' perception of the public shapes the communication process. Critical social scientists have developed conceptual models to describe the rather constrained ways in which scientists perceive the public.

1. *The cognitive deficit model*. This assumes that if only the lay public knew more about science and ceased to be in a state of knowledge deficit, a better relationship between science and the public would emerge (Gregory and Miller, 1998; Sturgis and Allum, 2004). In this model, the shortcoming is in the public itself, and this is the reason why the potential of science is thwarted.
2. *Injection of science model*. Scientific knowledge is developed by experts and implanted into the bloodstream of society. Here the delivery system constrains the application of science for society (Mooney, 2010).
3. *The loading dock model of science and policy*. The task of scientists is to develop and deliver scientific knowledge to policy makers, and their job, in turn, is to explain what it means – and how it should be supplied – to the public. This model assumes that if policy makers did their job properly, there would be less of a regulatory bottleneck and greater public support (Cash *et al.*, 2006).

These models do not criticize individual scientists, laboratories, discoveries or institutions. Instead, they critique the underlying assumptions that guide the actions of some scientific, political and commercial leaders and institutions who use science.

Institutions charged with advancing technological innovation can readily slip into simplistic thinking about the public and its views. Scientists' concerns about public understanding of science have, at times, been rendered as 'public appreciation for the technological products of science'. This would be based on the assumption that 'once a lay person learns about science and technology, she or he will automatically appreciate it'. The term 'public acceptance' of novel technologies carries with it the tacit message that an expert has determined that the risks are acceptable and that a choice has already been made 'for' the public (Barben, 2009). When scientific, industrial or government leaders use the term 'public acceptance', they assume that a technology has been proven (to their satisfaction) to be safe; therefore, the chief task is persuasion. Use of this term suggests that the public cannot rationally decide to reject a technology, or express the desire for restrictions upon it. These assumptions undermine effective communication.

These assumptions, which are embedded in the term 'public acceptance of science and technology', are also most pernicious. Efforts to mitigate, manage and communicate risk to the public – uninformed by how risk is perceived by the public – exacerbate public fears and mistrust. The failure of scientific regulatory institutions to understand that the public renders judgement based more on its perception of scientists' trustworthiness than on knowledge of science or risk, unwittingly creates public alienation from science, and this fundamental error is repeated (Irwin and Wynne, 1996). Avoiding this error requires scientists and their institutions to revisit their assumptions about the public (Yafee, 1997; Wynne, 2001). The position of regulatory scientists and their public agencies is key to effectively managing the communication between researchers and the public, for they are charged (in democracies) with representing the public's interest. Regulatory

institutions are given tremendous responsibilities, but are highly constrained in their resources for conducting the kind of research necessary to weigh risks versus benefits regarding the proposed use of a novel microorganism. They are also highly constrained by statute, regulations and administrative rules, both in making their decisions and in communicating to the public (Irwin *et al*., 1997). Ideally, regulatory agencies should function as a bridge to foster mutual understanding by scientific researchers and society but, in practice, they often comply with the interests of elected officials or their industrial clients, or at least are perceived as acting that way by some (Wynne, 2001).

To address public risk perception in ways that are meaningful to the lay public requires addressing the issue of trust in and trustworthiness of scientists and their institutions (Gregory and Miller, 1998; Warner *et al*., 2008). Evaluating the trustworthiness of others is something everyone can do. This poses two challenges. First, few scientific institutions think of themselves as needing to foster public trust; many resist doing so. Secondly, trust is hard to create but very easy to destroy. This is known as the Asymmetry Principle (Slovic, 1993). The 'new media environment', with the rise of the Internet, social media and other novel communication technologies (Press and Williams, 2010), when combined with the Asymmetry Principle, can exacerbate public mistrust of official decision making about risk, unless new communicative and deliberative strategies are implemented. In this social context, many typical communication practices used by the scientists of regulatory agency may unintentionally undermine public trust. This has the potential to block the introduction of a novel microorganism with more potential benefits than risks, but also, more broadly, to erode public confidence in regulatory agencies and their decision making on behalf of the public's interest.

Studies of public responses to nanotechnology risk communication have consistently found that public attitudes are contingent upon three elements: issue framing, evaluation of risks versus benefits and the perceived trustworthiness of the messenger (Priest, 2006; Kahan *et al*., 2007; Kahan and Rejeski, 2009). These generally apply to public risk communication regarding beneficial microorganisms. Understanding that the public holds a range of pre-existing attitudes towards novel technologies logically supports the need for a well-conceived communication strategy that presents microorganisms in the context of the benefits they are anticipated to supply, and the importance of developing messages for these diverse audiences. Risks should never be communicated to the public apart from the intended social benefits; as simple as this principle may sound, it is repeatedly disregarded by research scientists and regulatory scientists. The commonsensical recommendation to always communicate anticipated benefits *with* risks may be beyond the control of scientists and regulatory agencies. For example, under current rules in the USA, the benefits of a proposed biocontrol agent introduction cannot be considered by regulatory scientists; the administrative decision to award a permit for introducing a biocontrol agent can only be based on the potential risks.

22.4 From Risk Perception to Participatory Public Engagement

Many critical social scientists understand these 'risk controversies' as less about the uncertainties of natural science, and instead as challenges to democratic decision making in highly technological societies (Beck, 1992; Kleinman, 2000; Hackett *et al*., 2008). As divergent understandings of risk among the public, scientific experts, regulatory agencies and policy makers have become more apparent, a host of initiatives have sprung up to try to bridge these gaps: enhanced public communication, public outreach, public consultation and public participation. In practice, these terms are often used interchangeably or without distinct meanings (Rowe and Frewer, 2005). The initiatives generally share the assumption that the public should be engaged not as a passive audience but as responsible citizens (Whiteside, 2006).

Science communication scholars and others have advanced an alternative model, that of 'participatory public engagement'. This approach facilitates participation and mutual learning among members of the public, scientists and stakeholders with respect to the development and application of science and technology in modern society. It is usually presented as a 'dialogue' in which citizens and scientists both benefit from listening to and learning from one another, referred to as mutual learning (McCallie *et al*., 2009). Participatory public engagement requires that citizens invest effort in more than merely asking questions of experts. It requires that scientists to do more than merely present their data. Such a social or co-learning process brings scientists and non-scientist citizens together to learn from one another. It requires citizens to learn about science and policy, and scientists to learn what members of the public know and do not know about science. Perhaps most importantly, it imposes the expectation on all parties of listening, respecting others' views, and openness to dialogue as a precondition for making consensus-based decisions (Kleinman *et al*., 2007).

Participatory public engagement is designed to facilitate the expression of reasonable lay concerns from responsible citizens to scientists and regulatory officials with the intent of increasing the quality of deliberative decision making. Thus, it rests on the fundamental social value of democratic participation (Sclove, 1995). Another term for this is participatory technology assessment (pTA), and recent scholarship in this area has outlined specific strategic options for creating such a process in the USA (Sclove, 2010). The USA led the world in pTA from 1972 until the US Congress eliminated the Office of Technology Assessment in 1995. There are now more than a dozen public ministries in the European Union (EU) that use pTA approaches (Sclove, 2010), yet there are significant national differences in efforts to democratize novel technologies (Toumey, 2006). This chapter will use the term 'participatory public engagement', because it includes broader educational and communicative efforts, whereas pTA is a particular type of public engagement process to render a public decision about the application of one or more technologies.

Public engagement differs from public outreach or consultation in that it requires bidirectional communication between scientists, decision makers and citizens, and members of the public as a diverse audience (Rowe and Frewer, 2005; McCallie *et al*., 2009; Mooney, 2010). The following provides a typology of risk communication based on information flow between participants (adapted from Rowe and Frewer, 2005):

1. *Public communication*. Information flows from (research and regulatory) experts to the public. Examples: information broadcasts, public hearings, public meetings, web page information.
2. *Public consultation*. At the initiative of governmental bodies, information flows from the public to scientists and decision makers. Examples: opinion poll, referendum, survey, consultation document, electronic consultation (interactive web site), focus group, study circle.
3. *Participatory public engagement*. Information and social values are exchanged between scientific experts and citizens as representatives of the public. All parties exchange their understanding of science and its relationship to human values, and this information is transparent and made intelligible to broader public audiences. So the information flow is better understood as a negotiated dialogue through time. Examples: action planning workshop, citizen advisory panel, consensus conference, negotiated rule making, deliberative opinion poll, planning cell, town meeting (New England model) with voting.

The processes of participatory public engagement have to be structured in such a way as to allow for respectful dialogue, but also for the accountability of scientists, government and industry leaders, and citizen participants representing the broader public (Kleinman *et al*., 2007). Such a dialogue requires agreed-upon ground rules, and an active facilitator to hold the members accountable to these rules.

Participatory public engagement may appear more costly than public communication and consultation. It imposes costs on

citizens that participate on behalf of the broader public (Kleinman *et al*., 2011). The selection of appropriate citizens is key, as is the incentive system that might reward their participation – through personal interest, civic values, or financial compensation (Kleinman *et al*., 2011). However, most costly is the potential expense of scientists re-evaluating their research in light of public feedback, and scientific institutions re-evaluating how they relate to diverse public perceptions, social values and attitudes. Participatory public engagement may 'slow' the deployment of individual microbial projects, but within the overall context of research and application of microbiology for social benefit, participatory approaches will be more economical. For example, if public engagement had addressed and mitigated some public fears of crop biotechnologies in Europe, how much would this have been worth? Participatory public engagement can provide structure that encourages respectful inquiry by all parties into technological development, regulation and application. This can have spillover benefits by fostering public views regarding microorganisms that recognize and value their benefits.

The design of participatory public engagement should facilitate the deliberation of responsible citizens with reasonable concerns about what constitutes 'social benefit'. Social benefit cannot be effectively defined exclusively by scientists and government officials. Bringing democratic values to bear on public deliberation of the risks of novel organisms or technologies requires scientifically informed deliberation by citizens about the potential risks and benefits (Whiteside, 2006). This may require scientists and public agencies to explain their proposed actions differently. Participatory public engagement should be designed to filter out the expression of alarmist fears and ideologically driven obstructionism. Ideally, citizen concerns could address:

1. the assumptions that underlie the introduction of novel organisms or technologies;
2. the degree of knowledge about the broader ecological context in which these are introduced and their interaction with other organisms in the environment;
3. the distribution of social benefits and their impact on social equity;
4. the capacity of individuals to choose the technology; and
5. the time lag between the introduction, the realization of benefits and the possible unanticipated negative impacts.

Citizen participants are likely to ask these kinds of questions and, in the process, reveal their understanding of the public interest (Whiteside, 2006). Thus, the design of a public engagement process should take these kinds of concerns into account, and recruit citizens with the skills to participate in a public deliberation. These participants should be able to articulate the public's interest in the introduction of a proposed novel microorganism with the associated safety concerns (protection of human health and the environment). This suggests that those with professional skills, as well as stakeholders of various interest groups, should be recruited so that they can agree on the basic outline of the public interest (Kleinman *et al*., 2007, 2011). Any potential risks or benefits can best be evaluated in light of the public interest, or the common good. So scientists and their institutions may be challenged to consider both their assumptions and the potential areas of uncertainty in their proposed actions. They may also need to consider the breadth of what constitutes public interest, beyond the expressed desires of economic stakeholders, which are often quite narrow.

Scientists and regulatory institutions may also have to grapple more seriously with the social values that guide some people's resistance to novel organism and technology introductions. Participatory public engagement is designed to achieve multiple social goals through deliberative processes: to improve the quality of public input to shape scientific decision making; to foster appropriate public trust in scientific institutions; to reduce the overall costs of decision making by anticipating areas of social controversy; and to expedite the efficacy of public agency decision making.

Initiatives to foster 'upstream engagement' with nanotechnology through anticipatory public dialogues (Macnaghten *et al*.,

2005; Burri, 2009) are developing models for negotiating risk perception, evaluation and judgement. These can serve as models for upstream engagement with the use of novel microorganisms. The structure of public engagement is essential to a successful initiative, and science communication scholars have advanced training in designing such efforts.

Some scientists are reluctant to speak in public because of the distorting effect of mass media (The Pew Research Center, 2009), and the potential for messages being manipulated by activist groups (Mooney, 2010). The most fundamental cost of participatory public engagement is the requirement to initiate a fresh approach to fostering dialogue between scientists, their institutions and members of the public. This is costly because it requires revisiting assumed knowledge about the limits of the generic public, when in fact citizen participation has the potential to actually improve the application of scientific knowledge to the needs of society. To succeed, scientists and their institutions – and citizens – would have to garner more direct benefits from participating in such public engagement processes. This would require skills beyond that typical of natural scientists, and a fresh approach to configuring professional incentives to reward their participation (see Box 22.1). Yet the 'no action' alternative in this case risks public disengagement and alienation from science and technology, and the potential rejection of applications that could provide more benefits than harm.

22.5 Conclusion: Constructing Shared Understandings of Risks and Benefits

It is inevitable that values and culture will shape public perceptions of the risks of microorganisms, but it is not inevitable that the debate become polarized or negative. Public perception of the application of microbiological applications is contingent chiefly upon the efforts made by research and regulatory scientists and their institutions to engage the public. Four decades ago, social scientists were not able to provide a robust characterization of public risk perception. The misallocation of public resources in risk communication for nuclear power and hazardous chemicals is understandable in that historical context. But scholars now know much more about divergent perceptions of risks held by experts and the public. The dramatic polarization of risk perceptions of crop biotechnology should prompt fresh efforts across scientific research and regulatory institutions to engage the public regarding microbiological applications for social benefit. The fundamental communication errors of crop biotechnology can and should be avoided.

New participatory forms of public engagement, such as participatory technology assessment, can help to overcome the gaps in assumptions and knowledge of risks and benefits. These not only have the ability to improve the quality of public communication, but also to enhance democratic deliberation on the relative risks and benefits of microbiological applications. Appropriate professional incentives for scientists and their institutions will have to be configured so as to reward this form of service to society. Effective public communication across gaps in understandings of risk can foster shared understandings of scientific knowledge, risks and benefits, and social values and democratic decision making. Realizing the potential of microorganisms to provide benefits to humans and society is contingent, in part, on scientists engaging and transforming public perceptions of risk.

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Box 22.1. Recommendations for constructing participatory public engagement with microbiological applications (adapted from Mooney, 2010)

1. Research scientists and regulatory agencies should seek input from the public at the earliest stages of management action development and should continue to seek consensus through participatory processes.
- **(a)** A key metric of an effective participatory process will be for experts to demonstrate to the public that the scientific community is taking the public's views into account.

2. When assessing the risks and benefits of the use and release of microorganisms, research and regulatory scientists should account for the non-technical and value-based concerns of the public, in addition to technical concerns.
- **(a)** Research and regulatory scientists should perform a thorough and publicly accessible evaluation of non-technical concerns.
- **(b)** Research and regulatory scientists should clearly articulate the ethical values that will guide their work, build those values into all aspects of their work, and consequently build all relationships around those ethical principles and values.

3. The scientific community should appreciate and utilize data from social scientists in order to better understand the public attitudes towards science and technology that shape the social context for use of microorganisms.
- **(a)** Science and policy journals should include regular columns that present data from social science studies regarding public attitudes towards science and environmental policy.
- **(b)** Professional scientific meetings should include discussions of current public attitudes towards new scientific discoveries and why those attitudes are vital to scientific research.

4. Research scientists and regulatory agencies need to create more opportunities to engage the public so as to cultivate mutual trust.
- **(a)** Open forums, tours of facilities and science cafés are existing ways that the public can interact with the expert community; these options provide the expert community with an opportunity to build the trust of the public.
- **(b)** Scientists and policy makers should develop effective communication strategies based on authoritative information from independent scientists and government officials. This strategy can be used both when creating new regulatory guidelines and during times of crisis.
- **(c)** This will require new or reconfigured professional incentives.

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