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Preface

Biological control using biopesticides based on *Bacillus thuringiensis* and *Lysinibacillus sphaericus* has been employed for almost five decades in countries such as Brazil for the control of agricultural pests and vectors of tropical disease. Professionals in the academic community have undertaken in-depth studies on a variety of potential biological control agents including fungi, bacteria, viruses, helminths and others. As a result of these studies commercial products have been developed for use in the field. A great advantage of these products is that they show few of the undesirable, nonspecific, activities associated with more traditional insecticides that affect many arthropods not implicated in crop destruction or the spread of etiological agents of human or animal disease. Continuing research and dissemination of results will further the development of new products.

This publication by Springer highlights the scientific and technical progress made on two of the most important biocontrol agents – the bacteria *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. Brazil is such a major user of biological control agents that much of this book concentrates on the characterization, development and commercialization of these bacteria. Much of the information presented does, of course, have relevance on a worldwide stage. The seventeen chapters have been written by a body of highly experienced scholars, professors, scientists and researchers. Five of them have been written by experts from outside of Brazil.

In addition to natural selection increasing the range of bacteria effective as biocontrol agents, the contents of this book deal with how studies on the physiology, biochemistry, general biology, and cellular and molecular biology aspects of the considered bacteria can help us develop improved products. The characterization and current classification of *Bacillus thuringiensis* toxins are addressed, which provide a useful framework for the optimization of the entomocidal principles required for their successful use in the control of pests and vectors of human diseases.

Aside from the toxins, the characterization of *Bacillus thuringiensis* serovars by AFLP microsatellites, rep-PCR, and more recently genomic sequencing, is described. Such technologies greatly enhance our ability to identify new potential products and to understand, and potentially manipulate, the virulence of particular strains. The expression of insecticidal genes in baculovirus, or in plants, provides an

alternative way to study, and employ, the encoded proteins. In addition, understanding the interaction of the insect with the virulence factors of the bacterium, allied to the spectrum of activity of *Bacillus thuringiensis* in relation to the main orders of insect pests, provide useful information for the control of agricultural and agroforestry crops. Another important chapter show the expression of Cry proteins in insect cells using recombinant baculoviruses has been shown to be an alternative means of production of these proteins for functional and/or structural studies. Also the novel strategies include site directed mutagenesis, domain swapping among different Cry toxins where novel hybrid proteins were constructed containing domains or loop regions from different Cry proteins that resulted in improved toxicity against selected insect pests.

In selected chapters, many interesting aspects are highlighted, such as reports of biotechnological studies linked to genetically modified plants containing new *Bacillus thuringiensis* proteins, and the beneficial interactions between Bt-bioinsecticides and parasitoids and predators in agricultural ecosystems. Also interesting is the work presented on a range of microorganisms with biopesticidal action, other than the two main protagonists of this book, which the authors propose as new alternatives as active agents in the field aimed at targets such as *Acromyrmex* spp., *Nasutitermes ehrhardt* and *Rhizoctonia solani*. This also includes the potential of using synergistic interactions of *Bacillus thuringiensis* and *Bacillus subtilis* with *Purpureocillium lilacinus*, or even the Nuclear Polyhedrosis virus, among others.

Concerns over mosquito resistance to the entomopathogenic bacteria *Bacillus thuringiensis* and *Lysinibacillus sphaericus* are revisited with information on the current state of the art. Finally, aspects related to the role of the Brazilian governmental company "Empresa Brasileira de Pesquisa Agropecuária", which is focused on agriculture and which develops products for application at the field level, as well as studies to combat new pests, are addressed.

At the end of this preface, I am sure that the plethora of authors are convinced that they have done their best to make this book a reality, and that they have contributed greatly to the work, which will hopefully fulfill the curiosity of an increasing number of readers and students interested in this area.

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Contents

Chapter 1
Bacillus thuringiensis Characterization: Morphology, Physiology, Biochemistry, Pathotype, Cellular, and Molecular Aspects

Leon Rabinovitch, Adriana Marcos Vivoni, Vilmar Machado, Neiva Knaak, Diouneia Lisiane Berlitz, Ricardo Antonio Polanczyk, and Lidia Mariana Fiuza

Abstract In this publication, "*Bacillus thuringiensis* and *Lysinibacillus sphaericus* – characterization and use in the field of biocontrol," this chapter can be seen as a brief general and historical introduction to the central theme of the book, where data on the cellular physiology, biochemical, genetic, molecular, and toxicological aspects of the bacterium, *B. thuringiensis* (Bt), are reported. This predominant entomopathogenic prokaryote was discovered and denominated Bt around a century ago, between 1902 and 1911. From the microbiological point of view, this bacterium is ubiquitous, Gram-positive, produces ellipsoidal but predominantely cilindrical endospores (central to paracentral) and contains a parasporal inclusion body called crystal or δ-endotoxin. The crystal is constituted of Cry proteins with molecular weight between 30 kDa and 140 kDa, which are coded by *cry* genes. On the other hand, this bacterial species synthesizes several enzymes and toxins that give them a wide adaptation to natural habitats. Bt strains have been

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studied and, over time, characterized and described as toxic and specific for Lepidoptera, Diptera, Coleoptera, Nematoda, Protozoa, Trematoda, Acari, Hymenoptera, Hemiptera, Orthoptera, Isoptera, Mallophaga, and among other target pests. Globally, 82 Bt serovars sometimes called subspecies were described until 1999, which currently correspond to more than 700 *cry* genes distributed in about 70 classes. The nomenclature review of *cry* genes, which encode Bt Cry proteins, has been published by Crickmore et al. and has been constantly updated on the website: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/.

Keywords Bacteria • *Bacillus* spp. • *B. thuringiensis* • Physiology • Biochemistry • Genetics • DNA • Toxicology

1.1 The Bacterium *Bacillus thuringiensis* Berliner, 1911

Among prokaryotes, bacteria of the genus *Bacillus* (family *Bacillaceae*) have been used in the microbial control of pests. In this genus, the species *Bacillus thuringiensis* (Bt) is distinguished by its biopesticide potential.

Bt was discovered by Ishiwatta in 1902 in reared *Bombyx mori* L. (Lepidoptera, Bombycidae) in Japan. Later it was reisolated by Berliner, in 1911, from *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) larvae in Thuringia, which gave rise to its current name (De Barjac and Bonnefoi 1968). According to Van Frankenhuyzen (1993), the first biological control trials of Bt were conducted against *Ostrinia nubilalis* Hübner (Lepidoptera, Pyralidae) between 1920 and 1930 in Europe. Between 1930 and 1940, several trials were carried out with other lepidopteran species in Europe and in the USA. Regarding the biological control of insects today, Bt is the mostly used microorganism worldwide (Lacey et al. 2015).

Bt is a ubiquitous bacterium with a large enzyme complement, which allows it to be found in a variety of sites, such as: soil, insects and their habitats, stored products, plants, forest, and aquatic environments. It can remain latent in the environment even in adverse conditions for its development (Azevedo et al. 2000; Fiuza 2001). On the other hand, 18 Bt strains were isolated from *Simulium* sp. larvae and adult and of *B. sphaericus* (Cavados et al. 2001). Larvae were collected in different rivers of states of Rio de Janeiro and São Paulo. Between strains obtained, only two were shown to belong to serotype H-14, *B. thuringiensis* serovar *israelensis*. Nine strains were "autoagglutinating" ones and seven *B. thuringiensis* serovar *Oswaldocruzi* (H-39) were also indentified and it was the first report of the isolation of entomopatogenic *Bacillus* from *Simulium* sp. larvae and adult collected in Brazil (Cavados et al. 2001). Its entomopathogenic activity is highly dependent on the parasporal inclusion body that forms during sporulation, which consists of Cry proteins that are encoded by *cry* genes (Höfte and Whiteley 1989; Schnepf et al. 1998).

According to the literature, parasporal inclusion bodies (crystals) are composed of proteins of varying quantity and quality according to the bacterial strain (Hofte and Whiteley 1989). Strains containing these crystals were measured as being toxic

and specific to larvae of Lepidoptera, Diptera, Coleoptera, Nematoida, Protozoa, Trematoda, Acari (Goldberg and Margalit 1977; Dulmage 1981; Taylor et al. 1992; Zhong et al. 2000), Hymenoptera, Hemiptera, Orthoptera, Isoptera, and Mallophaga (Feitelson et al. 1992; De Maagd et al. 2001; Castilhos-Fortes et al. 2001; Cavados et al. 2001). Strong reviews about the host spectrum of Bt crystal toxins were provided by Van Frankenhuyzen 2009, 2013.

Globally, 82 Bt subspecies were described until 1999, and more than 700 *cry* genes distributed in about 70 classes have been described. The nomenclature of the *cry* genes encoding Bt Cry proteins was published by Crickmore et al. (1998) and has been constantly updated in the website: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/.

1.2 Cellular Morphology and Physiology

Bt is an aerobic Gram-positive and rod-shaped bacterium, with a vegetative cell of 1.0–1.2 μm wide and 3.0–5.0 μm in length, usually mobile by means of peritrichous flagella, naturally not numerous. The flagella may bind to insect cells and is important in virulence (Zhang et al. 1995). Also, there is strains of Bt that cannot be tested by the classical flagella serotyping due to "autoagglutination" that occurs in NaCl (0,85%) in the absence of specific autiserum (Lecadet et al. 1999; Chaves et al. 2008). The spore of this bacterium has an ellipsoidal shape but mostly are cylindrical and is located in the central or paracentral region when inside the mother cell. The species is non-strict aerobic with a temperature range of growth between 10–5 ºC and 40–45 °C. The main characteristic that distinguishes this species from the others of the same genus is the intracellular presence of a protein crystal (Höfte et al. 1986; Höfte and Whiteley 1989; Habib and Andrade 1998; Glare and O'Callaghan 2000); see Fig. 1.1. For instance, the protein cristal is plasmid borne and has been transferred to strains of *Bacillus cereus* and even to *Bacillus pumilus* (Selinger et al. 1998). Cells grown on glucose nutrient agar produce large amount of storage material, giving a vacuolated or foamy appearance. Like *B.cereus and* Bt gives egg-yolk reaction positive. Most strains are catalase-positive, oxidase negative, casein, gelatin and starch are hydrolyzed. Voges-Proskauer- positive and citrate is utilized as sole carbon source. Nitrate is reduced and tyrosine is decomposed. Phenylalanine is not deamineted. Most strains utilize saccharose and other sugars, but *Bacillus thuringiensis* serovars israelensis do not ferment this disaccharide (Claus and Berkeley 1986; De vos et al. 2009).

Sometimes, species of *Bacillus* presenting very similar phenotypic and genotypic characteristics are clustered by some researchers in groups. This is the case of *Bacillus cereus* and closely related species such as *Bacillus mycoides*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus cytotoxicus* and the psycro-tolerant *Bacillus weihenstephanensis*. Another important factor taken into account when clustering these species into a group is the width of the cells. For the large *Bacillus* group the cell width should be $\geq$ 1μm, at least. Bt is a member of this group. There is a very wide range of colonial morphology, both within and between species, and of course medium composition and other incubation conditions have strong influences.

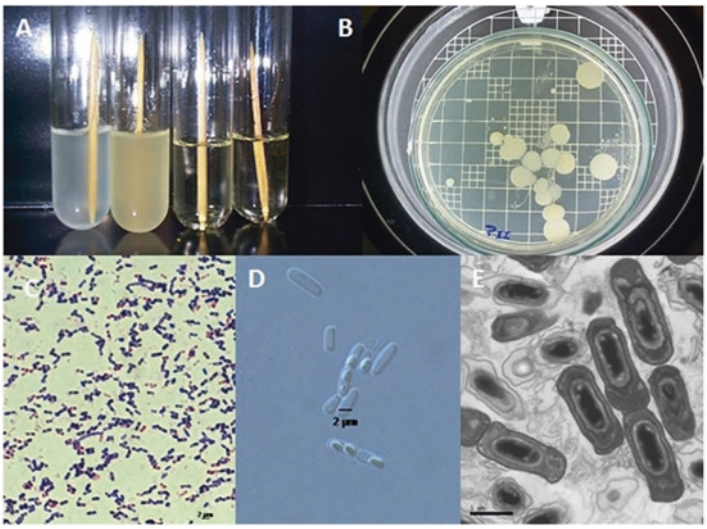

Fig. 1.1 *Bacillus thuringiensis*: (**a**, **b**) selective culture medium, (**c**) gram staining, (**d**) interferential phase contrast micrograph, and (**e**) transmission electron micrograph, Bar = µm

Colonial descriptions may include details of diameter, overall plan shape, elevation, any tendency to spreading or colonial motility, morphology of edges, surface texture (such as glossy or matte), color, consistency, any tendency to be adherent to the medium, and hemolysis (absent, slight or marked, partial or complete). Sporulation may be strongly associated with the spatial development of the bacterial community, and full details of the cultural conditions employed when making observations of colonial morphology are therefore essential (Logan et al. 2009), according to Fig. 1.1.

Bt has two distinct phases during cell development: vegetative cellular division and spore formation (Bulla et al. 1980, Ibrahim et al. 2010). The development of the spore and crystal involves seven distinct stages: (a) phase I – formation of axial filaments; (b) phase II – formation of forespore septum; (c) phase III – first appearance of parasporal crystals and formation of a forespore; (d) phases IV to VI – formation of exospore, primordial cell wall, and spore nucleoid transformation; and (e) phase VII – maturation of spores and cell lysis (Bulla et al. 1980; Bechtel and Bulla 1982; Ibrahim et al. 2010); see Fig. 1.2.

The classification of Bt does not reflect the pathotype of the bacteria, which is essentially defined by the delta-endotoxins that make up the characteristic crystalline inclusion of the strains. These insecticidal proteins are synthesized after stage II of sporulation and accumulate in the mother cell as a crystal, which can account for up to 25% of dry weight of the sporulated cells (Ribier and Lecadet 1973; Lereclus et al. 1993). The latter authors report that these crystals can have different forms: bipyramidal, pyramidal, cuboidal, flat rhomboid, spherical, and rectangular. The most common form is that of a bipyramidal crystal. The crystals may contain one or more delta-endotoxins, or Cry proteins, which have molecular weights between 30 kDa and 140 kDa, and which are converted into toxic peptides after ingestion by target pests, predominantly insects (Lereclus et al. 1993; Pinto et al. 2009; Fiuza 2009). In a study about application of

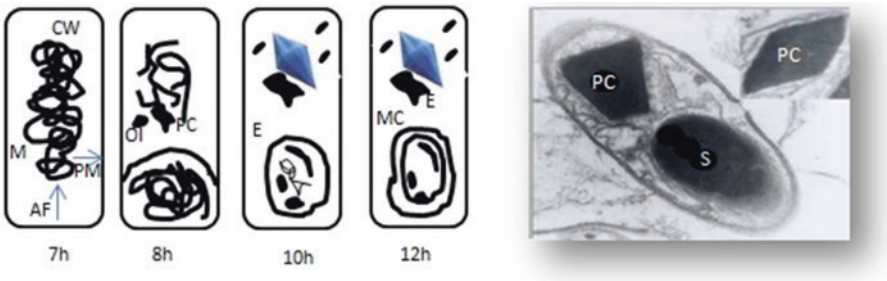

Fig. 1.2 Diagrammatic scheme of sporulation in *B. thuringiensis*. *M* mesosome, *CW* cell wall, *PM* plasma membrane, *AF* axial filament, *0I* ovoid inclusion, *PC* bipyramidal parasporal crystal, *E* exosporium, *S* spore (Based on Bulla et al. (1980))

gamma radiation to eliminate spores in biopesticides based on *B.thuringiensis* serovar *israelensis* it was shown that radiation ensures total inactivation of the spores in the insecticide without changing the larvicidal activity (Rabinovich et al. 2014).

In addition to Cry proteins, Bt isolates can synthesize proteins called Cyt, which have in vitro cytolytic activity and in vivo specificity to dipterans (Höfte and Whitheley 1989). According to Soberón et al. (2013), Cyt toxins are able to affect insect midgut cells and may increase the insecticidal activity of certain Cry toxins.

Proteins known as beta-exotoxins are also synthesized by some Bt isolates. One of these, thuringiensin is a nonspecific and thermostable protein toxic to vertebrates (Sebesta et al. 1981). Because it is nonspecific, isolates that are capable of producing, it cannot be used in the production of biopesticides (Bishop et al. 1999; Siegel 2001). Beta-exotoxin has been identified in different subspecies Bt, such as *Bt tenebrionis* (Perani et al. 1998); *Bt kenyae* and *Bt tolworthi* (Hernandéz et al. 2001); *Bt thuringiensis*, *Bt kurstaki* (Hernandéz et al. 2003), and *Bt darmstadiensis* (Tsai et al. 2003).

On the other hand, Vip proteins are divided into four families according to their amino acid identity. Vip1 and Vip2 proteins act as a binary toxin and are toxic to some coleopteran and hemipteran species (Chakroun et al. 2016). In addition to the aforementioned proteins, Bt can produce phospholipases, proteases, chitinases, and enterotoxins (Schnepf et al. 1998; Rabinovitch et al. 1998; Zahner et al. 2005). Enterotoxin is similar to that produced by *B. cereus*, whose ingestion in food results in intoxication (Tayabali and Seligy 2000). In health, Bt produces some classes of bacteriocins, which are important for the control of pathogenic microorganisms and food contamination (Salazar-Marroquin et al. 2016). According to these authors, the bacteriocins are peptides ranging from 1 to 12.4 kDa, and 18 bacteriocins produced by Bt have already been described.

1.3 Biochemistry and Molecular Biology

In the pioneering studies carried out by de Barjac and Bonnefoi (1962), 24 strains of Bt were evaluated on biochemical and serological tests. These authors found that the crystal-producing bacteria could be subdivided into six biochemical

groups. In addition, the reality of this subdivision was supported by the presence of an H antigen that was specific to each group (de Barjac and Bonnefoi 1968).

According to Logan et al. (2009), the methodology described for the identification of *Bacillus* described by Gordon et al. (1973), Claus and Berkeley (1986), and Logan and De Vos (2009) can now be considered valuable in differentiating aerobic bacteria from spore formers and is widely applicable across the many genera. The commercially available biochemical tools mostly used for the identification of *Bacillus* spp. and correlates are the API 20E and 50CHB systems (BioMerieux), the VITEK systems (BioMerieux), and Biolog. However, it should be noted that tests performed with traditional culture media, when compared to commercially available kits, may show different results for a more specific biochemical test. In this way, Logan et al. (2009) recommend that the tables of differential characters accompanying proposals for new taxa are often partly compiled from the literature. It is important to be aware of this potential lack of comparability, and the kinds of tests used for all the included taxa in such tables should be declared. For all characterization tests, the methodologies must be stated explicitly, and it is essential that results be verified using reference strains as positive and/or negative controls. An example of biochemical analysis considered essential by several researchers in this area is the reduction of nitrate, which has been widely and reliably used in the characterization of aerobic endospore producers for many years.

In molecular biology studies, one of the most important aspects of the *Bacillus* genus is its diversity. There are species that have a key role in medicine, industry and the economy. Some examples are *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, and *B. pseudomycoides*. Many studies based on molecular markers find it difficult to ultimately separate *B. cereus* and Bt. The most obvious difference between these two species is the presence of plasmids which encode toxic proteins for insects in Bt (Chen and Tsen 2002; Rasko et al. 2005). The differentiation between these species using molecular markers continues to be a hard task, regardless of the methods used (Manzano et al. 2009; Zwick et al. 2012).

Several taxonomic studies using different methods were conducted in the last decades looking to establish valid criteria for the differentiation and the diversity analysis interspecific and intraspecific for species of the genus (Bottone 2010; Logan 2012; Sanchis and Bourguet, 2009). In the specific case of Bt, since its description in 1915, the main method applied to the identification of subspecies was based on the flagellar H antigen reaction (de Barjac and Bonnefoi 1962, 1973). For example, using this method in the last few decades, more than 3.000 strains known as *B. thuringiensis* were gathered in 69 serotypes and 13 subtypes (Lecadet et al. 1999). The serotyping, although being valid, is not the most appropriate method to classify Bt strains, since strains in a given serotype do not always have the same biochemical, genetic, and/or toxic features. For an overview of the criteria used for the characterization of *Bacillus* species, consult Logan et al. (2009).

The application of molecular tools that make possible to differentiate the different strains of Bt in a more appropriate way is crucial for studies that seek to identify new strains to be used in pest control programs. Challenges arise from the great variability found in this species and the difficulty in consistently correlating the molecular patterns detected with the insecticidal activity in the strains. Among the tools mostly used, high-

lights include that based on 16S rRNA, RAPD, RFLP, REP-PCR, ERIC-PCR, and MLST. All of them make it possible to detect differences between the analyzed strains, but a debate remains on which of these tools are the most appropriate to be used as a standard method to molecularly characterize *B. thuringiensis* strains and to correlate the molecular patterns with toxicity to different insect species.

The 16S rRNA genes are considered one of the main molecular markers for studies in bacteria diversity. This marker is widely used for phylogenetic analysis and for studies in metagenomics (Woese et al. 1990; Joung and Cote 2001; Yarza et al. 2014). These genes have both conserved and variable regions which make its application possible for studies in different taxonomic levels. In recent decades, its application significantly extended our knowledge about diversity in prokaryotes. Data can be generated by sequencing some regions of this gene or by using the RFLP technique.

The main restriction of the use of 16S rRNA is the high similarity between the sequences of closely related species (Christensen et al. 1998), as happens in the *Bacillus* genus. To overcome these limitations, recent works have been using 16S rDNA analysis together with other markers, increasing the capacity to differentiate Bt strains and other species of *Bacillus* (La Duc et al. 2004, Soufiane and Côté 2009; Vidal-Quist et al. 2013; Bhandari et al. 2013; Prabhakar and Bishop 2014; Caamaño-Antelo et al. 2015).

Among the studies that use 16S rRNA gene with other markers are ones conducted by La Duc et al. (2004) and Soufiane and Coté (2009), who also analyzed the variation found in the gene for DNA gyrase. The gene for DNA gyrase has a single copy, with a constitutive expression, found in almost all bacteria and can be amplified by universal primers. Its evolutionary rate is higher than that of 16S rRNA, which gives it a greater capacity to accurately discriminate related species (Mun Huang 1996; Wang et al. 2007). For La Duc et al. (2004) and Soufiane and Coté (2009), despite this increases in power to differentiate between species of *Bacillus*, it is not enough to differentiate *B. cereus* from Bt. This result contrasts with that of Park et al. (2007), who successfully differentiated these and more species of genus *Bacillus* using a combination of primers only for the DNA gyrase gene. According to Soufiane and Coté (2009), although these genes cannot discriminate between *B. cereus* and Bt, they are efficient enough to differentiate the strains belonging to the same serovar of Bt. For these authors, the power to identify the strains was higher for the DNA gyrase gene.

The analysis of repetitive regions of the genome is one of the tools successfully applied to analyze the diversity among the species of *Bacillus*. Its use has made possible the differentiation between Bt and *B. cereus* strains (Reyes-Ramirez and Ibarra 2005; Cherif et al. 2007; Peruca et al. 2008; Sauka et al. 2012; Katara et al. 2013; García et al. 2015). The study of these regions is named fingerprint analysis and is based on amplification by PCR of the regions repetitive enterobacterial palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequence. The genetic variability found in these sequences makes its successful application possible to study the intra- and interspecific diversity of species belonging to the genus *Bacillus*. Its efficiency to differentiate the species is greater than the one found in the gene for 16S rRNA (Rai et al. 2015). The additional advantages of this technique are its easy and quick implementation, its simplicity, and the fact of that the results are reliable and reproducible. These regions are distributed along the bacteria genome, and their

amplification generates electrophoresis patterns which can be related to specific strains (Versalovic et al. 1994; Brumlik et al. 2001; Reyes-Ramirez and Ibarra 2005; Cherif et al. 2007). The use of specific primers for regions of interest amplifies DNA segments of different sizes and, when revealed by electrophoresis in an agarose gel, generates specific patterns. The primers are designed for sequences that are close to repetitive sequences (Versalovic et al. 1994; Shangkuan et al. 2001; Lima et al. 2002). The application of ERIC-PCR made it possible to differentiate the Bt strains coming from different sites and registered a good relation between the electrophoresis patterns and the area where the strains came from (García et al. 2015). Similar results were obtained by Sauka et al. (2012) using the REP-PCR technique to differentiate strains from different samples.

Multilocus sequence analysis (MLSA) was developed by Maiden et al. (1998) to study the genetic diversity in pathogenic microorganisms. It is based on the analysis of (housekeeping) genes that are expressed in a constitutive way, in other words, genes which have a central role in maintaining the cell's metabolism (Gevers et al. 2005). The alleles are identified through the sequencing of internal fragments of these genes. The new variants are created by mutations, synonymous or not, in the nucleotide sequence. This technique is considered an excellent tool to study the inter- and intraspecific genetic variability and to study the strains evolution (Van Belkum 2003; Cardazzo et al. 2008; Perez-Losada et al. 2013). The analysis of several genes minimizes the impact of horizontal and vertical gene transfer that occurs in bacteria and increases the capacity of resolution because of the number of variants that are analyzed; it is easy to reproduce and to allow the comparison of results obtained from different researchers (Maiden et al. 1998; Maiden et al. 2013; Glaeser and Kämpfer 2015). Studies about differentiation in *Bacillus* species using MLSA indicate a high similarity between *B. cereus* and Bt According to some authors, the two species should be considered as a single one (Helgason et al. 2004; Zahner et al. 2013).

The molecular techniques described above can differentiate strains of Bt with considerable efficiency, but it cannot be said that they are capable of safely differentiating the two species. Nowadays, despite of the great number of studies, the main way of differentiating *B. cereus* from Bt is the presence of proteins (Bt toxins) active against insect species. The studies also cannot associate molecular patterns of the chromosomal DNA with the insecticidal activity. Given this scenario, perhaps the best way to identify and characterize new strains of Bt is to analyze the plasmid genes responsible for the synthesis of these toxins. Several studies have used PCR to identify and characterize new strains of Bt (Salama et al. 2015; Katara et al. 2016; Boukedi et al. 2016). In this technique primers are used for known Cry genes, aiming to predict the proteins produced by the studied strains.

Besides molecular patterns based on using nucleic acids, some chemical markers are considered important for the analysis and description of the inter- and intraspecific variability in bacteria. Among the chemotaxonomic tools applied to the identification of bacteria, highlights include the so-called fatty acid methyl ester (FAME) and matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry.

The FAME technique is based on the analysis of short-chain fatty acids, which have between 9 and 20 carbons. The production of the fatty acids is considered a specific property of each microorganism and can be driven by environmental factors. These compounds are important for the adaptation process of microorganisms. All these properties have made the fatty acids analysis an important tool for the taxonomy of bacteria (Kampfer 1994; Yano et al. 1998; De Sarrau et al. 2012; Cardinali et al. 2015). According to Diomandé et al. (2015), fatty acids analysis is also a good marker for studies of the adaptation in *Bacillus* species, as their composition varies according to the environment.

The MALDI technique was implemented by Karas et al. (1989) and Tanaka et al. (1988). Because of the results obtained in different areas (medicine, food quality control, environmental ecology), this technique is considered one of the most important tools for the study of microorganisms. It is based on the use of a mass spectrophotometer to analyze the spectral patterns of ions present in macromolecules. In this technique, all proteins of the bacteria cell provide a specific pattern (fingerprint) which can be statistically analyzed (Seng et al. 2009; Allen et al. 2015).

According to Teramoto et al. (2007), the spectral pattern of bacteria is dominated by ribosomal proteins, which are conserved in an evolutionary way and represent a unique feature for each taxon. In some fields the results may be equivalent to those obtained using nucleic acids (Schumann and Maier 2014). Ziegler et al. (2015) adapted this technique to analyze ribosomal proteins of bacteria that inhabit root nodules. According to the authors, the results are obtained more quickly, and the process has lower cost, is less laborious, and has the same efficiency as the traditional techniques that use 16S rRNA (RFLP, AFLP, and sequencing). A recent study using the MALDI MS technique identified several proteins involved in important biological processes which might be used to identify species of the genus *Bacillus*, including *B. cereus* and Bt (Pfrunder et al. 2016).

A final analysis of the techniques described in this review, which are used to characterize the diversity of species of the genus *Bacillus*, shows us that many of them allow the differentiation of intraspecific strains. However, differentiation between *B. cereus* and Bt continues to be a problem for all techniques. Perhaps, this challenge might be overcome with the sequencing of genomes or transcriptomes of species from this group. Comparative analysis of the genome from *Bacillus* species will be important to establish a precise phylogeny of the genus, as well as to solve the taxonomic issues (Bhandari et al. 2013; Varghese et al. 2015).

All techniques introduced in this review provide good results when used for Bt strains. Therefore, a question remains: which one is more appropriate to be used as a standard for studies with this species? An answer remains to be given, but if we consider the importance of Bt in pest control, the chosen technique must be easily applied, have a low cost, and, most importantly, provide results regardless of the origin of the samples. Thus, we believe that the scientific community should concentrate their efforts in more detailed analyses and choose among the available techniques, one to be used as a standard. The creation of a database, gathering results and information generated by researchers from all around the world, would also be an important advance.

1.4 Pathogenicity and Virulence

Pathogenicity and virulence are two important parameters in target pest susceptibility bioassays to entomopathogen(s) in laboratory. However, due to an unclear definition of these terms, many bioassays are carried out with the purpose of estimating virulence without first checking whether the organism is pathogenic or not. It is also very common to find the term "more pathogenic" or even attempts to evaluate virulence in simple mortality bioassays.

Shapiro Ilan et al. (2005) pointed out that some definitions and uses of the terms pathogenicity and virulence have been a topic of discussion among several disciplines in the field of pathology. Although Thomas and Elkinton (2004) have proposed an innovative way to define pathogenicity and virulence (Table 1.1), Shapiro-Ilan et al. (2005) stated that Steinhaus and Martignoni's (1970) definitions are clearly distinguishable and remain useful. Therefore, these definitions should be

Table 1.1 Definitions of pathogenicity and virulence

Pathogenicity	Virulence
The quality or state of being pathogenic. The potential ability to produce disease[a]	The disease-producing power of an organism. Degree of pathogenicity within a group or species[a]
…the ability of a strain or species of microorganism to produce disease in various hosts. Indicated term is used qualitatively[b]	Degree of pathogenicity against a specific host species in controlled conditions within a group or species of microorganisms. Indicated term is used quantitatively[b]
The quality of being pathogenic[c]	The quality of being virulent, the quality of being poisonous, and the disease-producing power of a microorganism[c]
The ability to invade and injure the host's tissues. Applies to groups or species of pathogens[d]	The disease-producing power of the pathogen, the ability to invade and injure the host's tissues. The degree of pathogenicity within a group or species[d]
Nearly synonymous with virulence but applied to groups or species[e]	The disease-producing power of a microorganism. The ability of a microorganism to invade and cause injury to the host. The relative capacity of a microorganism to overcome the host defense mechanisms…. The degree of pathogenicity within the group or species[e]
The quality or state of being pathogenic. The potential ability to produce disease. Applied to groups or species[f]	The disease-producing power of an organism. Degree of pathogenicity within a group or species[f]
The number of dead individuals relative to the number exposed to the pathogen[g]	The number of dead individuals relative to the number infected[g]

Adapted from Shapiro Ilan et al. (2005): [a]Steinhaus and Martignoni (1970); [b]Aizawa (1971); [c]Cantwell (1974); [d]Tanada and Fuxa (1987); [e]Tanada and Kaya (1993); [f]Lacey and Brooks (1997); [g]Thomas and Elkinton (2004)

adhered to within the discipline of invertebrate pathology. Pathogenicity is the quality or state of being pathogenic, and virulence is the disease-producing power of an organism – the degree of pathogenicity within a group or species. Furthermore, for a given host and pathogen, pathogenicity is absolute, whereas virulence is variable. An organism is either pathogenic to a host or it is not. In contrast, virulence is a measurable characteristic of the ability to cause disease.

The SIP glossary (http://www.sipweb.org/resources/glossary.html) corroborates with Shapiro-Ilan et al. (2005); pathogenicity: the quality or state of being pathogenic. The potential ability to produce disease applied to groups or species of microorganisms, while virulence is used to express degree of pathogenicity. Pathogenicity is often genetically determined as the ability to produce disease, and virulence as the ability to produce disease that is not genetically determined. Pathogenicity is qualitative. Virulence: the disease producing power of a microorganism, i.e., the ability of a microorganism to invade and injure the tissues of its host. The relative ability of a microorganism to overcome the body's defenses of the host. The degree of pathogenicity within a group or species. Virulence can be quantified. Thus, it's possible to speak of lowly virulent, virulent, and highly virulent strains within a group or species of microorganisms that are pathogenic.

Measures of disease severity are linked with mortality dose response bioassays, such as LC_{50}, but may also include single-dose mortality assays, or non-lethal measures, e.g., ET_{50}, reductions in fitness, or extent of tissue damage (Shapiro-Ilan et al. 2005).

The screening of isolates with high virulence is the most important step in the development of biopesticides (Alves 1998). Pathogenicity and virulence are the most frequently evaluated parameters, although sublethal effects on surviving hosts should be also considered. For instance, surviving insects may have their physiological processes affected resulting in harmless larvae.

Polanczyk and Alves (2005) verified that Bt affected the biological parameters of *S. frugiperda* (Lepidoptera: Noctuidae), mainly in larval and female pupae weight, and in some instances, these effects were passed on to further life cycle stages affecting oviposition and egg fecundity. Although sublethal effects are difficult to evaluate, mainly under field conditions, they should be considered when the activity of an entomopathogen is measured. Santos Júnior et al. (2009) verified the Bt sublethal effects in *Helicoverpa zea* (Lepidoptera: Noctuidae) by the reduction of larvae and pupae weight. Sedaratian et al. (2013) reported the sublethal effect of Bt bioinsecticide Biolep® WP on the length of larval, prepupal and pupal stages of *Helicoverpa armigera* (Lepidoptera: Noctuidae).

Ghassemi-Kahrizeh and Aramideh (2014) reported the effect of Bt on the potato beetle (*Leptinotarsa decemlineata*) observing that only 15% of surviving individuals became adults, and the pupal mortality of the surviving larvae was around 50%. There was an assumption that large potato beetle larvae remain in plants after Bt application causing significant damage. But the large larvae are unable to feed themselves, and even if they feed, a high mortality was reported during the pupae stage, and there was a delay in development for the adult stage. Furthermore, even

if they reach the adult stage, these adults will not have a normal morphology and physiology. Only counting the mortality rate of the larvae in a short period cannot provide a good estimate of the level of protection of the cultures obtained through the Bt application. Therefore, the mortality caused by the application of Bt-based biopesticides will be higher than the estimated values.

Besides the sublethal effect, the effect of Bt on lepidopteran adults has few reports in the literature. The negative effects on adult longevity or even low mortality rates were observed for *Heliothis virescens* (Lepidoptera: Noctuidae) and *Spodoptera exigua* (Lepidoptera: Noctuidae) (Grove et al. 2001). Zhang et al. (2013) reported that Bt reduced the adult longevity, mating, spermatophyte transfer, and oviposition in *S. frugiperda* and *H. armigera.*

Star and Banks (2003) proposed a new method for assessing toxicity to a target organism because exposure to a toxic agent can result in mortality as well as multiple sublethal effects, as reported above. Demographic studies have been suggested as more desirable means to assess total toxic. In addition, demographic studies are generally conducted throughout the life span of an organism and thus provide a complete mortality report. Demographic toxicological studies or life table response experiments provide a measure of the effect on population growth rate.

Life tables are very useful instruments for this purpose, exposing groups of individuals that increase the doses or concentrations of a toxic substance over their lifetime. Daily mortality and reproduction are recorded, and these data are then used to generate life table parameters. As life tables are often developed in laboratory and data are generated on individuals, rather than populations, a realistic measure of population growth rate is not obtained. However, the intrinsic rate of growth has been shown to be a more accurate measure of the toxic effect than estimates of lethal concentration (LC_{50}).

Sedaratian et al. (2013) described Bt effects on *H. armigera* using a life table. The duration of the different phases of life in treated *H. armigera* was significantly affected by the treatments. In addition, fecundity was also negatively affected in female developed from larvae treated with Bt *kurstaki*, with egg-hatching rate reaching zero. Sublethal concentrations of Bt *kurstaki* reduced the net rate of reproduction (R_0), and there were also significant differences between R_0 values in all treatments. Intrinsic and finite rates of increase (r_m and k, respectively) were significantly lower in insects treated with Bt *kurstaki*. There was also a reported reduction in the development rate for *H. armigera* treated with Bt *kurstaki*. The mean time between generations (T) and doubling time (DT) were significantly higher in insects exposed to Bt.

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Chapter 2
The Biology, Ecology and Taxonomy of *Bacillus thuringiensis* and Related Bacteria

Ben Raymond

Abstract *Bacillus thuringiensis* produces a range of specialized virulence factors that enable it to infect invertebrate hosts. Despite the level of interest in this species, there have been a number of controversies and disagreements regarding its ecological niche, how it kills its hosts and benefits from the production of Cry toxins and whether *B. thuringiensis* constitutes a real species that is a distinct member of the *Bacillus cereus* group. Hypotheses arguing that *Bt* is a soil saprophyte, a gut or plant commensal or a specialized pathogen are critically evaluated. Evidence supporting the specialized pathogen hypothesis includes proteomic and genomic studies revealing adaptations to lyse cells and exploit peptide-rich resources. *Bt* infects insects and reproduces effectively in the field without obvious epizootics and uses plants to vector inocula from soil to the phylloplane. *Bt* Cry toxins, and other virulence factors, can be treated as cooperative public goods. Cooperative production of virulence factors has implications for dose-response curves and understanding which ecological factors can select for the maintenance of virulence. Finally, the taxonomy of *Bt* and the phylogeny of the *B. cereus* group are discussed. The genetic and ecological variation within the *B. cereus* group is substantial and argues against lumping all members of this clade into one species; a revised nomenclature of the group is suggested that includes restricting the use of *B. thuringiensis* to a single clade that contains the vast majority of invertebrate-adapted isolates and revising the use of the *cereus* and *anthracis* epithets.

Keywords Evolution of virulence • Phylogeny • Transmission

Bacillus thuringiensis is defined as a member of the broader *Bacillus cereus* that is capable of producing crystalline inclusion bodies. The *B. cereus* group contains a diverse array of pathogenic strains. *Bacillus cereus* sensu stricto can be a causative agent of two forms of human food poisoning: emetic (associated with the toxin cereulide) and diarrhoeal (associated with a broad range of enterotoxins) (Stenfors

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Arnesen et al. 2008). The *B. cereus* group also contains *Bacillus anthracis*, the aetiological agent of anthrax, which is predominantly a disease of vertebrate herbivores (Turnbull 2002). Without doubt *B. thuringiensis* (*Bt*) remains the single most important bacterial species in insect pest management. Its utility derives from the large quantities of proteinaceous toxins that form crystalline parasporal inclusions, termed the Cry (crystal) and Cyt (cytolytic) proteins (Schnepf et al. 1998). As described elsewhere in this book, the vast majority of commercially viable genetically modified (GM) insect-resistant crops express one or more Cry toxins. The total planted area of Cry toxin expressing crops exceeded 20 million hectares in 2015 (James 2015). Moreover, spore and toxin *Bt* formulations are also the most successful organic microbial pesticide, with target hosts ranging from mosquitoes to lepidopteran pests of agriculture, horticulture and forestry (Glare and O'Callaghan 2000). Nearly 100 years of research has meant that the diversity, structure and mode of action of *Bt* Cry toxins have been intensively studied.

However, despite this long period of interest the biology, phylogeny and ecology of the bacterium have been the subject of much controversy and disagreement. Controversies that have dogged our understanding of this bacterium include whether or not it is a specialized invertebrate pathogen (Jensen et al. 2003; Raymond et al. 2008a, 2010a), how it manages to benefit from the production of highly costly Cry toxins (Martin and Travers 1989), whether it can infect vertebrates (Siegel 2001; Federici and Siegel 2007) and whether *Bt* should be regarded as a species or a mere plasmid carrying subtype of *Bacillus cereus* (Helgason et al. 2000; Didelot et al. 2009). One highly cited studied has even claimed that *Bt* requires gut bacteria to assist in killing hosts, although the results of that study have not proved repeatable by other groups and were caused by confounding the removal of gut bacteria with the use of broad-spectrum antibiotics (Broderick et al. 2006; Johnston and Crickmore 2009; Raymond et al. 2009; van Frankenhuyzen et al. 2010). These disagreements have implications outside of the purely academic sphere. As this book goes to press, the European Union regulators are debating whether the safety regulation of *Bt* microbial products needs to be tightened. This revision of its status as one of the safest pest control products on the market was prompted by a controversial review arguing that *Bt* is biologically and ecologically indistinguishable from *B. cereus* (EFSA 2016). In addition to safety issues, understanding the details of pathogenicity and the infection process and how *Bt* benefits from the production of its toxins may help inform strategies for both the discovery of new strains and the improvement of existing products.

This chapter will critically examine some of the controversies surrounding the biology of this important microbe and address some of the recent advances in our understanding of the fundamental biology of *Bt.* In particular, the evidence base to support the view that *Bt* is a specialized invertebrate pathogen is now substantially stronger than it was at the time of the last review on this topic (Raymond et al. 2010a). A caveat here is that *Bt* as a species is in need of taxonomic revision. Key developments in this area covered in this review include fundamental field ecology experiments demonstrating benefits of Cry toxins for bacterial reproduction and fitness (Raymond et al. 2010b, 2012); better genomic and phylogenetic data on *Bacillus* including an understanding of biological and ecological variation across distinct clades (Cardazzo et al. 2008;

Guinebretière et al. 2008; Didelot et al. 2009; Alcaraz et al. 2010; Raymond et al. 2010b; Raymond and Bonsall 2013), application of evolutionary theory to explain how selection for high virulence is maintained in *Bt* (Schulte et al. 2010; Raymond et al. 2012; Zhou et al. 2014; Cornforth et al. 2015; Deng et al. 2015; van Leeuwen et al. 2015), an appreciation of the prevalence of *Bt* strains pathogenic to nematodes (Ruan et al. 2015) and an increased understanding of the relationship of *Bt* with plants (Monnerat et al. 2009; Raymond et al. 2010b; Vidal-Quist et al. 2013).

2.1 Competing Hypotheses Regarding the Fundamental Ecology of Bt

How did these controversies arise? For spore-forming bacteria, a common source of confusion is that viable material can be readily isolated from habitats that are largely unsuitable for growth. *Bt* is readily isolated from soil and plants (Delucca et al. 1981; Martin and Travers 1989; Smith and Couche 1991; Kaur and Singh 2000; Hendriksen et al. 2006). Another source of confusion is the fact that *Bt* strains have a variable ability to grow and sporulate within particular insect species, despite often having high pathogenicity (Prasertphon et al. 1973; Suzuki et al. 2004). Moreover, disease outbreaks or epizootics are very rare in the field (Porcar and Caballero 2000) although they occur readily in grain stores and in insect culture in the laboratory (Burges and Hurst 1977; Delucca et al. 1982; Itova-Aoyolo 1995; Federici and Siegel 2007). Effective transmission of *Bt* between larvae has also been difficult to demonstrate experimentally (Takatsuka and Kunimi 1998) and can require a high density of hosts and/or cannibalism (Knell et al. 1998).

Although Bt is readily recovered from the environment, an early influential paper reported a lack of correlation between host abundance and the abundance of entomopathogenic *Bt* (Martin and Travers 1989). The combination of high prevalence, but the difficulty in observing transmission, has led to a wide speculation on the ecological niche of *Bt.* It has been suggested that *Bt* is a soil micro-organism with incidental insecticidal activity (Martin and Travers 1989), that *Bt* is part of the phylloplane microbiota and has evolved to provide symbiotic protection against insect attack (Smith and Couche 1991; Elliot et al. 2000) or that *Bt* may be part of the commensal gut microbiota of many insects without causing overt disease (Jensen et al. 2003). Many of these ideas are persistent although several have been tested and failed to gain support in a number of studies.

2.2 *Bt* Grows Poorly in Soil and Is Poorly Adapted to the Nutritional Resources Prevalent in Soils

To begin with *Bt* is not a soil bacterium, in the conventional sense, as it has a very poor ability to grow in unamended soil (West et al. 1984, 1985; Yara et al. 1997). Growth in autoclaved soil, which both removes saprophytic competitors and release

additional nutrients, is not particularly convincing evidence for having a saprophytic niche. For *B. cereus* sensu stricto, which can be chromosomally very similar to entomopathogenic *Bt* (see discussion below), growth in sterile filtered media made of soluble soil nutrients is also questionable support for saprophytism (Vilain et al. 2006). In the field, *Bt* populations decline slowly in soil over several years (Addison 1993; Eskils and Lovgren 1997; Hendriksen and Carstensen 2013). In one case, the evidence suggests that cycles of germination, growth and sporulation occur seasonally in soil, although this is against a background of reducing population size, and this observational study could not preclude the contribution of insect mortality (Hendriksen and Carstensen 2013).

One of the most convincing lines of evidence against *Bt* or *B. cereus s.s.* being soil saprophytes comes from comparative genomics (Alcaraz et al. 2010). Typical members of the genus *Bacillus* (e.g. *B. subtilis* group species) have a large number of genes involved in processing carbohydrates, particularly complex carbohydrates that derive from plants (Wipat and Harwood 1999; Alcaraz et al. 2010). Processing of complex plant carbohydrates is a key adaptation for saprophytes and plant commensals (Badri et al. 2013). *Bt* and *Bc* are unusual in that they possess relatively few carbohydrate-processing genes and lack the capacity to use many simple sugars such as mannose, arabinose, and in some cases, sucrose (Rasko et al. 2005; Alcaraz et al. 2010). In contrast, bacteria in the *B. cereus* group are rich in enzymes involved in peptide and amino-acid processing in comparison to members of the *B. subtilis* group (Read et al. 2003; Rasko et al. 2005; Alcaraz et al. 2010). For example, *B. cereus* and *B. anthracis* possess six amino-acid efflux systems, which prevent the accumulation of amino acids intracellularly to levels that can inhibit growth (Read et al. 2003). In addition, a substantial proportion of the secretome of *Bt* and *B. cereus* (70% of secreted stationary phase proteins) is composed of proteases or other enzymes/virulence factors with putative roles in cell lysis and disrupting membranes (Gohar et al. 2005). In short, the *B. cereus* group is dominated by meat-eaters. Any readily culturable bacterium with a large genome and a flexible metabolism will be able to grow when nutrients are provided, so some germination and growth in the soil may be possible. Nevertheless, the balance of evidence indicates that *Bt* is not well adapted to soil conditions and is poor at competing in this environment.

2.3 *Bt* Is Not a Commensal Bacterium

The idea that *Bt* might be able to reproduce as a commensal originated from observations of the occurrence of vegetative cells in the midguts of soil invertebrates (Hendriksen and Hansen 2002), rather than from experimental work. *Bt* can grow and germinate vegetatively in the insect midgut in the process of lethal infections, although the number of cells can be very few in some hosts (Chiang et al. 1986; Zhou et al. 2014). *B. cereus* group strains have also been regularly recovered from the guts of Lepidoptera and other invertebrates, and the specialized ‘Arthromitus’

form of *B. cereus* appears to have adaptations specifically for attachment to the midgut (Margulis et al. 1998; Jung and Kim 2006; Raymond et al. 2008b). However, an explicit experimental test of the commensal hypothesis using *Bt kurstaki* and larvae of the diamondback moth, *Plutella xylostella*, showed that *Bt* does not replicate in sublethal infections and cannot be transmitted vertically from female to eggs (Raymond et al. 2008a). In fact, this study showed that survival of spores in the midgut is lowest when ingested *Bt* strains carry Cry toxins with suitable receptors in the insect midgut (Raymond et al. 2008a), the inference here being that germination of spores is increased in susceptible hosts but that without a successful lethal infection, vegetative cells pass out through the hindgut via peristalsis. Growth in the gut prior to invasion of the haemocoel is ecologically very different, first because the action of the Cry toxins paralyses the gut (Endo and Nishiitsutsujiuwo 1980), ensuring that germinating material remains in the digestive tract, and second because pore formation leads to leakage of haemolymph into the gut, which increases nutrient supply (Heimpel and Angus 1959). The available evidence is therefore that *Bt* is an 'obligate killer', a pathogen that requires host death for reproduction (Ebert and Weisser 1997).

2.4 Transmission and the Relationship of *Bt* with Plants

While we have an expectation that *Bt* has to kill invertebrates in order to reproduce, we also have a pathogen that rarely causes disease outbreaks. For *Bt* strains attacking herbivorous hosts, one way of reconciling these facts is to consider that *Bt* transmission may not typically occur directly from cadaver to larva but may use plants as vectors. High concentrations of spores and toxins can deter feeding (Knell et al. 1998), and concentration of cultivable spores above 10^3 per cm^2 is rare on plant tissue in the field (Maduell et al. 2002; Collier et al. 2005; Raymond et al. 2010b). Thus the majority of naturalistic infections may be initiated from relatively low doses. Instead of being randomly distributed onto plants by rain splash, bacteria in soil may invade plant tissue through the xylem (Monnerat et al. 2009). Experimental inoculation of sterilized seeds with *Bt* spores shows that this bacterium is capable of colonizing plants endophytically directly from the soil (Bizzarri and Bishop 2008; Monnerat et al. 2009; Raymond et al. 2010b). Colonization from soil is sufficient to ensure that bacteria are present on growing leaf material at doses high enough to kill insects (Bizzarri and Bishop 2008; Monnerat et al. 2009); precise analysis of dose-response curves shows that doses in the region of ten spores can cause detectable levels of mortality in susceptible hosts such as *P. xylostella* (Cornforth et al. 2015).

Moreover, the ability to colonize plants is not universally distributed across the *B. cereus* group (Vidal-Quist et al. 2013); strains of *Bacillus weihenstephanensis*, which have a presumed niche in the plant rhizosphere, are poor leaf colonists, while a range of pathogenic isolates of *Bt kurstaki* ST8 are efficient leaf colonizers (Raymond et al. 2010b). While *Bt* can be readily recovered from plant surfaces, this does not necessarily mean that *Bt* is a specialist epiphyte using the plant exudates as

a primary resource for growth. In comparison with many bacterial epiphytes, *Bt* proliferates weakly on the leaf surface, sporulates readily and persists quite well when humidity is low (Maduell et al. 2008). This weak epiphytic proliferation suggests that colonization of plant material takes place in order for these bacteria to reach a habitat where infection of hosts is likely to take place. While there is no particular evidence to indicate that plants are maintaining *Bt* populations as symbiotic bodyguards, *Bt* can colonize leaf tissue and evades plant immunity in roots (Vidal-Quist et al. 2013) indicating the presence of adaptations that allow *Bt* to use plants to effectively vector bacteria from the main spore reservoir in the soil to tissues where hosts are likely to be feeding.

Critically, a manipulative field experiment has shown that populations of *Bt kurstaki* in the environment increase in the presence of larval hosts, without the existence of obvious epizootics (Raymond et al. 2010b). The increase in total numbers of bacteria in the *B. cereus* group and in the proportion of strains carrying Cry toxins was detectable in the top 1 cm of soil but not on leaf tissue. Experimental cages that excluded Lepidoptera larvae showed no such increase. Application of biopesticides to this experiment (DiPel WP) resulted in a very transient increase in *Bt* density on leaf tissue, while adding hosts to experimental cages had a more substantial impact on *Bt* density in the long term (Raymond et al. 2010b). This work corroborates earlier observational studies reporting increases in the abundance of dipteran herbivores can increase the prevalence of *Bt* strains pathogenic to Diptera (Damgaard et al. 1998). These data also support the indirect transmission hypothesis. An increase in *Bt* density in the top layer of soil suggests that infected and paralysed insects quickly fall off plants into leaf litter, from which spores may then enter soil and persist long enough to be taken up into plants endophytically where they may sporulate and produce Cry toxins (Raymond et al. 2010a, b). Notably this proposed life cycle does not require adaptations for efficient attachment of spore and crystals or efficient persistence of Cry toxin in soil, relevant because there do not seem to be any specific adaptations for spore crystal attachment for *Bt kurstaki* and many other strains (Deng et al. 2015). Roughly 50% of *Bt* colonizing plants from soil were located endophytically (Raymond et al. 2010b) so both spores and crystals may be located in stomata or co-localized within plant tissue. This life cycle is similar to the one proposed for *B. anthracis*. Anthrax spores must persist for long periods before finding a new host; infections in ungulates are typically acquired orally when animals consume contaminated plant material, allowing bacteria to enter the host through abrasions in the mouth (Dragon and Rennie 1995). The selective advantages of an association with plants may explain the ability of *B. anthracis* to persist in the rhizosphere (Saile and Koehler 2006) and why *B. anthracis* may be able to promote the growth of some plant species (Ganz et al. 2014). In contrast, direct cadaver host transmission may be more important for nematode-infecting *Bt* strains (Ruan et al. 2015), while *Bt israelensis* spores and toxins are efficiently concentrated by aquatic filter feeding in blackflies and mosquitoes (Lacey et al. 1978; Charles and de Barjac 1981), so that direct transmission of spores in aquatic habitats is very plausible.

2.5 *Bt* as a Specialized Pathogen

To paraphrase the old adage, if it looks like a pathogen, kills insects like a pathogen and reproduces like a pathogen, it probably is a pathogen. In addition to the production of Cry toxins, *Bt* has numerous adaptations associated with being an efficient specialized invertebrate pathogen (Raymond et al. 2010a). To summarize, *Bt* has to be able to disrupt or pass through the peritrophic membrane surrounding the gut, cross the midgut epithelium, evade host immunity and suppress competition from bacteria in the gut. Adaptations to survive on a peptide-rich diet have been discussed above; these are complemented by the secretion of iron-scavenging siderophores. *Bt* can produce a range of additional virulence factors such as Vip and Cyt toxins that enable it to cross the midgut epithelium (Yu et al. 1997; Perez et al. 2005; Bravo et al. 2007). The PlcR-papR quorum-sensing system, possibly active at the level of the microcolony very early in infection, coordinates the release of a large array of enterotoxins, proteases and phospholipases with a role in assisting invasion from the midgut (Salamitou et al. 2000; Gohar et al. 2002, 2008; Slamti et al. 2014; Zhou et al. 2014). Immune inhibitors such as the InhA1 and InhA3 metalloproteases may help evade haemocytes and break down antimicrobial peptides (Ramarao and Lereclus 2005; Guillemet et al. 2010). A second later acting quorum-sensing system, NprR, helps coordinate efficient resource use and sporulation during proliferation in late infection in the cadaver and activates degradative enzymes such as lipases, proteases and chitinases (Dubois et al. 2012, 2013; Slamti et al. 2014). While a number of the above systems might be general virulence factors which occur commonly in both invertebrate and vertebrate pathogens, the mass of evidence certainly points to a pathogenic lifestyle, albeit one in which plants may be exploited as vectors. Since *Bt* populations can increase in response to the presence of insect hosts (Ohba and Aratake 1994; Damgaard et al. 1998; Raymond et al. 2010b), a simple explanation for why we might not always see a correlation between the presence of insects and *Bt* abundance in the field is that *Bt* spores are persistent, readily dispersed (Damgaard et al. 1997), and the availability of hosts transient. *Bt*, as we discuss below, is genetically heterogeneous, and given its prevalence in the environment and the concomitant selection pressure imposed on hosts, we might not expect all bacterial genotypes to do equally well in all invertebrate species (Schulte et al. 2010). Adaptations to overcome resistance in one particular genetic background can also trade off and reduce efficacy in other host genetic backgrounds (Soberon et al. 2007). As we have described previously, experiments using biopesticide-derived strains might also underestimate the transmission and replication potential of *Bt* relative to freshly isolated wild-type strains (Raymond et al. 2010b, 2013).

2.6 Virulence, Cooperation and How Investment in Cry Toxins Is Maintained in the Field

Although we are now better informed as to how *Bt* populations can benefit from the presence of invertebrates hosts, the magnitude of the cost of investment in Cry toxins, up to 25% of dry weight at sporulation (Agaisse and Lereclus 1995), is difficult to comprehend. The persistence of this high-cost investment is harder to understand when it is appreciated that the Cry toxins must be solubilized in the insect midgut before being activated and able to bind to receptors (Schnepf et al. 1998). Thus, these metabolically costly products are not even privately available to benefit of the bacteria that produce them. Cry toxins would be described by economists as 'public goods', a term now widely employed by evolutionary biologists (Sachs et al. 2004; West et al. 2007a). For example, *Bt* spores may coexist with *B. cereus* spores on the leaf surface (Collier et al. 2005; Raymond et al. 2010b). If *B. cereus* spores are ingested with *Bt*, then in some cases the *B. cereus* strain can exploit the action of Cry toxins by invading the host haemolymph, where they can outcompete *Bt* by virtue of the fact that they do not have to invest in Cry toxin production in the cadaver (Raymond et al. 2008b). Similar results have been observed in experiments competing *B. anthracis* (essentially a Cry-null *B. cereus* biovar) against a Cry5B expressing *Bt* strain in nematodes (Kho et al. 2011). Note that this ability to outcompete *Bt* in the host does not extend to all members of the *B. cereus* group, such as the more saprophytic *B. weihenstephanensis* (B. Raymond unpublished data). However, if we cure *Bt* strains of the plasmids carrying Cry toxin genes, these approximately isogenic strains can outcompete Cry producers in the cadaver (Raymond et al. 2012).

The conceptual problem of cooperation, i.e. producing goods or investing in behaviour that is beneficial to groups but costly to individuals, may be unfamiliar to many in invertebrate pathology, but has been a long-standing subject of interest to evolutionary biologists (Hamilton 1964a; West et al. 2007a). Cooperation is broadly defined in evolutionary biology as a behaviour that provides a benefit to another individual (Hamilton 1964a; West et al. 2007a). While there are many different forms of cooperation, the most conceptually challenging form to explain is altruism, in which the cost to individuals exceeds the direct benefit to that individual (West et al. 2006). Altruism presents a challenge to evolutionary theory since this form of cooperation can be exploited by 'cheats', individuals that freeload on the cooperative behaviours of others and do not cooperate or cooperate less than expected in return. Cheats, such as our Cry toxin-null mutants, are expected to have higher fitness than cooperators in mixed populations, especially when the frequency of cooperators is high and there are abundant available public goods (Griffin et al. 2004; Ross-Gillespie et al. 2007; Raymond et al. 2012). Over the last two decades, the idea that microbes engage in cooperative and altruistic behaviours has been widely developed. These altruistic behaviours, usually based on the secretion or release of extracellular factors, can have a wide range of functions including biofilm formation, nutrient acquisition, quorum sensing, host-cell lysis, antimicrobial activity and

immune evasion (West and Buckling 2003; Brockhurst et al. 2006; Diggle et al. 2007; West et al. 2007a). Examples of microbes altruistically laying down their lives or sacrificing all future reproduction for their group mates include the autolytic self-destruction of colicin-producing *Escherichia coli* (Cascales et al. 2007) and the non-reproductive role of stalk forming cells in fruiting bodies of microbes that serve to increase the height of dispersal of their reproductive colleagues (Velicer et al. 2000; Strassmann and Queller 2011).

The conditions that can lead to the persistence of altruistic behaviours, such as the production of Cry toxins, are now relatively well understood (Hamilton 1964a, b; Frank 1998, 2010; West et al. 2007a, b). In simple terms, individuals should tend to show altruistic behaviour towards their relatives and individuals also likely to share genes for cooperation; for bacteria this generally means clonemates (Hamilton 1964a, b; Frank 1998). Secondly, there should be enough spatial structure to facilitate competition between groups of individuals (Taylor 1992; Griffin et al. 2004). In other words, if some groups contain high levels of cooperators, individuals in this group should have an improved access to resources compared to groups with a lower level of investment in cooperation. While this may seem a relatively abstract point, for pathogens such as *Bt*, the implications are clear: groups of bacteria with higher levels of investment in Cry toxins are going to be better at establishing infections in hosts, everything else being equal (Raymond et al. 2012). For *Bt* many of the predictions made for the evolution of cooperation seem to hold true for the production of Cry toxins. While *Bt*- and *B. cereus*-like strains can be found in the field, in general *Bt* persists in patches that show a high level of clonality, particularly in places on plants where *Bt* is likely to be eaten by insects (Raymond et al. 2012). While cheater mutants do well when virulent *Bt* has high density and Cry toxin producers are at a high frequency, Cry toxin producers have an advantage at low population density and when Cry toxin production is rare (Raymond et al. 2012). Thus, even in the presence of high levels of competition from social cheaters, Cry toxin producers can invade and have high fitness in the field, once given the presence of selective pressure from hosts. These results could provide another explanation for the relative rarity of *Bt* epizootics: as the density of Cry producers increases, the invasion of non-pathogenic cheaters is more likely and could curtail the spread of disease. Environments in which the invasion of cheating *B. cereus* strains is less likely (grain bins, insect culture in the laboratory) are precisely those environments in which *Bt* seems best able to produce epizootics (Burges and Hurst 1977; Delucca et al. 1982; Itova-Aoyolo 1995; Federici and Siegel 2007). Bottlenecks occurring in the colonization of plants probably play a large part in the near-clonal population structure we see in the field. However, structured and near-clonal populations of *Bt* can emerge simply through the process of invading hosts (van Leeuwen et al. 2015). Not only do *Bt* populations pass through a tight population bottleneck when colonizing the gut (Zhou et al. 2014), but strong competitive interactions based on how quickly competing genotypes invade the host can also limit genetic diversity in the cadaver and increase clonality (van Leeuwen et al. 2015).

2.7 Bt as a Useful Model in Pathogen Evolutionary Ecology

Cooperation and social evolution have broad implication for understanding the ecology and evolution of a range of pathogens and of *Bt* in particular (Raymond and Bonsall 2013). While cheating and social conflict are strongly in evidence in competition between Cry producers and non-producers, similar conflicts may exist for the production of the other major groups of *Bt* virulence factors: those regulate by the PlcR-papR quorum-sensing system (Zhou et al. 2014). Here, competition between wild-type bacteria and signal-null or signal-blind mutants shows the frequency and density-dependent fitness characteristic of social interactions (Zhou et al. 2014). However null PlcR or papR mutants are not effective cheats in that they do not have higher fitness than wild-type strains when in competition in the cadaver (Zhou et al. 2014). Here, the group-level competition required to stabilize cooperation seems to occur at the level of microcolony within the insect gut, so that patches of microbes with increased investment in quorum-regulated virulence factors are better able to invade the host and potential cheaters fare less well (Zhou et al. 2014).

Cooperation and cheating models may also be relevant for understanding the evolution and maintenance of virulence in other invertebrate parasites. Entomopathogenic nematodes and their bacterial symbionts also rely on a wide range of secreted virulence factors (Forst et al. 1997; Ffrench-Constant and Bowen 2000; Daborn et al. 2002; Eleftherianos et al. 2007). Entomopathogenic strains with high virulence can be hard to maintain without serial propagation in hosts, a process that can lead to attenuation or 'deterioration' (Wang and Grewal 2002; Bai et al. 2005; Bilgrami et al. 2006). Notably, attenuated nematodes, which have reduced ability to infect hosts, have shown reduced expression of secreted proteases with putative roles in suppression of host immunity and tissue invasion (Simões et al. 2000; Adhikari et al. 2009). One hypothesis that could explain the loss of virulence during host passage is that conditions in the laboratory (high doses) could favour cheater mutants with reduced virulence. A test of this hypothesis using experimental evolution showed that conditions that would be expected to favour cheats led to rapid loss of virulence, while low-dose serial propagation regimes maintained a high level of virulence (Shapiro-Ilan and Raymond 2016).

More broadly, understanding that investment in virulence may be a cooperative group-level activity can affect how we might model or understand dose-response mortality curves (Cornforth et al. 2015). Traditionally dose response has been understood to be the result of multiple infectious agents acting individually, each with an independent probability of causing infection – the independent action hypothesis (Haas 1983). This hypothesis has a number of important applications in terms of modelling disease risk, especially for those that extrapolate dose response from limited data (Haas 1983; Haas et al. 1997; Jones et al. 2009). This model of infection has also been widely applied in invertebrate pathology, for instance, in constructing quantitative methods to examine the existence of synergistic interactions in mixed infections or between *Bt* toxins (Tabashnik 1992). However, experiments with *Bt* provide one of the first direct tests of the independent action theory

and do not find good evidence to support its main assumptions (Cornforth et al. 2015). In essence, the mortality/dose-response curves of *Bt* toxin spore mixtures and Cry toxins are too threshold-like to be explained by independent action (Cornforth et al. 2015). Threshold-like dose-response curves, i.e. relatively more efficient infections when pathogens are at high doses, might be expected to drive a greater reliance on cooperation and collective action (Raymond and Bonsall 2013).

2.8 Phylogeny and Relationship to *Bacillus cereus* and Wider Group

The final controversy addressed here is that of the identity of *Bt* as a species. Since *Bt* is currently defined on the basis of the expression of Cry toxin parasporal inclusions, and because the genes responsible for these proteins are typically located on conjugative plasmids (Gonzalez et al. 1982; Gonzalez and Carlton 1984; Vilas-Bôas et al. 2008), it is not surprising that the taxonomy of *Bt* is unconventional if not downright messy. If we define phylogenetic clades based on chromosomal genes, at least two well-defined groups contain *Bt* isolates, giving us the problem of polyphyly (Fig. 2.1). In addition, both of these clades are comprised of both *Bt* and *B. cereus* making *Bt* also paraphyletic (Priest et al. 2004; Cardazzo et al. 2008; Didelot et al. 2009; Raymond et al. 2010b; Raymond and Bonsall 2013). While these facts are not in doubt, disagreement remains on the issue of what exactly we should do about it. One view is that the entire *B. cereus* group containing *Bt*, *B. cereus s.s.*, *B. anthracis*, *B. mycoides*, *and B. weihenstephanensis* should be treated as one species (Helgason et al. 2000; Tourasse et al. 2006), while a recent whole-genome sequencing paper suggested breaking up the group into 19 or 20 species (Liu et al. 2015). Alternative options include leaving things as they are or taking a more moderate view in terms of splitting the group.

While it is going to be difficult to untie this particular Gordian knot, there are a number of convincing arguments against lumping the entire group under one species name. First, only the early protein electrophoresis methods have supported the view that the *B. cereus* group is genetically homogeneous (Helgason et al. 2000). All subsequent phylogenies based on chromosomal sequencing, particularly multilocus sequencing typing and whole-genome methods, have shown that there are several well-supported genetically distinct clades in the *B. cereus* group (Vilas-Boas et al. 2002; Priest et al. 2004; Sorokin et al. 2006; Vassileva et al. 2006; Cardazzo et al. 2008; Guinebretière et al. 2008; Didelot et al. 2009; Raymond et al. 2010b). Analyses of the patterns of horizontal gene transfer suggest that there are at least three major clades and that most recombination occurs within rather than between clades, making these groups something akin to 'biological species' (Didelot et al. 2009). In addition, there is abundant evidence for substantial ecological differentiation between clades, either in terms of their ability to colonize plants (Raymond et al. 2010b; Vidal-Quist et al. 2013), their carriage of virulence factors such as enterotoxins (Cardazzo et al. 2008), the risks they pose to vertebrates (Cardazzo et al. 2008; Guinebretière et al. 2010; Raymond and Bonsall 2013) or their metabolic and

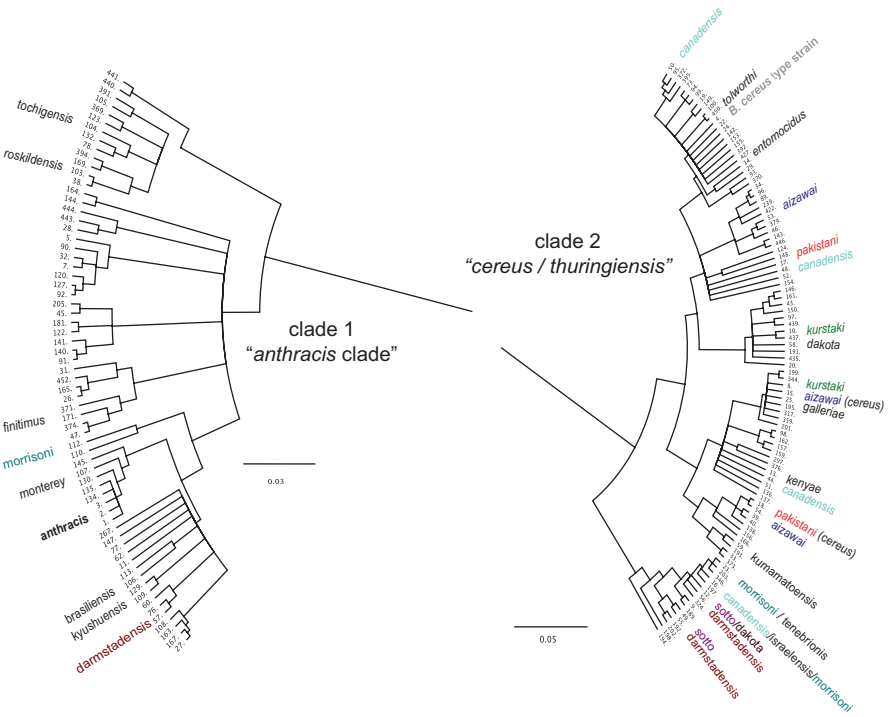

Fig. 2.1 The distribution of Cry-producing strains in two major clades of the *B. cereus* group. The tree is redrawn from the MLST study in Raymond et al. (2010b) using sequences from seven housekeeping genes and data from PubMLST (https://pubmlst.org/). Serovar names have been colour coded to indicate how widely distributed they are across clades

growth characteristics (Guinebretière et al. 2008). Analysis of the surface layer protein *csaB* gene, which is important in eliciting host immune responses, also indicates that host-specific factors may have driven diversification between clades (Zheng et al. 2013). Lumping clades, therefore, seems to be poorly justified on the basis of genetics, biology, ecology and reproductive isolation.

Attributing particular clades to particular species names could be relatively straightforward in some cases. For examples, strains in the clade containing *B. anthracis* are far more likely to have been isolated from acute vertebrate infections (Raymond and Bonsall 2013). *B. anthracis*, for historical reasons, applies to a clone specialized on ungulates, with a handful of SNPs to distinguish isolates (Keim et al. 2009). The justification for retaining this name for a tiny subset of the genetic diversity of *B. cereus s.l.* seems poor when there are other strains with a similar niche and which possess the key virulence plasmids of *B. anthracis* (Hoffmaster et al. 2004). All the potentially lethal emetic strains of *B. cereus* are also situated within the *anthracis* clade (Raymond and Bonsall 2013). A convenient albeit potentially unpopular revision would be to retain *anthracis* as a specific epithet for the whole *anthracis* group and as subspecies specific epithet for strains currently classed as *anthracis.*

While Cry toxin-expressing strains can be found within the 'anthrax clade', they have an elevated ability to cause infection in vertebrates (Hernandez et al. 1998), and most of them (*konkukian, brasiliensis, monterrey, pulsiensis, roskildiensis, tochigiensis*) (Fig. 2.1) have no known invertebrate host. *Bt roskildiensis,* despite being isolated in Denmark, has some activity against termites (Castilhos-Fortes et al. 2002), and one strain of *Bt kyushuensis* has some activity against mosquitoes (Ragni et al. 1996). The antisera standard for *Bt brasiliensis* (BGSC 4AY1/T39001) does not appear to actually produce clear inclusion bodies (B. Raymond unpubl. dat.), while the characterization of *Bt morrisoni* (biovar *san diego*) as ST 112 and therefore a member of the anthrax clade (Kim et al. 2005) is almost certainly an error. The *san diego* biovar is expected to be biological and genetically similar to biovar. *tenebrionis.* which is consistently and clearly related to the other entomopathogenic *Bt* strains (Raymond and Bonsall 2013). *Bt finitimus* HD3 (BGSC 4B2) is one of very few Cry-producing strains in the anthrax clade (Didelot et al. 2009) with an association with insects, as it was isolated from the lepidopteran *Malacosoma disstria* (Zeigler 1999). In short, the Cry-producing strains in the *anthracis* clade are a mixed bag of isolates with poorly characterized biology and host range that may be further confounded by misclassification and contamination. We could withdraw the name *thuringiensis* from this group without any great loss (Fig. 2.1); in fact redefining them as *B. anthracis* could help emphasize that any strain in this group is likely to be far too dangerous to ever be produced as a biopesticide. This one major revision would at least resolve the problem of polyphyly for *B. thuringiensis.*

B. weihenstephanensis was originally defined as a cool-adapted psychrotolerant member of the *B. cereus* group (Lechner et al. 1998). Strains in the *B. weihenstephanensis* clade or clade 3 have almost exclusively isolated from plants and soil (Raymond et al. 2010b; Raymond and Bonsall 2013); this group is depauperate in many enterotoxin genes (Cardazzo et al. 2008), is consistently adapted to low temperatures (Sorokin et al. 2006; Guinebretière et al. 2008) and has a poor ability to grow in insects; evidence that strongly points to this being the most saprophytic clade in the group. Cry-producing strains are typically not found in this group (Raymond et al. 2010b), although psychrotolerant adaptations can be found widely in *B. cereus s.l.* (Stenfors and Granum 2001; Bartoszewicz et al. 2009). Thus, while psychrotolerance should not be seen as sufficient to define a strain as *B. weihenstephanensis* all members of this 'clade 3' could usefully be called *B. weihenstephanensis.* Note that this clade does include isolates of *Bacillus mycoides,* a common saprophytic variant showing hyphal-like colonies on solid media. However, the distinctive *mycoides* phenotype seems to be distributed widely across the group and therefore may be an unreliable species name (Cardazzo et al. 2008; Raymond et al. 2010b; Liu et al. 2015). This is in contrast to *Bacillus pseudomycoides,* which makes for an apparently coherent and distinct lineage (Cardazzo et al. 2008; Guinebretière et al. 2008).

The final major clade was originally named 'clade 2' by the first MLST scheme (Priest et al. 2004) (Fig. 2.1). While this clade is relatively diverse, it contains nearly all the *Bt* isolates that have been well characterized as insect or nematode pathogens and certainly all the well-studied strains (Cardazzo et al. 2008; Didelot et al. 2009; Raymond et al. 2010b; Raymond and Bonsall 2013). Genotypes from this clade tend not be associated with acute vertebrate infections (Raymond and Bonsall

2013), a finding consistent with what we know of very low risk imposed by *Bt* biopesticides for mammals (Siegel 2001; Federici and Siegel 2007) and assessment of clade-level variation in cytotoxicity (Guinebretière et al. 2010). The *B. cereus*-type strain is very closely related to *Bt* serovar *entomocidus* (Federici and Siegel 2007) (Fig. 2.1) and sits within an ecologically diverse sub-clade of this group (Raymond and Bonsall 2013; Liu et al. 2015). Nevertheless, stable exchange of Cry toxin-bearing plasmids seems relatively rare, and the vast majority of genotypes are stably associated with Cry toxin production or not (Raymond et al. 2010b). One taxonomic solution is to retain the designations *cereus* and *thuringiensis* as useful terms and accept that these are paraphyletic species but to restrict the use of these specific epithets for clade 2 bacteria only (Fig. 2.1). The current practice using *B. cereus* as a catch-all species name for any strain with limited characterization – or using the terms *cereus* I, II and III to denote particular clades – is only going to lead to confusion.

2.9 Concluding Remarks

The nature of horizontal gene transfer in bacteria is such that phylogenies of global collections are likely to give a confusing picture. The use of multiple gene or genomic data can lead to better justified clades, but more and more sequencing is not necessarily going to resolve our taxonomic difficulties. More data will almost certainly reveal more isolates with intermediate phenotypes/genotypes and can lead to confusing over-splitting of species (Liu et al. 2015). Nevertheless, at a local scale, *Bt* and *B. cereus* strains are highly differentiated, and groups such as *B. weihenstephanensis* are genetically and ecologically coherent, ensuring that these species names are useful and informative (Vilas-Boas et al. 2002; Raymond et al. 2010b). The assumption that all members of the *B. cereus* group are genetically and ecologically homogeneous can have misleading consequences in terms of assessing safety risks (EFSA 2016) and does not accurately represent what we see in the field. Social interactions may mean that selection for cheating can produce *B. cereus* strains when *Bt* is cured of toxin-producing plasmids; however, this does appear to happen very frequently. Moreover, the production of Cry toxins has profound consequences on the growth and sporulation characteristics of *Bacillus*; it is likely therefore that it will have considerable direct implications for its realized niche. A key barrier for *B. cereus* establishing infections in the vertebrate gut is competition with existing microbes (Ceuppens et al. 2012). The production of Cry toxins substantially weakens the competitive ability of vegetative cells in vivo (Raymond et al. 2007, 2012), and this is likely to make *B. thuringiensis* substantially less fit in the gut of vertebrates, where Cry toxin production is not adaptive. Thus, not only does the production of Cry toxin facilitate the invertebrate pathogenic niche; it may also largely preclude strains from efficiently exploiting vertebrates. As such the designation *B. thuringiensis* remains valuable, and its link to the phenotypic production of Cry toxins is sensible, given the caveats discussed above.

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Chapter 3
Bacillus thuringiensis Toxin Classification

Neil Crickmore

Abstract Since the first insecticidal crystal toxin genes from *Bacillus thuringiensis* (Bt) were cloned and sequenced in the late 1980s, there have been various attempts to classify these proteins in ways that would be useful for scientists working with them. Such methods have generally involved grouping them by either sequence similarity or by toxicity spectra. The former method has the advantage that it is relatively simple to perform but does not necessarily provide information on the biological properties of the protein. Classifying toxins by their activity spectrum is much more informative but is considerably more difficult to achieve due to the need to test the toxin against a range of different target species. More recently, it has been possible to group toxins by structure as the number of proteins with solved structures increases and the methodology for being able to predict structures improves. In this chapter, I will describe how the Bt toxins have been classified since those early days and then move towards our current understanding of toxin classification and characterization.

Keywords Nomenclature • Structural similarity • Cry, Cyt and Vip toxins

The first *Bacillus thuringiensis* toxin gene was cloned in 1985 (Schnepf et al. 1985) from a strain of HD-1-Dipel. No name was given to the toxin at that time although as other toxins were cloned from HD1, they were classified according to the size of the *Hin*dIII fragment on which they were found (Kronstad and Whiteley 1986). It then became the 4.5 kb gene although when the same gene was later cloned from another strain, it was called *cry1-1* (Shimizu et al. 1988). In 1989, a nomenclature was proposed that classified the toxins according to both their sequence and their known specificity (Hofte and Whiteley 1989). In this initial nomenclature, there were just four classes. The first class were lepidopteran-active toxins which were around 130–140 kDa in size. These were given the mnemonic Cry (for crystal) and called the CryI toxins. The three closely related toxins from HD1 were called CryIA(a), Cry1A(b) and CryIA(c), the former being encoded by the so-called 4.5 kb gene. Other,

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less-related, toxins were named CryIB, CryIC and CryID. The second class were smaller (ca 65 kDa) toxins that had activity against either Lepidoptera or Lepidoptera and Diptera and consisted of just two members CryIIA and CryIIB. The third class constituted the coleopteran-active toxin CryIIIA and the fourth two dipteran active toxins from Bt subsp. *israelensis* CryIVC and CryIVD. A separate nomenclature was proposed for another toxin from Bt subsp. *israelensis* that had dipteran activity in vivo but a general cytolytic activity in vitro (Ward et al. 1986). This toxin was given the mnemonic Cyt (for cytolytic) and named CytA. While this nomenclature was well received at the time, it soon ran into problems. Since there was no body overseeing it, newly cloned sequences were being named by their discoverers, and often two completely unrelated toxins were given the same name – as was seen with CryV. A gene was cloned that encoded a toxin, which, despite having significant sequence similarity to the CryI class, had a unique activity to both Lepidoptera and Coleoptera, and so warranted a new class (Tailor et al. 1992; Gleave et al. 1992). Elsewhere toxins with reported activity against nematodes (Sick et al. 1994) were also being labelled CryV.

3.1 Development of a Sequence-Based Nomenclature

In 1998, a new nomenclature was published that classified toxins solely on their amino acid sequence, with no regard for their biological activity (Crickmore et al. 1998). By this time, the number of different toxin genes that had been cloned numbered around 70. The vast majority of these were clearly related and contained most, if not all, of the five conserved sequenced blocks identified by Höfte and Whiteley in 1989. Using a phylogenetic approach, a dendrogram was constructed that was then used to name the toxins. A slightly modified naming system was used in which each toxin was given a name incorporating four levels, e.g. Cry1Aa2. The first level (in this case 1) represented the primary rank, and generally speaking, toxins that shared at least 45% sequence identity were given the same number. The second rank (A) was used to distinguish sequences sharing between 45% and 78% identity. Those toxins that shared between 78% and 95% identity were distinguished at the level of the tertiary rank (a). The final, quaternary, rank was used to distinguish between different clones that shared at least 95% sequence identity. It is worth noting that all newly reported genes were given a unique name even if they were identical to a sequence already in the database. Part of the logic for this was that sequencing technology was not robust enough to guarantee complete accuracy (at least in a cost-effective manner), and it was known that small changes could affect the activity of a toxin. Thus, by giving each toxin a unique identifier, it could be unambiguously referred to in publications or patents. The revised naming system was very similar to that proposed by Höfte and Whiteley, and so many of the original names did not change significantly, for example, CryIA(a) became Cry1Aa and CryIVB Cry4Ba. Some names did have to change, although CryIVC came from the same strain as CryIVA, and both were active against Diptera; their sequences were not particularly close so CryIVC became Cry10Aa. The decision was also made to include the entire sequence of the toxin in the analysis, despite the

knowledge that for many of the toxins, the functional toxin was found in the N-terminal half. The logic here was that it couldn't be assumed that this was true for all the toxins, using the entire sequence ensured that different toxins were given different names, even if those names didn't accurately reflect functional relationships between the proteins. Although the nomenclature did not set out to classify according to function, it was at least hoped that proteins with similar sequences would be functionally similar. A number of criteria were laid down to determine whether or not a newly discovered toxin would be included in the nomenclature. Originally, any sequence which showed obvious similarity to an existing sequence would be included – even if there was no direct biological evidence that it was a functional toxin. That criterion has now been revised, and only sequences that represent novel quaternary ranks (e.g. Cry1Aa30) would be included without supporting evidence. The main reason for this change was that the advent of next-generation sequencing meant that data could be much more easily generated, and the nomenclature could have been swamped with sequences that encoded pseudogenes or gene fragments and not functional toxins. To be included in the nomenclature now, the discoverer must provide evidence that the protein is a functional Cry toxin. Normally, this would involve purifying the protein, or producing recombinant protein, and demonstrating toxicity towards a target. However, if it could be shown that the protein encoded by the gene was present in substantial amounts in the crystal of the native Bt strain, then it can be considered to be a bona fide Cry protein. The nomenclature was designed to classify a family of proteins and there was no restriction on host organism. Although most originated from Bt, others were isolated from other species such as Cry16Aa and Cry17Aa from *Clostridium bifermentans* (Barloy et al. 1996, 1998) and Cry18Aa from *Paenibacillus popilliae* (Zhang et al. 1997). As with the original nomenclature, the 1998 revision included a separate classification for the Cyt toxins, and later a third one was added for the Vip toxins. These vegetative insecticidal proteins represented a new family of secreted toxins (Chakroun et al. 2016). Within the main Cry family, there were three outlying sequences (Cry6Aa, Cry15Aa and Cry22Aa) that did not contain any of the five conserved sequence blocks or bear much resemblance to the other toxins. We now know that these toxins are structurally dissimilar from the other toxins (see below), and in hindsight, it would have been better to assign them to their own families. To ensure the stability of the nomenclature, it is overseen by a steering committee which maintains a website providing users with information about the toxins (Crickmore et al. 2017).

3.2 Classification by Homology Group

Although the 1998 nomenclature continues to allocate unique names to each newly discovered toxin based on sequence similarity, the increasingly diverse set of toxins can be grouped in a number of different ways. The phylogenetic approach used to derive the nomenclature can be used to identify such groups, and a review in 2003 described three such groups (de Maagd et al. 2003). The main one represented the large group of toxins now known to have an active core consisting of three domains

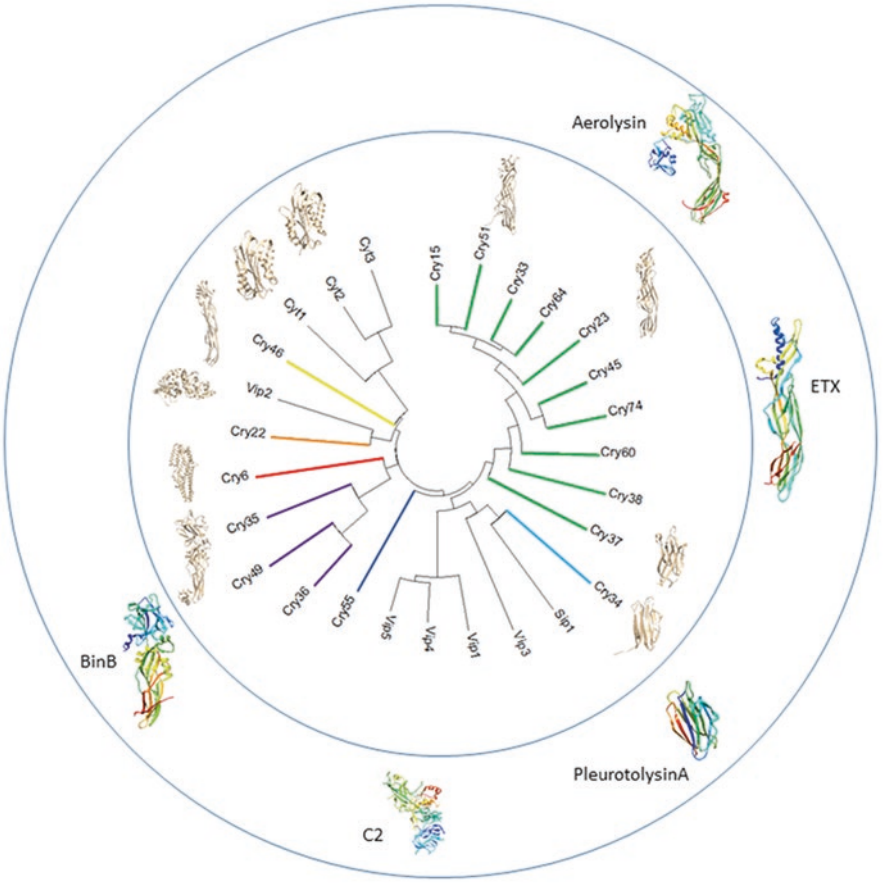

Fig. 3.1 Classification of the non-three-domain toxins by homology group. In the centre, a neighbour-joining tree shows the relationship between the toxins, the coloured braches representing different homology groups. Where structures have been solved, these are shown in the *inner circle*, while the *outer circle* shows structures of other toxins within the same protein family

(Pardo-Lopez et al. 2013). Another contained the Cry35 and Cry36 toxins which resemble the binary (Bin) toxin of *Lysinibacillus sphaericus* (Berry 2012) and a third contained Cry15, Cry23, Cry33 and Cry38 which resembled other toxins such as the clostridial epsilon (ETX) toxin (Alves et al. 2014) and aerolysin (Iacovache et al. 2016). A more recent analysis (Berry and Crickmore 2016) identified nine distinct homology groups within the Cry toxin family. Aside from the three-domain, Bin and ETX groups described above, the others consist only of a single type of toxin (Cry6, Cry22, Cry34, Cry37, Cry46 and Cry55). These homology groups are shown in Fig. 3.1. Mention should also be made at this point of a Bt toxin separately classified (by its discovers) as Sip1A (Donovan et al. 2006), a secreted, coleopteran-active, toxin that from Fig. 3.1 can be seen to cluster with many of the Vip proteins but also with Cry34.

3.3 Classification by Structure

Figure 3.1 shows the structures that have been published for a number of the non-three-domain toxins. What is most noteworthy is that many of the toxins from different homology groups have very similar structures. As recently reviewed (Berry and Crickmore 2016), it can be seen that the Bin and ETX groups as well as Cry46 all have an extended beta-sheet domain and a distinct alpha/beta "head" domain. This structural homology would suggest a common mechanism of action as beta-pore-forming toxins (Dal Peraro and van der Goot 2016). The Cyt toxins have long been known to share a common fold and presumed mechanism of action (Xu et al. 2014). Vip1 and Vip2 share sequence homology with the two component iota toxin from *Clostridium perfringens* and the C2 toxin from *Clostridium botulinum* (Knapp et al. 2016). In these toxins, the A subunit (Vip2) has ADP-ribosyltransferase enzymic activity, while the B subunit (Vip1) is believed to facilitate entry of the active subunit into the cell. Two of the smaller toxins Cry34 and Cry37 are part of binary toxins (with Cry35 and Cry23, respectively). How they function is unclear; despite the fact that Cry34 has been reported to have a weak activity by itself (Herman et al. 2002), one could speculate that they primarily work in conjunction with the head domain of the beta-pore-forming toxins to facilitate binding to specific cell surface receptors, analogous perhaps to the GPI-binding domain of aerolysin (Wuethrich et al. 2014). The structure of Cry6A has recently been solved (Huang et al. 2016; Dementiev et al. 2016) and reveals an extended alpha-helical protein, a unique structure among the Cry toxins but consistent with a pore-forming mechanism of action. Of the other homology groups discussed above, no structural information exists for either Cry55 or Cry22. While the latter contains a number of putative lectin-like pfam domains – which potentially could be involved in receptor binding – no clues exist as to the structure of Cry55.

3.4 Classification by Size

The Cry toxins vary significantly in size from the 1344 amino acid Cry43Aa down to the 119 amino acid Cry34Aa (Fig. 3.2). It has long been known that the three-domain class of Cry toxin exist in two distinct forms, one of 130–140 kDa and another of 60–70 kDa. Both forms contain the three domains of the functional toxin at their N-terminus (see Fig. 3.3) with the larger proteins containing an additional domain known to be involved in crystallization within the host bacterium (de Maagd et al. 2003). For a number of the short-form toxins, for example, Cry19A, the C-terminal crystallization domain is found as a separate gene, and its encoded protein is believed to act in the same way as the extension on the long-form toxins (Barboza-Corona et al. 2012). Not all of the short-form toxins are found to have an associated crystallization protein encoded with it. Figure 3.3 shows examples of three toxins, one of which is a long-form toxin with attached crystallization domain

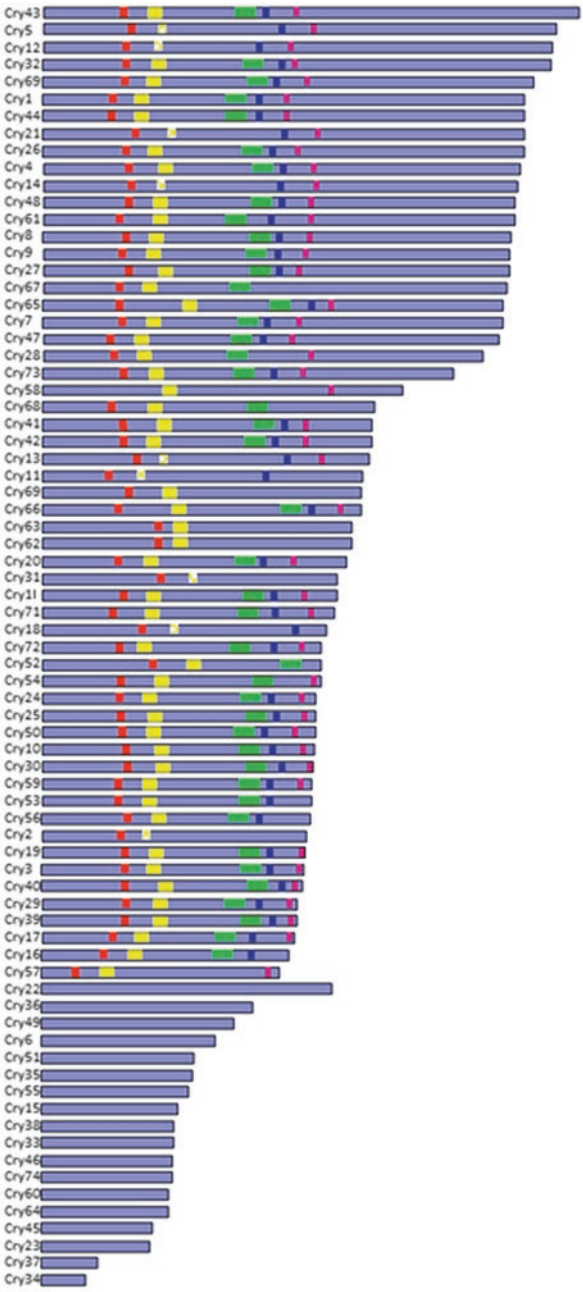

Fig. 3.2 Classification of the Cry toxins by size. The three-domain toxins are shown above the non-three-domain ones. The *coloured boxes* in the three-domain toxins represent the conserved blocks 1–5

(Cry1Ac) and two short-form toxins (Cry2Aa and Cry3Aa). As can be seen, there are no major differences in structure between the toxin portions of each, yet for the latter two, there is no obvious need for the crystallization domain in order to form crystals in the host Bt cell. It is possible that subtle differences in structure could

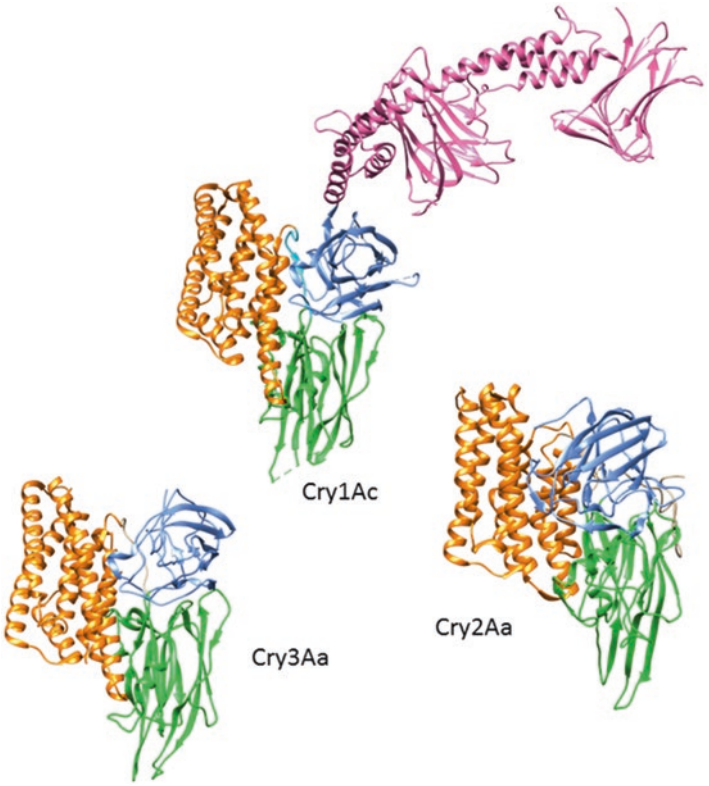

Fig. 3.3 Structures of three-domain toxins with differing requirements for in vivo crystallization

negate the need for a separate crystallization factor or that a different factor is required. For Cry2Aa, such a factor has been identified, an additional protein completely unrelated to the C-terminal extension of the long-form toxins, but required for crystallization within Bt (Crickmore and Ellar 1992). For Cry3Aa, no such crystallization factor has been identified although the expression of this toxin is unusual in that it is expressed earlier in the cell cycle than other Cry toxins, and an additional regulatory element has been identified (Agaisse and Lereclus 1996). An unusual situation was described for Cry65Aa where the toxin contains a truncated C-terminal extension domain, but a complete domain is present as a separate downstream gene. Both are reported to contribute to crystallization (Peng et al. 2015). For the three-domain toxins, one of the conserved sequence blocks (block 5) normally marks the end of the active toxin (Schnepf et al. 1998). A number of these toxins have sequences other than the crystallization domain beyond this conserved block. The partial crystallization domain of Cry65Aa has been mentioned above, and a previous review of Bt toxins identified the presence of repeat units, of unknown function, at the C-terminus of a number of toxins such as Cry11Ba, Cry20Aa and Cry27Aa (de Maagd et al. 2003). Two of the toxins (Cry41 and Cry42) have acquired a beta-trefoil ricin-like domain at their C-terminus, although at least for Cry41,

there is no evidence that this plays any functional role in toxicity (Krishnan 2013). For other toxins such as Cry58, the additional sequence beyond block 5 shares no obvious similarity with any other reported protein. For the non-three-domain toxins, the longer length of Cry22 appears to be due to the presence of lectin-like repeat domains (de Maagd et al. 2003) although as mentioned above, the structure and function of this protein remains unclear. The small size of Cry34 could represent the fact that its primary role is to assist the binding of its Cry35 binary partner, although it has reported activity of its own and appears related to the similarly small-sized aegerolysin toxins (Berne et al. 2009).

3.5 Classification by Host Organism

Although most of the three-domain type Cry toxins originate from *Bacillus thuringiensis*, homologues have been found in other species of bacteria. As mentioned above, Cry16Aa and Cry17Aa were isolated from a *Clostridium* (Barloy et al. 1996, 1998). Mosquitocidal activity was found for the former toxin which was found to be secreted from both the host strain and a recombinant Bt strain rather than from a crystal during sporulation. In contrast, the Cry18Aa toxin from *Paenibacillus popilliae* does form crystals similar to those found in Bt (Zhang et al. 1997). Various genes for Cry43 toxins were subsequently cloned from strains of *Paenibacillus lentimorbus* (Yokoyama et al. 2004). With the advent of next-generation sequencing, the possibility of finding Cry toxin genes in other bacteria increased. For example, a toxin gene related to *cry8* has been reported from a strain of *Brevibacillus laterosporus* (GenBank accession number AGU13849). Many three-domain *cry* genes have been annotated in GenBank as originating from strains of *Bacillus cereus* (e.g. EOO42768) although in many cases, this could be due to problems differentiating between this bacterium and Bt. The ETX-type beta-pore formers are a ubiquitous class of proteins (Moar et al. 2016), and so it is likely that insecticidal homologues will be found in other species. Similarly, Vip1- and Vip2-type toxins are widely distributed, particularly among bacilli.

3.6 Classification by Cellular Location

By definition, the Cry toxins are found in the crystalline inclusion body that forms during sporulation of the mother cell. However, as described above, some of the insecticidal toxins produced by Bt are not laid down in crystals but instead are secreted from the cell. Most notable among these are the Vip proteins which contain an N-terminal signal peptide to direct them out of the cell (Chakroun et al. 2016). A perplexing toxin however is the short-form three-domain Cry1Ia; this was initially thought to be a silent gene in Bt until traces of an N-terminally truncated form were found in the extracellular medium of a culture of Bt strain AB88 (Kostichka et al.

1996). A precursor, with an intact N-terminal signal peptide, was then found within the cell and being expressed in early sporulation. The evolutionary path by which toxins with similar structures are expressed in different compartments remains unknown.

3.7 Classification by Target Specificity

Potentially, the most useful way of classifying toxins is through their biological activity, which is why the initial nomenclature incorporated this property (Hofte and Whiteley 1989). Due to the difficulty of establishing whether a particular toxin had activity against a wide range of insect targets, this parameter was dropped from the revised nomenclature. Instead separate databases were compiled listing the known activities of individual toxins (van Frankenhuyzen 2009, 2013). This topic has also been covered in a more recent review (Palma et al. 2014) as well as in a later chapter of this book. One difficulty of classifying toxins in this way is the lack of reliable data from which to draw reliable conclusions. Often this is because single reports of an activity perpetuate in the literature, despite the fact that no corroborative evidence exists. As an example, the Palma et al. review suggests that Cry1Ab has activity against gastropods despite studies indicating no significant effect of Cry1Ab maize on nontarget gastropods (de Vaufleury et al. 2007). Another problem with classifying by activity is the presence of contradictory data in the literature, for example, while one report indicates that Cry2Aa is highly toxic to the mosquito *Culex quinquefasciatus* (Moar et al. 1994), another reports a lack of activity (Lima et al. 2008). Such discrepancies can be due to a number of different factors including the exact toxin used (a few amino acid differences between two different Cry2Aa toxins could affect activity), the manner in which the toxin was prepared, the population of insect used, the larval stage used, the nature of the bioassay, etc. As a result, any attempt to derive structure/function relationships for toxins based on historical literature should be done with great caution.

3.8 Concluding Remarks

Bt Cry toxins are formally classified based on sequence similarity (Crickmore et al. 1998), a system which has the advantage of being relatively simple to operate and provides a unique name to each toxin. However, an increasing number of toxins do not come from Bt or are not naturally found in the crystal. This is not necessarily a problem if the nomenclature is really considered to describe a family of related proteins exemplified by the Bt Cry toxins. To some extent, the name given to a toxin can indicate its relatedness to other toxins, for example, Cry1Aa is closely related to Cry1Ab and slightly less so to Cry1Ba. Toxins with different primary ranks can be considered unrelated even though they may seem similar, e.g. Cry60Aa and Cry61Aa.

In particular, the names give no indication of structural relatedness in that toxins with quite different structures (e.g. Cry6, Cry7 and Cry15) are all given the same mnemonic. This latter distinction is probably something that should be addressed in any future revision of the nomenclature. The other ways of classifying the toxins discussed in this chapter provide useful means of comparing the proteins but would not provide a feasible alternative to the formal sequence-based classification due to the lack of sufficient data for many of the toxins to be able to make informed decisions. What is clear is that there is a great diversity of toxins out there, many with biotechnological potential, and continued efforts to analyse their sequences, structures and activities will ultimately assist in the development of new products.

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Chapter 4
Insecticidal Proteins from *Bacillus thuringiensis* and Their Mechanism of Action

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Abstract *Bacillus thuringiensis* (Bt) are Gram-positive bacteria that produce different insecticidal proteins, named Cry, Vip, and Cyt, during the sporulation phase of growth. Here we will describe each one of these classes of protein, their mechanism of action, and their three-dimensional structure if it is available. We will also describe the different strategies that have been used to find novel insecticidal genes that could be used in biological control of insect pests as well as the strategies to evolve known genes to produce proteins with improved toxicity against selected insect pests. These novel strategies include site-directed mutagenesis and domain swapping among different Cry toxins where novel hybrid proteins containing domains or loop regions from different Cry proteins were constructed, resulting in improved toxicity against selected insect pests. Finally we will describe high-throughput systems that have been used to evolve Cry toxins in vitro. Overall, Bt toxins represent one of the most successful strategies for the biocontrol of insect pests.

Keywords *Bacillus thuringiensis* • Cry toxins • Vip toxins • Cyt toxins • Insect control

Bacillus thuringiensis (Bt) are Gram-positive bacteria that produce different insecticidal proteins, named Cry, Vip, and Cyt, during the sporulation phase of growth (Fig. 4.1). Bt represents the biological control strategy most used nowadays for insect control worldwide. The insecticidal proteins produced by Bt are toxic to different insect orders such as Lepidoptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga as well as nematodes. The first product based on Bt toxins was commercialized in 1938 in France for the control of flour moths, and in 1958 Bt products were commercially available in the USA (Lambert and Peferoen 1992). Most of the commercial Bt products for insect control in agricultural fields are powders or concentrated liquid suspensions containing a

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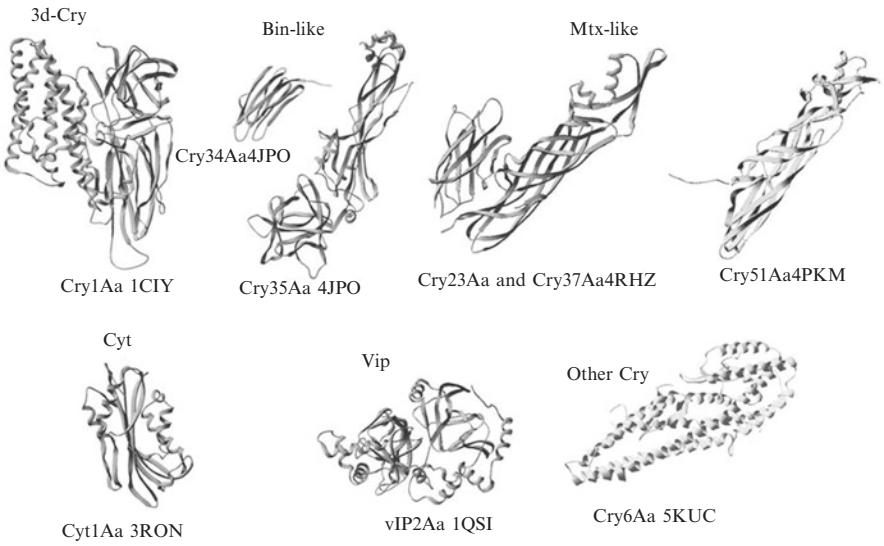

Fig. 4.1 Crystal structure of different proteins produced by *Bacillus thuringiensis* bacteria

mixture of spores and toxin crystals. These formulations are applied directly to the plant leaves or other parts of the plant where the larvae feed. The most successful application of Bt toxins for control of agricultural pests are the genetically modified plants or Bt plants, where the Bt genes coding for specific Cry or Vip toxins have been genetically introduced into the genome of important crops such as cotton, maize, and soybean. Bt plants were commercialized for the first time in 1996, since then the use of Bt plants has been growing every year, and now the estimated crop area is more than 178 million hectares in 28 countries (James 2015). The expression of Cry toxins in Bt plants has resulted in important reductions in the spraying of chemical insecticides, especially in developing countries (Qaim and Zilberman 2003).

In addition, Bt also produces insecticidal proteins that are highly specific against dipteran insects, which are vectors of several diseases in humans such as dengue fever, chikungunya, Zika, and malaria, among others. A commercial formulation of Bt subsp. *israelensis* (Bti) was first available in 1981 (Guillet et al. 1990), and it was extensively used in West Africa by the World Health Organization (WHO) to solve the problem of onchocerciasis or river blindness, transmitted by *Simulium* populations that become resistant to chemical larvicides such as the organophosphate (Guillet et al. 1990). Bti formulations have also been successfully used in mosquito control programs in many other countries as Brazil and Germany resulting in reduction by more than 90% of the mosquito population without evidence of any harmful impact on the environment (Becker 1997).

4.1 Strategies for the Discovery of Novel Bt Genes Coding for Insecticidal Proteins

Numerous screening programs worldwide have been established to isolate thousands of Bt strains. Bt strains can be easily identified in soil or phylloplane samples due to their production of parasporal crystals that can be observed by optical microscopy and analyzed in electrophoresis performed in polyacrylamide gels (SDS-PAGE). Initially, these collections of Bt strains were mainly screened by bioassays testing the insecticidal activity of individual strains against different target insects. Although this method of characterization was effective in finding interesting strains, it is tedious and slow. Thus, other strategies were implemented to speed up the discovery and identification of putative novel genes that code for interesting Bt toxins. Among the experimental approaches that have been used to identify and clone novel Bt genes, we can mention the polymerase chain reaction (PCR), hybridization, and microarray analyses that were extensively used for many years. However, the novel sequencing technologies allowed the cloning of large DNA libraries and identification of novel genes by using high-throughput sequencing strategies.

Traditional PCR has been used widely to amplify specific DNA fragments and to determine the presence or absence of target genes allowing the identification of previously reported Bt genes. This strategy is efficient and practical since it is highly sensitive, requires very little DNA for analysis, and allows for large numbers of samples to be processed in a relatively short period of time (Carozzi et al. 1991; Cerón et al. 1995). The disadvantage is that only known genes or closely related genes could be characterized.

Other PCR strategies have been developed to identify putative novel *cry* and *vip* genes. For example, the multiplex PCR could identify specific and novel genes. This technique is rather simple; it consists of using a set of several oligonucleotides primer pairs each specific against a particular gene. Control genes will produce a precise pattern of PCR products between 100 and 1000 bp. A different pattern may indicate a novel *cry* gene related to the selected target gene. By using this method, the novel *cry1Cb1* gene was cloned and novel *vip3AcAa* gene was identified (Kalman et al. 1993; Fang et al. 2007). Sequence analysis of the novel *vip3AcAa* gene suggested that it resulted by sequence swapping between the N-terminal region of Vip3Ac1 and the C-terminal region of Vip3Aa1 (Fang et al. 2007). A different PCR strategy linked to restriction fragment length polymorphism (RFLP) could detect both known and novel *cry* genes. In this strategy two pairs of oligonucleotide primers that amplify conserved region of *cry* genes were used to amplify a PCR product that is then digested with restriction enzymes for RFLP analysis (Kuo and Chak 1996). A rather related strategy is the PCR linked to high-resolution melting analysis (HRMA) where a PCR product amplified in the presence of a specific fluorescence dye such as LCGreen was analyzed afterward for its DNA melting pattern, and analysis of temperature transitions allowed the identification of novel *cry* gene variants (Li et al. 2012).

DNA hybridization was a techniques mainly used for identification of *cry* genes. An interesting variant of this strategy was reported by Berad et al. (2001). Complete or partial gene sequences from ten different *cry* genes were combined and used as a single probe for hybridization to genomic DNA from several Bt strains. This method was shown to be rapid and sensitive for the detection of known *cry* genes (Beard et al. 2001). Hybridization in microarrays containing high number of probes could detect target genes in a single hybridization step. This strategy was used to characterize *cry* genes from a Bt-strain collection (Letowski et al. 2005).

Recently, new sequencing technologies improved the detection of novel *cry* gene sequences from complex samples. For example, high-throughput 454 sequencing was used to determine the sequences of all PCR products amplified from a template pool, containing DNA from 2000 strains (Chen et al. 2014). Similarly, high-throughput sequencing has been used in Metagenomic-PCR where plasmid-enriched DNA was prepared from 235 soil samples. This sample was used then as a template to amplify *cry*2 genes which were sequenced by a 96-capillary 3730xl DNA analyzer. Using this strategy, 177 *cry*2 genes were sequenced, and a novel *cry2Ah* gene was cloned (Shu et al. 2013). The high efficiency and low cost of second-generation DNA sequencing technologies such as ion semiconductor (ion torrent sequencing), pyrosequencing 454, and sequencing by synthesis (Illumina) has significantly improved the efficiency of novel *cry* gene discovery by sequence analysis of complete genome from selected Bt strains. However, a disadvantage is that most of Bt strains contain multiple *cry* genes in their genome, and in many cases, these *cry* genes are rather similar (Crickmore et al. 2016). However, since the read lengths of these strategies are short (50–400 bp), identical sequences may come from different genes. This problem will be important in highly related *cry* genes since they would be considered to be repeated sequences resulting in errors in genome assembly. The third generation of DNA sequencing technology such as single-molecule real-time sequencing (Pacific Bio) has overcome these problems since reads are from 5000 to 10,000 bp, significantly reducing the probability of splicing mistakes and allowing a straightforward assembling of full-length *cry* genes (Eid et al. 2009). However, a disadvantage is that the cost is still relatively high.

4.2 Insecticidal Proteins Produced by Bt

As mentioned above, Bt produces different kinds of insecticidal proteins. By using the different techniques highlighted above, close to 950 different toxin genes have been cloned and classified in 74 groups of Cry, 3 groups of Cyt, and 3 groups of Vip proteins (Crickmore et al. 2016). The criteria for inclusion of a new sequence in the web page of Bt toxin nomenclature is that the reported sequence "has significant sequence similarity to one or more toxins within the nomenclature or be a *Bacillus thuringiensis* parasporal inclusion protein that exhibits pesticide activity or some experimentally verifiable toxic effect to a target organism" (Hofte and Whiteley 1989; Crickmore et al. 2016).

Cry, Vip, and Cyt proteins produced by Bt are not related phylogenetically. Among the Cry toxins, there are three main groups that are also not related phylogenetically and have been annotated as the three-domain Cry (3d-Cry) toxins, Bin-like Cry toxins, and Mtx-like Cry toxins (Crickmore et al. 2016).

4.3 The 3d-Cry Toxins

The 3d-Cry toxin family is the biggest Bt toxin group containing more than 55 subgroups; among them some proteins are active against Lepidopteran, Coleopteran, Dipteran insects, and nematodes. Cry31 and Cry41 belong to this family, but they are also named parasporins 1 and 3 since they showed activity against human cancer cells and showed no toxicity to various insects (Ohba et al. 2009).

The crystal structures of several 3d-Cry toxins have been solved (PDB numbers: 1CIY, 4ARX, 1I5P, 1DLC, 1JI6, 2C9K, 1W99, 4D8M, 3EB7); all of them are composed of three domains, where domain I is formed by seven alpha-helices forming a closed bundle and is proposed to be involved in toxin oligomerization and pore formation. Domains II and III are formed mainly by beta-strands and are proposed to be important for binding and recognition of toxin receptors (Hofte and Whiteley 1989; Pardo-López et al. 2013) (Fig. 4.1). More specifically, beta-16 from domain III of Cry1A is a binding region important for alkaline phosphatase (ALP) receptor binding in the insect gut, while loop3 of Cry1A domain II binds to aminopeptidase (APN) receptor (Pacheco et al. 2009; Arenas et al. 2010). In addition it was shown that loops 2 and 3 and alpha-8 from Cry1A domain II interact with the cadherin (CAD) receptor located in apical microvilli of midgut cells (Gómez et al. 2002, 2003). The similarities in crystal structure suggest that these proteins may function using similar mechanism of action in the different insect orders. Similar receptors to APN, ALP, and CAD have been described in Lepidopteran, Coleopteran, and Dipteran insects (Pardo-López et al. 2013).

It was proposed that protoxins are ingested by the larvae and immediately solubilized due to the high pH and reducing conditions of insect gut. The protoxins are cleaved by midgut proteases resulting in the formation of toxin fragments resistant to further proteolysis with a size of 60 kDa (Pardo-López et al. 2013). The activated toxin binds to different receptor molecules in a sequential manner to finally insert into the membrane of midgut cells, forming pores that burst these cells leading to the death of the larvae. The activated toxin first binds with low affinity to ALP and APN, two proteins that are highly abundant in the membrane. This interaction places the toxin close to microvilli membrane where the next receptor, CAD protein, is located. The interaction with CAD protein triggers a cleavage of amino terminal region of the toxin including helix alpha-1 and part of helix alpha-2a. A conformational change follows resulting in toxin oligomerization (Gómez et al. 2002; Pardo-López et al. 2013). The toxin oligomer structure remains unknown, but it is proposed to be formed by assembling of three to four toxin molecules. It is known that helix alpha-3 is very important in toxin oligomerization, and mutants in

this region lose completely their toxicity but can still bind to toxin receptors with similar affinity such as CAD (Jiménez-Juárez et al. 2007). It was shown that toxin oligomer showed increased affinity to APN and ALP receptors (Pacheco et al. 2009; Arenas et al. 2010), and it was proposed that this interaction is necessary to help in the insertion of oligomeric toxin into the membrane to form a pore that kills the insect midgut cells. Also, it was shown that helices alpha-4 and alpha-5 are inserted into the membrane during pore formation, and mutations in helix alpha-4 resulted in proteins that lost toxicity and pore formation activity even though they are still able to bind to apical cell membranes and CAD receptor (Girard et al. 2008; Zavala et al. 2011; Pardo-López et al. 2013).

The construction of modified Cry1A toxins (Cry1AMod) engineered to lack the N-terminal end including helix alpha-1 supported this model since these mutants were able to form oligomers in solution after protoxin activation with trypsin in absence of receptors and were toxic to Cry1A-resistant population with mutations in cadherin gene (Soberón et al. 2007). The Cry1AMod proteins did not increase their spectrum of action since it was shown that Cry1AMod still require binding to APN and ALP for membrane insertion (Muñóz-Garay et al. 2009).

It is remarkable that besides the great number of 3d-Cry toxins that have been reported, only few of them such as Cry1Ab, Cry1Ac, Cry 1Fa, Cry2Ab, Cry3Aa, and Cry3Bb are currently expressed in Bt plants (James 2015).

4.4 Bin-Like Cry Toxins

Cry35 and Cry34, Bin-like Cry toxins, form a binary toxin highly effective against coleopteran larvae such as *Diabrotica virgifera,* which is a major corn pest in the USA. This insect feeds on corn roots and its management is complicated since they are protected from insecticide contact. Cry35 and Cry34 toxins are also pore-forming toxins. Cry35 is a 44 kDa protein that shares some similarities with the binary Bin-toxins produced by *Lysinibacillus sphaericus* that are active against dipteran larvae (Neves et al. 2014). The Cry34 toxin is a 14 kDa protein. Together both proteins are able to form ion channels in synthetic membranes (Baum et al. 2004; Masson et al. 2004) and individually showed no toxicity to the insect larvae (Masson et al. 2004).

The structure of Cry35 revealed that it is composed by two β-strand domains (PDB 4JP0). As expected it shows similarities with BinB toxin from *L. sphaericus* (PDB 3WA1) but a region of this protein also showed some similarities with the structure of a ricin B-lectin (PDB 3PHZAa). The structure of Cry34 was also solved (PDB 4JOX) showing a β-sandwich structure (Fig. 4.1). Structure similarity analysis indicated that Cry34 resembles the pore-forming toxin fragaceatoxin C (PDB 4WDC).

The Bin-like Cry34/Cry35 toxins have been expressed in corn and have been available to growers since 2003. These toxins were also pyramided in plants in combination with Cry3Bb, the plants that expressed both proteins showed a more

efficient control of the *D. virgifera* larvae, and it is proposed that combination of these two proteins would help in delaying development of resistant insects (Prasifka et al. 2013).

4.5 Mtx-Like Cry Toxins

Several Cry proteins are members of Mtx-like Cry family such as Cry15, Cry23, Cry33, and Cry38 that are active against coleopteran insects, Cry60 that is dipteran specific, and Cry51 that is active against coleopteran and hemipteran larvae (Crickmore et al. 2016). Cry45 and Cry64 are also named parasporins 4 and 5 and show activity against cancer cells (Crickmore et al. 2016). This family of proteins was named Mtx-like because Cry15 showed some similarities with Mtx2 and Mtx3 from *L. sphaericus*.

Cry23 associates with Cry37 forming a binary toxin with pore-formation activity (de Maagd et al. 2003). The three-dimensional structure of the binary protein complex of Cry23 and Cry37Aa was solved (PDB 4RHZ). Cry23 is a 29 kDa protein showing an elongated structure composed by β-strands that resembles the structure of epsilon toxin from *Clostridium perfringens* (PDB 3ZJX), which is a potent pore-forming toxin. Cry37 is a 14 kDa protein that displays some similarities with the structure of perforin (PDB 4Y1S), also a pore-forming protein.

The structure of Cry51 was also solved (4PKM); it is a 35-kDa protein active against nymphs of the sucking insect *Lygus hesperus* and *L. lincolaris* that are important hemipteran pests in cotton in the USA. This toxin is also active against the coleopteran *Leptinotarsa decemlineata*. Cry51 has high structural similarities with Cry23 and epsilon toxin (Fig. 4.1). This protein has been expressed in cotton plants and its toxicity was demonstrated (Baum et al. 2012). Its mechanism of action remains unknown, but due to the high similarity with Cry23 and epsilon toxin, it is proposed that it is most likely a pore forming toxin.

4.6 Other Cry Toxins

Some Cry toxins are not related with the three-domain Cry family, the Bin-Cry family, or the Mtx-Cry family. Among them is Cry6Aa that is also a pore-forming toxin that shows insecticidal activity against coleopteran larvae and also against nematodes (Dementiev et al. 2016). The structure of this protein was recently solved showing to be composed mainly by alpha-helices (PDB 5KUC) (Fig. 4.1). Cry6A showed a structure with some similarities with other pore-forming toxins such as hemolysin E HlyE (PDB 1QOY) from *Escherichia coli* and the nonhemolytic toxin, HBL-B (PDB 2NRJ), from *Bacillus cereus*.

4.7 Cyt Toxins

Three groups of Cyt toxins have been reported. Cyt1 and Cyt2 are active against dipteran larvae and also show hemolytic activity when assayed in vitro with different red blood cells (Soberón et al. 2013). The Cyt3 specificity is unknown (Crickmore et al. 2016).

The crystal structures of these proteins have been solved (PDB 3RON Cyt1Aa, 2RCI Cyt2Ba, 1CBY Cyt2Aa) showing a single domain (Fig. 4.1). Cyt toxins have structural similarities with the fungal volvatoxin A toxin (PDB 1PP0) that is a membrane pore-forming toxin. Cyt toxin also display sequence similarities with toxins produced by different bacteria such as *Dickeya dadantii, D. zeae, Aeromonas salmonicida*, and *Clostridium kluyveri*, and that are important in the virulence phenotype of these organisms (Soberón et al. 2013). Cyt proteins are 27 kDa protoxins that after proteolytic activation give a 25 kDa protein that has pore-formation activity. The toxin interacts with lipids forming oligomers of more than 16 subunits (Chow et al. 1989). It was shown that helix α-C is involved in oligomerization, and point mutations in this region resulted in nontoxic proteins that are unable to oligomerize (López-Díaz et al. 2013). It is proposed that long β-strands are the regions that insert into the membrane during pore formation (Promdonkoy and Ellar 2000).

One of the most important characteristics of Cyt toxins is their capacity to synergize the activity of Cry4Aa, Cry4Ba, and Cry11Aa toxins (Crickmore et al. 1995) and to overcome resistance to these Cry toxins in resistant *Culex quinquefasciatus* populations (Wirth et al. 1997, 2005). It was shown that Cyt1Aa (specifically loop β6-αE and part of β7) interacts with two loops from domain II of Cry11Aa and Cry4Ba (loop α-8 and loop 2) (Pérez et al. 2005; Cantón et al. 2011) inducing their oligomerization (Pérez et al. 2007).

4.8 Vip Toxins

Three main groups were described; the first one include the Vip1/Vip2 toxins that form a binary toxin active against coleopteran larvae and aphids, while the second group are the Vip3 toxins active against lepidopteran pests (Crickmore et al. 2016). These proteins are produced during the vegetative phase of growth and are secreted outside of the bacteria.

Vip1 is a 100 kDa protein and Vip2 is 52 kDa. Vip1A binds to a receptor of 50 kDa located in the midgut cell. This protein forms oligomers that insert into the membrane and forms pores (Crickmore et al. 1995). It is proposed that Vip1A catalyze the translocation of Vip2A across the membrane. The three-dimensional structure of Vip2A (PDB 1QS1) was solved showing high similarity with NAD-dependent ADP-ribosyltransferase (PDB 1QS2) (Fig. 4.1). Once inside the cell, the Vip2A binds an ADP-ribose moiety to G-actin, affecting its polymerization and the cytoskeleton (Han et al. 1999). The three-dimensional structure of Vip1 has not been solved yet.

The binary Vip1/Vip2 was transformed into corn plants, but the expression of these toxins was lethal to the plant (Jucovic et al. 2008). A Vip2 zymogen was constructed where a pro-peptide sequence was added at the C-terminal end of Vip2A toxin to block Vip2A activity in the plant cell, but once ingested by the larvae, the pro-peptide could be cleaved by the midgut proteases recovering the insecticidal activity (Jucovic et al. 2008).

Vip3 is an 88 kDa protein that is secreted to the medium as a protoxin. Vip3 is proteolytically activated by the larval midgut proteases into an active toxin of 62 kDa. The identity of Vip3A receptors is unknown, it binds to different proteins in several insects, and these binding sites are different from Cry toxin-binding sites; thus, no competition with Cry toxins has been observed (Lee et al. 2003; Mahon et al. 2012). Also, Vip3A is a pore-forming toxin (Lee et al. 2003). This protein was expressed in corn plants showing protection to the attack of *H. zea, O. nubilalis*, and *S. frugiperda* (Burkeness et al. 2010). The pyramided expression of Vip3A with Cry1Ab increases the efficacy of insect control in these Bt plants (Burkeness et al. 2010).

4.9 Evolution of Bt Toxins Insecticidal Activity

Bt toxin gene discovery has provided new toxins that have the potential to be used for insect control and for resistance management. However, many important pests show low susceptibility to the known Cry toxins, and evolution of resistance to Cry toxins in many insect pests is threatening the continuous use of Bt technology. An alternative is to evolve a Cry toxin's insecticidal activity by genetic engineering. Recently different strategies to improve Cry toxicity by genetic engineering were reviewed (Bravo et al. 2013). Below we will discuss recent advances in the engineering of Cry or Cyt toxins that resulted in enhanced toxicity, modifying toxin specificity or countering resistance to Cry toxins.

Specificity of Cry toxins depends largely on the recognition of larval gut proteins (Bravo et al. 2011). Thus, increasing binding affinity to these receptor molecules or evolution of toxins to bind new receptor molecules will provide toxins with new insect specificities or with increased toxicity. Therefore, mapping the binding regions in the toxin and in the receptors is crucial information that is necessary for their evolution toward improved insecticidal activity. As mentioned previously, domains II and III of 3d-Cry toxins are involved in the recognition of larval gut proteins. The phylogenetic analysis of the 3d-Cry family revealed that domain III swapping between different Cry toxins has participated during the natural evolution process of these proteins (Bravo 1997; de Maagd et al. 2001). Different examples of domain III exchanges among different Cry toxins that have resulted in increased toxicity of the Cry toxins have shown that this is an effective strategy for in vitro evolution of Cry toxins (Bravo et al. 2013). A hybrid protein constructed with Cry1Ab domains I and II and domain III from Cry1C resulted in a six-fold increase in toxicity to *S. exigua* compared

to Cry1C (de Maagd et al. 2000). Another example of the success of this strategy was the construction of a hybrid protein containing domains I and II from the coleopteran-specific Cry3Aa and domain III from the lepidopteran-specific Cry1Ab (eCry3.1Ab) resulted in a protein with high toxicity to the corn pest *D. virgifera virgifera* in contrast to Cry3Aa and Cry1Ab that are nontoxic to this pest (Walters et al. 2008). In the case of domain II, it has been shown that exposed loop regions of this domain are involved in receptor binding (Bravo et al. 2011). Thus, loop exchange between different toxins is likely to be an interesting strategy for improving toxicity (Bravo et al. 2013). A nice example of this strategy was the modification of the mosquitocidal specificity of Cry4Aa by exchanging domain II loop 3 with Cry4Ba loop3 sequence (Abdullah et al. 2003). The hybrid protein gained toxicity to *Culex* sp. and retained toxicity to *Aedes aegypti* (Abdullah et al. 2003).

Also, it has been shown that mutations of domain II loop sequences could result in toxins with increased toxicity (for review see Bravo et al. 2013). Another example involving mutagenesis of loop regions of insecticidal proteins to change insect specificity was done in the Cyt2Aa toxin (Chougule et al. 2013). A peptide sequence that binds to an aphid gut APN was introduced into the different exposed loop regions of Cyt2Aa. Some of the hybrid proteins gained toxicity to the pea aphid *Acyrthosiphon pisum* and to the green aphid *Myzus persicae* (Chougule et al. 2013).

Finally, few examples of high-throughput systems for evolution of Cry toxins have been described. Phage display allows the display of multiple variants of the toxins in the surface of the phage, and the selection of binders to brush border membranes or to purified receptors has resulted in the identification of novel Cry toxins with improved toxicity against different insect species (Bravo et al. 2013). Recently, a novel phage-assisted continuous evolution system (PACE) that rapidly evolves high-affinity protein-protein interactions was used to evolve the Cry1Ac toxin to bind a non-cognate cadherin-like receptor (Bardan et al. 2016). Evolved Cry1Ac toxins using PACE bind *Trichoplusia ni* cadherin with high affinity and kill susceptible and resistant *T. ni* insects (Bardan et al. 2016). This system has the potential to evolve the toxin to improve its toxicity and to overcome resistance to Bt toxins (Bardan et al. 2016). Finally, to counter resistance to Cry1A toxins, Cry1Ab and Cry1Ac were engineered to lack the amino terminal end that is cleaved after cadherin binding. These modified toxins (Cry1AbMod or Cry1AcMod) were shown to form oligomers in solution and to kill Cry1A-resistant insects linked to different resistant mechanisms (Soberón et al. 2007).

Overall, the identification of the rate-limiting steps of Cry toxicity in different insect pests and the mapping of the binding regions involved in receptor recognition are likely to provide strategies to modify Cry toxins to change specificity, enhance toxicity, or counter resistance.

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Chapter 5
Effect of *Bacillus thuringiensis* on Parasitoids and Predators

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Abstract Advances in biotechnological studies have led to the development of genetically modified (GM) crops. The commercial release of transgenic plants producing *Bacillus thuringiensis* (Bt) insecticidal proteins has contributed to the management of several insect pests worldwide. Additionally, the use of selective products such as Bt-bioinsecticides allows for the conservation of beneficial organisms, including parasitoids and predators, in agricultural ecosystems, thus reducing chemical insecticidal applications. The use of these bioinsecticides reduces production costs, improves product quality, and can serve as a good strategy to slow the evolution of resistance in insect pest populations. Numerous studies have investigated the effects of Bt on insect pests and on their natural enemies. Here we review the effects of Bt on parasitoids and predators and emphasize that although Bt should be selective for natural enemies, special attention should be paid to the sublethal effects of these products on the biology and/or behavior of natural enemies. Thus, this chapter describes the possible effects of Bt on some predators and parasitoids species, including Bt-bioinsecticides and GM plants.

Keywords Integrated pest management • Bt-bioinsecticides • GM crops • Natural enemies

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Pesticides are the most common strategy used to control pests in agriculture due to their low cost and high availability in the market. However, pesticide misuse can reduce the populations of natural enemies, affect nontarget organisms, cause environmental contamination, and promote the development of resistance in insect populations (Sparks and Nauen 2015). Sometimes pesticides are combined with resistant plant varieties, biological control, cultural control, and other approaches that typically constitute an integrated pest management (IPM) program (Abrol 2013), and it is necessary to emphasize the development of other less-toxic control methods to the consumers, farmers, and environment.

Advances in biotechnology have led to the development of genetically modified (GM) crops. The commercial release of transgenic plants expressing *Bacillus thuringiensis* (Bt) insecticidal proteins against pests in agriculture which began in 1996 in the USA, followed by other countries, has contributed to the management of insect pests worldwide (Mohankumar and Ramasubramanian 2013).

Furthermore, the use of selective products such as *Bacillus thuringiensis*-based biopesticides, also known as Bt-bioinsecticides, to preserve beneficial organisms, including parasitoids and predators, decreases pesticide applications (Lacey 2017). The use of these biopesticides reduces production costs, improves product quality, and can serve as a good strategy to slow the evolution of resistance in insect pest populations.

Numerous studies have investigated the effects of Bt on insect pests and on their natural enemies. For instance, Glare and O'Callaghan (2000) reviewed the effects of Bt on parasitoids and predators. The authors emphasized that although Bt should be selective for natural enemies, special attention should be given to the sublethal effects on the biology and/or behavior of natural enemies. Thus, Bt-bioinsecticides should be much more selective than chemical insecticides (Lacey 2017), and this chapter describes the possible effects of Bt plants and Bt-bioinsecticides on predators and parasitoids.

5.1 Effect of Bt Plants on Parasitoids and Predators

Natural enemies can play important roles in pest control in GM crops (Chen et al. 2008) so the compatibility of GM crops and natural enemies is a very important issue (Mohankumar and Ramasubramanian 2013). Parasitoids and predators can be susceptible to GM crops, but in general, the effects of tritrophic interactions on the life history and behavior of natural enemies are limited (Han et al. 2016).

Considering the pathways of exposure, GM crops can affect natural enemies in several ways: directly – by feeding on GM plant tissues, such as in the case of omnivorous predators (Torres and Ruberson 2006, 2008; Veiga et al. 2014), or indirectly – genetically modified plants induce changes in the agroecosystem, in the amount and nutritional quality of prey, and can alter the emission of volatile compounds that attract natural enemies (Lundgren et al. 2009). GM crops can also indirectly affect natural enemies due to changes in tritrophic interactions (plant-herbivore-parasitoids/predator) in different food chains (Han et al. 2016).

5.1.1 Life History

Generalist predators, such as stink bugs, are abundant in several crops and can be affected by Bt plants because they can consume eggs and/or larvae (Symondson et al. 2002). Many species are omnivorous and can also feed directly on the plant, nectar, or pollen. Thus, these species are potentially exposed to Bt proteins from GM plants.

Survival, development, fecundity, and fertility were similar when the predators *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) and *Geocoris punctipes* (Say) (Hemiptera: Geocoridae) consumed larvae in cotton containing Cry1Ac and Cry2Ab proteins or corn containing Cry1F, compared to prey that fed on cotton or isogenic corn. Moreover, continued exposure during the second generation did not result in different characteristics in the life history of predators when they consumed prey that fed on Bt or non-Bt plants (Tian et al. 2014).

The *O. insidiosus* nymphal period and nymphal survival were similar between those insects feeding on *Aphis gossypii* Glover (Hemiptera: Aphididae) reared in conventional cotton or GM cotton containing the Cry1Ac protein, ranging from 16.8 to 16.9 days and from 68.3% to 70.0%, respectively (Veiga et al. 2014). The same parameters were evaluated for *Orius sauteri* (Poppius) (Hemiptera: Anthocoridae) feeding on *A. gossypii* reared on GM (Cry1Ac/Ab) and conventional cotton, were also similar, ranging from 9.8 to 10.0 days and from 92.3% to 96.3%, respectively (Zhang et al. 2008). The same effect was verified for *Orius majusculus* (Reuter) (Hemiptera: Anthocoridae), feeding on *Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae) in GM (Cry1Ab) and conventional corn (Zwahlen et al. 2000).

The developmental period, survival, fecundity, or viability of *Orius albidepennis* (Reuter) (Hemiptera: Anthocoridae) that fed on larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) reared with an artificial diet containing Cry1Ac, Cry1Ab, and Cry2Ab proteins did not change due to the presence of Bt toxins (González-Zamora et al. 2007).

Longevity of females and males of *O. insidiosus* feeding on *A. gossypii* was similar in GM and conventional cotton, ranging from 16.7 and 12.4 days to 15.2 and 13.9 days, respectively (Veiga et al. 2014). Furthermore, *O. sauteri,* feeding on *A. gossypii* reared in GM and conventional cotton, also showed similar longevity, ranging from 13.8 to 14.4 days, respectively (Zhang et al. 2008).

The biological parameters nymphal duration (13.0–13.6 days), longevity of the females (34.1–35.7 days), and fecundity (37.1–38.9 eggs/female) remained unaffected in *Orius tantillus* (Motschulsky) (Hemiptera: Anthocoridae) feeding on thrips, a nontarget of GM plants, and pollen from Bt rice (Cry1Ab) (Raen et al. 2016). These results indicate that Bt toxins do not affect Anthocoridae predators, especially when exposed to the proteins through their prey.

Cry1Ah and Cry2Ab toxins added to the artificial diet of the aphid *A. gossypii* did not affect development (11.2–11.6 days), the percentage of formed pupae (91.4–97.1%) of the ladybird *Propylea japonica* (Thunberg) (Coleoptera: Coccinellidae) (Zhao et al. 2016).

The consumption of prey *Chrysoperla rufilabris* (Burmeister) (Neuroptera: Chrysopidae), was not affected by Cry1A, Cry1Ab, or Cry1F proteins expressed in GM plants evidencing the selectivity of these proteins to the predator (Tian et al. 2013).

Parasitoids can use honeydew produced by other insects as food. The quality of this compound produced by *A. gossypii* that was fed Bt cotton and the possible effects on the development of the parasitoid *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) (Hagenbucher et al. 2014) were evaluated. Low gossypol concentration in the "honeydew" excreted by aphids that fed on GM plants had an effect on longevity and reproduction of the parasitoid.

5.1.2 Behavior

Several studies have shown that Bt plants do not affect the behavior of natural enemies, and these studies have focused on predatory mites as well as on insects. The predatory mite *Amblyseius andersoni* (Chant) (Acari: Phytoseiidae) did not exhibit a preference to feed on *Tetranychus urticae* (Acari: Tetranychidae) reared on Bt cotton (Cry1Ac and Cry2Ab) or Bt corn (Cry1F) compared with non-Bt plants (Guo et al. 2016).

Regarding predation behavior, studies of functional response are important for the evaluation of prey consumption at different densities; therefore, the ingested Bt can affect the functional response of natural enemies. The predator *O. insidiosus* feeding on *A. gossypii* reared in conventional and transgenic (Cry1Ac) varieties, exhibited similar prey consumption. In this case, the ingestion of *A. gossypii* by *O. insidiosus* remained unaffected by the GM variety (Veiga et al. 2014).

The Cry toxin expressed in the plant reached the third trophic level through the herbivore, but there was no negative effect on the predators, such as *O. insidiosus* (Torres and Ruberson 2008). The same species remained unaffected when the predators were exposed to Bt toxin through the prey indicating that Bt plants did not affect nontarget organisms (predators) (Veiga et al. 2014).

5.1.3 Biodiversity

The study of the effect of Bt plants on nontarget organisms was a science that arose due to the great adoption of these plants by farmers around the world. In field conditions, the potential effect of Bt plants on the biodiversity of natural enemies was evaluated in crops such as corn, cotton, rice, and potato (Ferry et al. 2007; Yang et al. 2015; Resende et al. 2016; Schoenly and Barrion 2016). However, most of them have been conducted with corn and cotton.

The biodiversity in conventional and transgenic corn (Cry1Ab, Cry1F, and the combination of Cry2Ab2 and Cry1A105) was similar in a study carried out in seven cities in Minas Gerais State (Brazil) (Resende et al. 2016). The authors pointed out

that insect richness and diversity depends on local and other factors, such as pesticides use. Other studies conducted with Bt corn only containing the Cry1Ab protein verified that the effect of Bt corn on natural enemies was minimal and lower than the effects caused by pesticides (Rose and Dively 2007; Alcantara 2012).

The number of larvae and adults of dominant predators have remained unaffected by Bt cotton in Brazil (Thomazoni et al. 2013), but in China, Yang et al. (2015) described changes in agricultural fauna over the last 15 years due to the adoption of cotton expressing Bt Cry1A toxins.

Otherwise, Lu et al. (2012) verified a pronounced increase in the abundance of three types of generalist predators (ladybugs, lacewings, and spiders) and a decrease in the abundance of aphid pests associated with generalized adoption of Bt cotton and decrease in pesticides sprayings, based on data collected between 1990 and 2010 at 36 places in six provinces of Northern China. The conservation of some tritrophic interactions in Bt cotton contributed to a more sustainable management of nontarget pests by enhancing their natural biological control.

5.2 Effect of Bt-Bioinsecticides on Parasitoids and Predators

The presence of several toxins in Bt-bioinsecticides and spores makes the potential effect of these products on arthropods different to the effect of the toxins expressed in transgenic plants. Thus, it is necessary to evaluate the two technologies separately.

Nonselective pesticides can control several pest species, but they can also cause pest outbreaks by eliminating beneficial insect species (Pimentel 2013). Bioinsecticides based on organisms such as bacteria can usually reduce the nontarget effects caused by conventional pesticides (Kalha et al. 2013).

5.2.1 Biological Development

The bioinsecticide Agree® did not impair the use of the parasitoid *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) against *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) in Brassicas. A study conducted with two generations showed that the total number of eggs parasitized by *T. pretiosum* differed significantly in the first generation, which was higher in the control (22.3 eggs), compared to Bt-exposed eggs (9.5 eggs). However, the Bt treatment (19.6 eggs) did not differ from the control (20.3 eggs) in the second generation (Goulart 2010).

Bt provided with honey to *Trichogramma pratissolii* Querino and Zucchi (Hymenoptera: Trichogrammatidae) using eggs of *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae) as the host, indirectly affected the emergence of the parasitoid progeny (Pratissoli et al. 2006). However, the authors did not describe any negative effect of Bt on total parasitism.

Polanczyk et al. (2006) did not observe any Bt interference on *T. pratissolii* longevity, but some isolates decreased the time necessary to obtain 80% parasitized eggs. Those authors concluded that Bt accelerated parasitism, but did not affect the total number of parasitized eggs. Bt isolates can be used together with *T. pratissolii* in IPM programs. Therefore, these results are important for the development of IPM strategies involving such control agents, mainly because Bt-bioinsecticides are mostly used to control Lepidoptera pests (Glare et al. 2017).

Regarding nontarget pests such asaphids, the Dipel® bioinsecticide caused only 30% of mortality of *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae) females that were exposed to biopesticide dried residues for 48 h, which is considered "harmless" according to the "International Organization for Biological Control" (Garantonakis et al. 2016).

Species of the subfamily Asopinae (Hemiptera) are beneficial nontarget insects, often used in bioassays because of their importance against Lepidoptera pests, and have a zoophytophagous feeding habit. *Podisus nigrispinus* (Dallas) (Hemiptera: Pentatomidae) is one of the most important species in this group.

There were negative effects of Bt-infected larvae of *P. xylostella* on *P. nigrispinus* biological characteristics, such as an increase in the nymphal period (5 days longer), lower survival until adult (48.3%), and fecundity was 88% lower than the control. Besides that, when *P. nigrispinus* consumed Bt-infected larvae of *P. xylostella* daily, a decrease in the number of progeny was observed resulting in population growth reduction (Goulart et al. 2015). Nascimento et al. (1998) also observed that the development and reproduction of *P. nigrispinus* were negatively affected when the predators continuously received Bt-treated larvae of *Bombyx mori* (L.) (Lepidoptera: Bombycidae) as food.

However, according to Carvalho et al. (2012), the Bt HD1 strain did not affect the biological characteristics of *P. nigrispinus* when the predator consumed infected larvae and water or healthy larvae and Bt suspension. Adverse effects on the life history of *P. nigrispinus* may be due to adverse indirect factors caused by Bt toxins, which may have affected the nutritional quality of the infected prey. Prey quality may have decreased due to their susceptibility to Cry proteins, resulting in a decrease in growth and in the development of natural enemies (Romeis et al. 2006).

Additionally, Cry toxin receptors were not observed in the *P. nigrispinus* midgut (da Cunha et al. 2012), which can indicate that the predator midgut environment is inadequate for intoxication. The Bt-bioinsecticide formulation could have also been partially responsible for the possible secondary effects of adjuvants, and inert in this nontarget organism (Magalhães et al. 2015a). Thus, it is possible to use *P. nigrispinus* with Bt-bioinsecticides against *P. xylostella* in Brassicas (Carvalho et al. 2012; Dibelli et al. 2013; Magalhães et al. 2015a, b).

Characteristics such as duration of the second instar, nymphal consumption, and longevity of females of *O. insidiosus* were affected by the presence of Bt in the predated eggs of *P. xylostella*. Females of *O. insidiosus* that were fed eggs of *P. xylostella* treated with Bt suspension produced lower progeny, decreasing the population growth rate (Goulart et al. 2015).

Torres and Ruberson (2008) verified the transfer of Bt toxins between trophic levels, by *O. insidiosus* that acquired 17% of the toxin from its prey *Frankliniella occidentalis* (Pergande) (Thysanoptera, Thripidae). Besides this, nymphs consumed more Bt treated prey. Even so, these factors were insufficient to affect the reproductive characteristics of the females, which were similar between the treatments described by Goulart et al. (2015).

5.2.2 On Behavior

Bt-bioinsecticides can alter the behavior of natural enemies. Considering the feeding behavior of omnivorous generalist predators, which can feed both prey and plants, the impact of Bt biopesticides on the *P. nigrispinus* species has been studied by several groups. This insect can be exposed to Bt toxins by feeding contaminated prey or by sucking the suspension directly after plant pulverization.

Predators move the stylet to reach different plant tissues, such as the xylem and parenchyma (Torres et al. 2010). Consequently, Bt-bioinsecticides reduce the risk of predators feeding the suspension, since they are able to locate a feeding site inside the plant tissue. The impact of Bt-bioinsecticide, HD1 isolate, and *B. thuringiensis* var. GC91 (Agree®) on feeding larvae of *P. xylostella* and phytophagy of *P. nigrispinus* was evaluated by Magalhães et al. (2015b) who described that nymphs and adults fed most on larvae and sought a lower intensity of food resources in the leaves when treated with bioinsecticides.

Dibelli et al. (2013) evaluated the interaction among *P. xylostella*, *P. nigrispinus*, and Agree® and observed that the predation capacity was not affected due to ingestion of the product by prey. Additionally, *P. nigrispinus* did not exhibit a preference for *P. xylostella* larvae in double preference bioassay, when the prey fed on leaves treated or untreated with the HD1 isolate (Carvalho et al. 2012). Thus, the addressed studies demonstrate the compatibility between Bt and predatory stink bugs.

5.2.3 Selectivity

Studies on selectivity in IPM are important because they are fundamentally based on the preservation of beneficial flora and fauna that are responsible for the natural biological control of pests in agriculture (Goulart et al. 2012).

Nunes et al. (1999) demonstrated that the application of Bt to cotton to control larvae reduced the predator population, in which 2.87 and 1.40 predators were observed at 7 days after application and 2.63 and 6.50 predators at 14 days after application in the Bt and control treatments, respectively.

The selectivity of the Bt-bioinsecticide (*B. thuringiensis* var. *kurstaki*) was evaluated in adults and pupae of the egg parasitoid *Telenomus remus* Nixon (Hymenoptera: Platygastridae), under laboratory conditions, according to the protocol proposed by

the International Organization for Biological Control (IOBC) (Silva et al. 2016). The Bt-bioinsecticide was classified as harmless in a contact bioassay with adults (Class 1), thus the Bt-bioinsecticide is selective to *T. remus*.

The relative toxicity of the Bt-bioinsecticide (*B. thuringiensis* var. *kurstaki*) was assayed to adults and "mummies" of *A. colemani*. The Bt-bioinsecticide was classified as harmless (<30% mortality) according to the IOBC and considered selective to the natural enemy (Garantonakis et al. 2016).

Ksentini et al. (2010) described moderate toxicity of Bt on *Trichogramma cacoeciae* Marchal, *T. bourarachae* Pintureau, and *T. evanescens* (Westwood). This was not observed in other studies, such as that reported by Goulart (2010) using *T. pretiosum* and Agree® Bt insecticide.

The parasitism rate and adult emergence of *T. pretiosum* in a host treated with Bt-bioinsecticides (*B. thuringiensis* var. *aizawai* and *B. thuringiensis* var. *kurstaki*) remained unaltered. According to the IOBC classification, the Bt bioinsecticides were classified as harmless or slightly toxic, which were thus selective for *T. pretiosum* (Laurentis 2017).

5.3 Final Considerations

Since the first commercial attempt to use Bt bioinsecticides in 1938 (Glare and O'Callaghan 2000), many products and toxins have been evaluated regarding the impact on nontarget species (Romeis et al. 2006; Chen et al. 2008; Carvalho et al. 2012). However, the effect of Bt-bioinsecticides on natural enemies is minimal or significantly lower than that of traditional pesticides (Glare and O'Callaghan 2000; Lacey 2017). Nevertheless, this subject is still far from reaching a definite conclusion due to the high number of Cry toxins still untested against pests or beneficial arthropods.

The studies described in this chapter demonstrate that the integration of parasitoids and predators that naturally occur at agricultural crop sites using Bt bioinsecticides is positive, in which the use of Bt and natural enemies significantly increased the crop yield and the impact of parasitoids and predators on pest populations in several cases (Furlong et al. 2008).

To provide an example of the economic impact of biological control, socioeconomic technical surveys were conducted at cruciferous production places of the Da Lat mountains in Vietnam, where farmers have access to up to 16 Bt-bioinsecticides. The potential of using multiple biological control agents was evaluated, such as Bt and the parasitoids *Diadegma semiclausum* (Hellen) and *Diadromus collaris* (Gravenhorst) (Hellen) (Hymenoptera: Ichneumonidae) to control *P. xylostella*. Based on both biological parameters (abundance of the parasitoids and of *P. xylostella*) and socioeconomic performance indicators, such as increase in yield and decrease in pesticide costs and gross margin for 1 year, farmers that adopted biological control for *P. xylostella* management reduced their production cost by US$ 133–513 ha, a 30% decrease compared to those that only used pesticides. Farmers

that used Bt and preserve parasitoids ensure sustainable cruciferous production in their production systems (Nga and Kumar 2008). The combined use of Bt-bioinsecticides or transgenic Bt plants and natural enemies, predators, and parasitoids allows the maintenance of a balanced agroecosystem and contributes to the safety and health of food production for human consumption.

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Chapter 6
Characterization of *Bacillus thuringiensis* Using Plasmid Patterns, AFLP and Rep-PCR

Fernando Hercos Valicente and Rosane Bezerra da Silva

Abstract Molecular characterization of *Bacillus thuringiensis* (Bt) strains may be used to characterize DNA, protein, and genetic variability among Bt isolates. Different techniques have been used to discriminate between different isolates with different properties such as genetic profile, genetic variability, and proteins. The most common technique used for characterization of genetic profile and toxicity prediction is the polymerase chain reaction (PCR); for genetic diversity it is the repetitive element polymorphism (Rep-PCR) using ERIC, REP, and BOX primers. Amplified fragment length polymorphism (AFLP) is used to understand genetic variability and detect molecular markers, and plasmid characterization is important to detect the number and plasmid patterns. *B. thuringiensis* proteins can be characterized according to their size in SDS PAGE. In general, for most techniques the advantage is that it is not necessary to know about the genome of the species to be studied, it is not so expensive, and the results are reproducible.

Keywords Genetic variability • Genetic profile • Proteins • DNA

6.1 *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a Gram-positive bacterium, Bacillaceae family, aerobic, that produces protein crystalline inclusions called Cry proteins during the stationary phase encoded by different *cry* genes (Angus 1954; Bechtel and Bulla 1976). *Bacillus thuringiensis* is a ubiquitous bacterium that can be found in different substrates such as soil, water, plant surfaces, dead insects, grain dust, spider webs, and stored grain (Glare and O'Callaghan 2000; Valicente and Barreto 2003; Federeci 1999). Crystal proteins are composed of one or more proteins, Cry or Cyt, and are called delta (δ) endotoxins, and these are the primary factors determining Bt

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pathogenicity (Schnepf et al. 1998). Many Bt strains also produce other types of insecticidal proteins, such as the Vip proteins (vegetative insecticidal proteins) that are synthesized during the vegetative phase growth, do not form crystals, and were first identified by Estruch et al. (1996). The identification of a Bt strain to a subspecies is done using the flagellar antigen H, e.g., *Bacillus thuringiensis* sv *kurstaki*. But this type of characterization does not consider the genes present in these strains, e.g., strain HD-1 (Bt sv *kurstaki*) harbors the genes *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa*, and *cry2Ab*, whereas strain HD-73 *(*Bt sv *kurstaki)* harbors only *cry1Ac* gene.

6.2 Cry Proteins

Cry proteins are toxic to insects from the orders Lepidoptera, Coleoptera, Hemiptera, Neuroptera, Orthoptera, Siphonaptera, Thysanoptera, Isoptera, (Glare and O'Callaghan 2000), and also nematodes. *Bacillus thuringiensis* may be used as a biological pesticide and also may be used as a source of toxins for transgenic plants (Bt plants). Crickmore et al. (1998) proposed a new nomenclature for Cry and Cyt proteins. Cry and Cyt protein nomenclature is based on the identity of the primary sequences among proteins. The nomenclature and sequences are available at the website http://www.lifesci.susx.ac.uk/home/Neil_Crickmore/Bt/ (2016). Most of *cry* genes are present on plasmids and not the chromosomes.

6.3 Genetic Variability and Molecular Characterization of *Bacillus thuringiensis*

Molecular characterization of Bt strains may be used to characterize DNA, protein, and genetic variability among Bt isolates. Different techniques have been used to discriminate between different isolates with different purposes. The most common techniques used are polymerase chain reaction (PCR), repetitive element polymorphism (REP-PCR), and amplified fragment length polymorphism (AFLP) and plasmid pattern characterization. Bt proteins can be characterized according to their size in SDS PAGE.

6.3.1 Polymerase Chain Reaction (PCR)

PCR has been used in order to characterize the genetic profiles of Bt isolates (Cerón et al. 1994, 1995; Lima et al. 2002; Bravo et al. 1998; Valicente et al. 2010). This method is advantageous because genes of interest may be detected in Bt isolates, building a genetic profile of a strain or a group of strains and also predicting mortality activity against an insect or an insect order based on the genetic profile. This

prediction is important when you have limited access to some insects, because some insects are difficult to find and/or expensive to rear. Valicente et al. (2010) found 28 strains harboring the *cry1D* gene, before testing these strains against some specific lepidopteran pests. Also, some countries have restrictions to rear insects. After PCR reactions, these strains showed a high mortality toward these insect pests. PCR is the most common and used technique because it is not so expensive and the results are reproducible.

6.3.2 Plasmid Patterns

The genes encoding for the Cry proteins are found mainly on plasmids of different sizes (4–150 MDa), not only in different combinations or multiple copies within a plasmid but also in combinations of these plasmids in different strains of Bt (Lereclus et al. 1993).

Plasmids are extrachromosomal genetic elements found in many species of bacteria and some yeast. Molecules of plasmid DNA are circular and double stranded. Compared to the bacterial chromosome, plasmids are able to autonomously replicate. During cell division, it is observed that at least one copy of each plasmid segregates to each daughter cell. In nature, some plasmids show functional incompatibility with other plasmids; this important factor avoids simultaneous presence of both plasmids in the same cell (Birge 1994).

A standard plasmid profile seems to be related with each strain. Two different groups of plasmids can be recognized: those that are $\leq$30 MDa, and those that are $\geq$30 MDa, the latter being called megaplasmids. For practical purposes, each group is divided according to the chromosomal band in an agarose gel. Smaller plasmids are below that band, and megaplasmids are above it. Smaller plasmids are generally present in higher copy numbers and megaplasmids present in low copy numbers (Ramírez and Ibarra 2008). Most of the smaller Bt plasmids are still referred as cryptic plasmids, since no specific function has been attributed to them. As for the megaplasmids, their main recognized function is harboring *cry* genes (Berry et al. 2002; Loeza-Lara et al. 2005; Roh et al. 2007).

Several techniques for the extraction and purification of plasmids have been optimized because of the importance of *cry* genes in host cells and their use as molecular tools (Gitahy et al. 2005). The most used technique is extraction using alkaline lysis and purification under gradient ultracentrifugation in cesium chloride (Sambrook et al. 1989). This was one of the first biochemical methods developed for obtaining plasmids of various microorganisms (Gitahy et al. 2005). Despite several adjustments, this technique is still slow and laborious, with a high level of contamination when ethidium bromide is used. Ramirez and Ibarra (Ramírez and Ibarra 2008) developed a more practical and faster protocol to obtain the plasmid DNA of Bt. Fagundes et al. (2011) tested the plasmid patterns of efficient and inefficient strains of *B. thuringiensis* against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), and found megaplasmids in 13% of the evaluated strains. These authors could characterize 59 strains based on the migration of bands in an

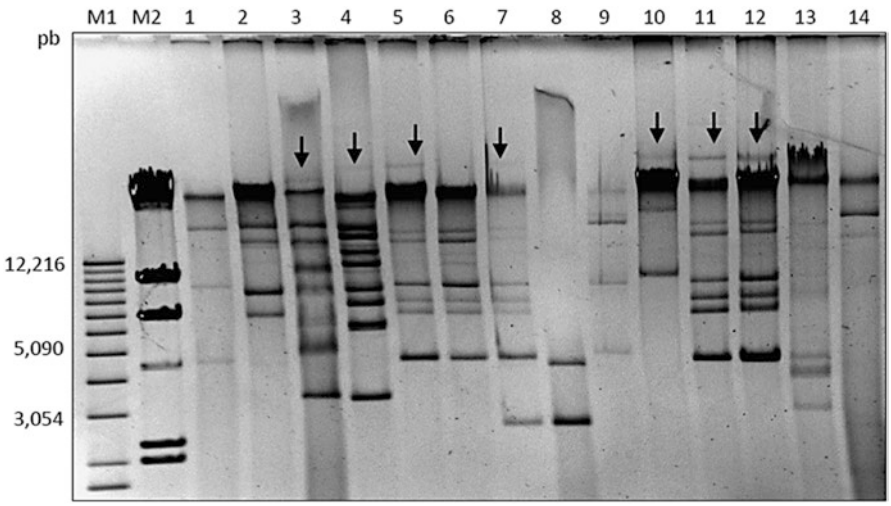

Fig. 6.1 Lanes: *1* HD4 (Bt *alesti*), *2* 348B (Bt *alesti*), *3* HD11 (Bt *aizawai*), *4* T07 (Bt *aizawai*), *5* T09 (Bt *tolworthi*), *6* 344 (Bt *tolworthi*), *7* 426 (Bt *tolworthi*), *8* 461A (Bt *tolworthi*), *9* HD29 (Bt *galleriae*), *10* 474 (Bt *galleriae*), *11* 348L (Bt *galleriae*), *12* 462A (Bt *galleriae*), *13* 460 (Bt *darmstadiensis*), *14* T10 (Bt *darmstadiensis*). *M1* (1Kb DNA ladder), *M2* (λ DNA Hind III marker), (↓) megaplasmids

agarose gel. Strains belonging to the same serovars showed different plasmid sizes (from 1636 to 23,200 bp), except for two strains belonging to serovar *galleriae*. The strain T09 Bt *tolworthi* showed a plasmid migration pattern identical to strains belonging to serovar *galleriae* (Fig. 6.1). Plasmid patterns differed for 46 strains, confirming that this is a useful tool to discriminate specific strains. However, it was not possible to associate the plasmid pattern or the occurrence of particular plasmids with the pathogenicity of a given species toward *S. frugiperda* larvae.

6.3.3 Repetitive Element Polymorphism Rep-PCR

Rep-PCR has become a frequent method to discriminate bacterial species analyzing the distribution of repetitive DNA sequences in several prokaryotic genomes (Versalovic et al. 1991). It is reliable, reproducible and simple, and of rapid implementation, in addition to high efficiency with the discrimination of microorganisms, even among populations of the same species (Versalovic et al. 1994; Rademaker and De Bruijin 1997; Louws et al. 1999).

Rep-PCR is based on the observation that outwardly facing oligonucleotide primers, complementary to interspersed repeated sequences, enable the amplification of differently sized DNA fragments, consisting of sequences lying between these elements (Versalovic et al. 1994). Multiple amplicons of different sizes can be

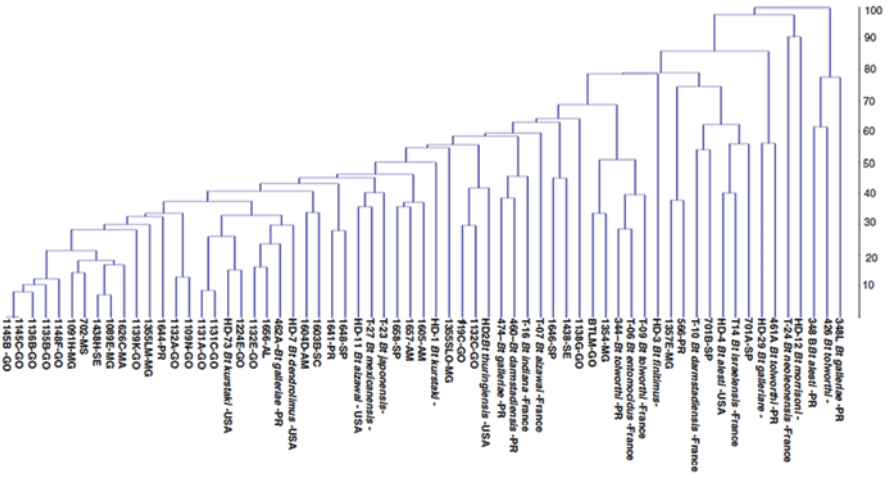

Fig. 6.2 Dendrogram obtained by using Rep-PCR on purified DNA from *Bacillus thuringiensis* species followed by evaluation using UPGMA clustering method

resolved by electrophoresis, establishing specific DNA fingerprint patterns for bacterial strains (Rademaker and De Bruijin 1997). Several of these interspersed repetitive elements are conserved in diverse genera of bacteria and, therefore, enable single primer sets to be used for DNA fingerprinting in many different microorganisms (Versalovic et al. 1994; Rademaker and De Bruijin 1997). Moreover, prior knowledge of the genomic sequence of a species is not necessary, with an initial selection of primers, to have this approach generate results in a short period of time (Shangkuan et al. 2001; Lima et al. 2002).

Palindromic units (PUs) /repetitive extragenic palindromes (REP) constitutes the characterized family of bacterial repetitive sequences. PUs are present in about 500–1000 copies in the chromosome of *Escherichia coli* and of *Salmonella typhimurium.* PU sequences consist of a 35–40 bp inverted repeat and are found in clusters in which successive copies (up to six) are arranged in alternate orientation (Higgins et al. 1982; Gilson et al. 1984). There is not much information published using rep-PCR to study the genetic diversity of *B. thuringiensis* isolates. Silva and Valicente (2013) studied the genetic divergence of 65 strains of Bt using Rep-PCR. Results showed that the repetitive sequences for the BOX primer were the most informative with 26 fragments, followed by ERIC (19), and REP (10), generating a total of 55 fragments. Figure 6.2 shows that ten groups were formed when 45% was the average distance of the population: group 1 with 41.5% of the isolates, 33.8% of the isolates were distributed in other groups, and 24.6% did not form a distinct group. 53.2% of the isolates from Embrapa (Brazilian Agricultural Research Corporation) are in the group 1, and 29.8% of the isolates are distributed in other groups. Bt strains from USDA (HD strains) and Institut Pasteur (T strains) showed more variability.

However, Silva and Valicente (2013) showed that when ERIC, REP, and BOX are used together, the profiles generated are not related to the subspecies of the *B.*

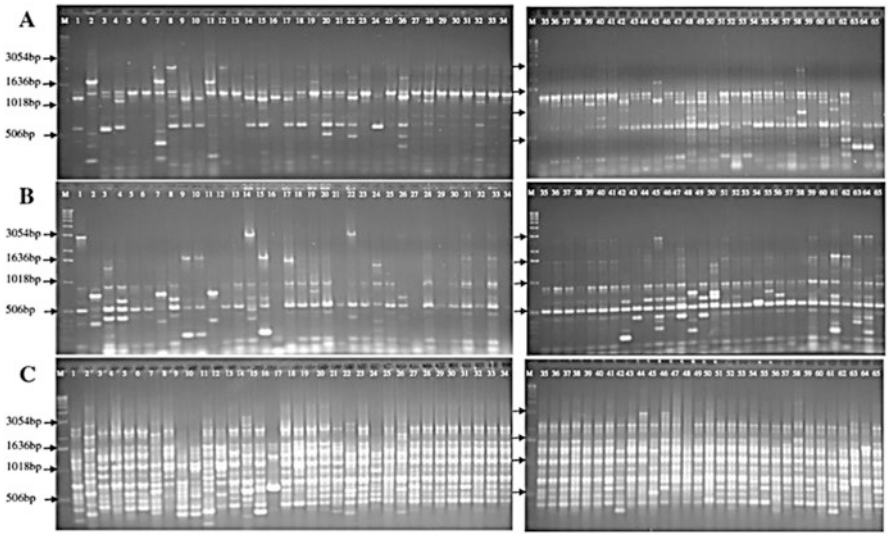

Fig. 6.3 Rep-PCR fingerprint patterns of *B. thuringiensis* and 65 reference strains. (**a**) REP, (**b**) ERIC, (**c**) BOX, and (M) 50 bp DNA ladder

thuringiensis isolates, since there were bands in the profile very similar to some isolates of the same subspecies. Figure 6.3 shows a general aspect of Rep-PCR fingerprinting patterns of *B. thuringiensis* of 65 reference strains (A) REP, (B) ERIC, (C) BOX, and (M) 50 bp DNA ladder (Da Silva and Valicente 2013).

A second family of repetitive elements, called intergenic repeat units (IRUs) or enterobacterial repetitive intergenic consensus (ERIC), has been described (Versalovic et al. 1991). IRUs are 124–127 bp long and are present in about 30–50 copies in *E. coli* and 150 copies in *S. typhimurium*. Although IRU resembles PU in several features, the nucleotide sequence is entirely different, and PU IRU appears to occur singly. Both PU and IRU families are similarly located in noncoding, probably transcribed, regions of the chromosome. The consensus BOX element is constituted, from 5′ to 3′, of three subunits, boxA (59 bp), boxB (45 bp), and boxC (50 bp), and it is 154 bp long present in about 25 fragments of the *S. pneumoniae* chromosome (Stern et al. 1984; Sharples and Lloyd 1990; Versalovic et al. 1991; Shuhaimi et al. 2001).

6.3.4 Amplified Fragment Length Polymorphism (AFLP)

This technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity (Vos et al. 1995). AFLP may be used as a tool for bacterial taxonomy and has shown utility in detecting molecular variability in very closely related bacterial strains (Burke et al. 2004; Grady et al. 2001). The fluorescent amplified fragment length polymorphism

(fAFLP) uses markers with fluorescent substances, the use of primers labeled with fluorochrome, associated with automatic sequencers and a large capacity for computational analysis (Ryu et al. 2005; Burke et al. 2004).

The ALFP technique has advantages such as: (a) it is not necessary to know about the genome of the species to be studied, (b) it provides the largest number of possible fragments in the same analysis, and (c) many of these fragments may be polymorphic. The main disadvantage of AFLP is the dominant characteristic, and this results in a low information output per locus. Some other characteristics of this technique are: (a) it needs pure DNA to avoid any changes in the patterns of the bands in the gel; (b) infrastructure should be appropriate because it is a little more laborious than some other techniques, because it demands some steps to get the final result; (c) in general it uses radioactive material (Ferreria and Grattapaglia 1995; Vos et al. 1995). AFLP technique uses restriction enzymes with "rare" and "frequent cutter," with *Eco*RI (rare cutter) and *Mse*I (frequent cutter) being the most common (Ridout and Donini 1999; Hill et al. 2004; Burke et al. 2004).

The number of fragments in the gel varies from a dozen to more than a hundred, and polymorphisms are identified by the presence or absence of a band (Ridout and Donini 1999). AFLP markers have been useful to see genetic differences among individuals, populations, and species (Muller and Wolfenbarger 1999, Arnold et al. 1999; Ticknor et al. 2001; Hill et al. 2004; Abreu et al. 2007). Few studies have been published using the fAFLP technique to characterize *B. thuringiensis* isolates. Valicente and Silva (2014) found that a total of 495 scorable fragments were generated, ranging from 50 to 500 bp, in 65 *B. thuringiensis* strains when five primer combinations were used. Out of 495 fragments, 483 were found to be polymorphic, and only 12 fragments were monomorphic.

Overall, these techniques should be more used to characterize and generate results in order to better understand the diversity of *B. thuringiensis*.

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Chapter 7
New Sequencing Technologies and Genomic Analysis Applied to *Bacillus thuringiensis*

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Abstract Despite being the most commercially successful biological control agent of insect pests, some *Bacillus thuringiensis* strains have had only recently had their genome sequenced. In this chapter we discuss the current state of next-generation sequencing technology and provide examples of applications of these techniques to uncover some aspects of *B. thuringiensis* biology, such as taxonomy and toxin mining.

Keywords Genomics • High-throughput sequencing • Pangenome • Phylogenomics

Since the publication of the first bacterial genome sequence over 20 years ago – *Haemophilus influenzae* in 1995 (Fleischmann et al. 1995) – there has been a huge increase in the generation of bacterial genome data, mostly due to the dramatic reduction in the cost of high-throughput sequencing techniques (Loman and Pallen 2015). DNA sequence data have allowed scientists to uncover relevant information about organisms, including microorganisms, helping researchers to better characterize them, understand their evolutionary relationships, and discover new genes and their functions (Liu et al. 2012; Wenfei et al. 2014).

In 2004, the complete genome of the *B. thuringiensis serovar konkukian* strain 97–27 was released, and now there are 91 genome assemblies deposited in the NCBI Assembly database, but only 37 are completed (Table 7.1). *B. thuringiensis* strains that were first commercialized as pesticides did not have a complete genome sequence available in the database until recently (Rang et al. 2015; Zhu et al. 2015). Despite the increase in genome sequences available for *B. thuringiensis*, the number of trained bioinformaticians is still limited, and many nonspecialists started to use

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Table 7.1 List of *Bacillus thuringiensis* complete genomes available in GenBank (accessed 20 January 2017)

Strain	Size (bp)	Proteins	GenBank	Method	Date
konkukian/97-27[a]	5.237.682	5197	AE017355	Sanger	2004
Al Hakam[a]	5.257.091	5288	CP000485	Sanger	2006
BMB171	5.330.088	5491	CP001903	454	2010
finitimus/YBT-020	5.355.490	5605	CP002508	I**b**	2011
chinensis/CT-43	5.486.830	5943	CP001907	454	2011
*israelensis*HD-771	5.886.036	6294	CP003752	I	2012
israelensis/HD-789	5.495.278	6261	CP003763	I	2012
MC28	5.414.494	6355	CP003687	I	2012
Bt407	5.500.501	6184	CP003889	454	2012
kurstaki/HD73	5.646.799	5801	CP004069	454, I	2013
thuringiensis/IS5056	5.491.935	6485	CP004123	454, I	2013
YBT-1518	6.002.284	6275	CP005935	I	2013
kurstaki/YBT-1520[a]	5.602.565	6207	CP004858	I	2014
kurstaki/HD-1	5.631.672	6463	CP004870	I	2014
kurstaki/YBT-1520[a]	5.607.837	6200	CP007607	454, I, P[c]	2014
galleriae/HD-29	5.701.188	6101	CP010089	I	2014
YWC2–8	5.674.369	5534	CP013055	P	2015
XL6	5.308.217	6532	CP013000	I, P	2015
HD1011	5.232.696	6041	CP009335	454, I	2015
HD571	5.256.240	5290	CP009600	454, I, P	2015
Al Hakam[a]	5.229.095	5323	CP009651	454, I, P	2015
HD682	5.213.295	5294	CP009720	454, I, P	2015
97-27[a]	5.235.838	5299	CP010088	I; 454	2015
HD1002	5.491.311	6471	CP009351	I	2015
morrisoni/BGSC 4AA1	5.652.292	5944	CP010577	I	2015
YC-10	5.675.007	6496	CP011349	P	2015
HS18–1	5.292.526	6129	CP012099	I, P	2015
indiana/HD521	5.429.688	5996	CP010106	I	2015
CTC	5.327.397	5306	CP013274	I	2015
tolworthi/IP Standard	5.896.839	6556	AP014864	I, P	2015
Bt185	5.243.635	5894	CP014282	P	2016
HD12	5.776.895	6192	CP014847	P	2016
Bc601	5.627.121	5931	CP015150	I	2016
alesti/BGSC 4C1	5.400.819	5635	CP015176	I	2016
MYBT18246	5.867.736	6413	CP015350	454, P	2016
KNU-07	5.344.151	5743	CP016588	P	2016
coreanensis/ST7	5.665.360	5675	CP016194	P	2016

[a]Strains sequenced twice
[b]Illumina
[c]PacBio

high-throughput sequencing techniques without prior knowledge (Vincent et al. 2016). Therefore, in this chapter we discuss the current state of next-generation sequencing technology and provide examples of applications of these techniques to uncover some aspects of *B. thuringiensis* biology.

7.1 Overview of Next-Generation Sequencing Technology

The history of genome sequencing began with Dr. Frederick Sanger and colleagues in 1977, when they developed a method based on incorporation of dideoxynucleotides during the process of DNA extension by DNA polymerase. Several advances were made to Sanger sequencing in the following years, and although laborious and expensive, it was the dominant sequencing technology for almost 30 years (Liu et al. 2012; Mardis 2013; Metzker 2010). Such limitations brought out the need to develop new, and more efficient, technologies for genome sequencing.

The methods that emerged after Sanger were called next-generation sequencing technologies (Metzker 2010; McGinn and Gut 2013). These newer, second-generation technologies (Roche 454, Ion Torrent, and Illumina) produced considerably shorter reads than Sanger sequencing, but the throughput was massively improved. There is a 2.5th generation, which is the Pacific Bioscience (PacBio) sequencing, while the third is represented by the Oxford Nanopore method.

Illumina is the preferred method for DNA sequencing within the second-generation technologies, which can be explained by its properties and generated data. Such characteristics result from the utilization of polymerases able to perform a single-base primer extension through the addition of modified nucleotides (A, T, G, C+ specific fluorophore). The amplification of DNA strands occurs during submission to different temperature cycles while linked to reversible terminators. The fragments generated by Illumina present a relevant increase in size (up to 300 nt), accuracy (>99.9%), and number of reads per run (1.8 Tb), when compared with first-generation methods (Bently et al. 2008; McGinn and Gut 2013; van Dijk et al. 2014).

Despite the increase in read lengths, the fragments produced by Illumina are still considered short, which makes genome assembly more complex than with long reads. The assembly with short reads is made through the use of software and bioinformatics tools, which do not work well with repeats, thus paired reads have been used as a way to minimize the repeats problem. Although this approach helps to increase genome reconstruction quality, it is still common to notice the presence of gaps, errors in assembly, and noncharacterized regions of the genome (Bentley et al. 2008; Koren and Phillippy 2015). Moreover, there is a version of the technology called Illumina synthetic long-read sequencing (Molecule) that is able to generate longer reads (up to 18 kb), facilitating genome assembly. However, Molecule still produce a large amount of broken sequences (short-reads) that can lead to the formation of gaps in the genome (Koren and Phillippy 2015; van Dijk et al. 2014).

On the other hand, the 2.5th sequencing technology (PacBio) makes use of primer extension from an individual DNA molecule; thus DNA amplification is not

needed. The method consists in the use of a genetically modified DNA polymerase, fluorescent-labeled nucleotides, dideoxynucleotides, and a structure called zero-mode waveguide (ZMW). During sequencing, the enzyme is anchored to the ZMW, and the nucleotides pass through the ZMW. By the time that the correct nucleotide is linked to the DNA polymerase, its fluorescent label emits a color. This allows the determination of the nucleotide position within a DNA molecule, and it makes real-time sequencing possible (Edi et al. 2009; Mardis 2013; Schadt et al. 2010).

With the introduction of PacBio into the market, it was possible to obtain longer reads, with a length that went up to 20 kbp, which led to a simplified genome assembly process (Koren and Phillippy 2015; van Dijk et al. 2014). Nevertheless, the accuracy achieved by this method is approximately 85%, evidencing a higher error rate when compared with Illumina (McPherson 2014). Besides that, the maximum throughput achieved by this next-generation technology is low, just 500 Mb (van Dijk et al. 2014).

Considering the features of PacBio, it is correct to say that it came as a way to improve sequencing and also to reduce the amount of time required (3 h). However, the low throughput and accuracy associated with the long reads ends up raising the cost per Mb ($2–17). Summarizing, PacBio is more expensive than Illumina, which makes its use not viable for small industries or labs (van Dijk et al. 2014). Even presenting considerable disadvantages, the facilities in genome assembly due to the production of long reads turned the technology in question very appreciated and broadly used for genome sequencing.

7.2 Phylogenomics Applied to *B. thuringiensis* Taxonomy

The genus *Bacillus* is a large and diverse taxonomic group including a wide variety of bacteria that exploit organic and inorganic substrates (Ravel and Fraser 2005). This genus is a phylogenetically incoherent taxon as the members of the group lack a common evolutionary history (Bhandary et al. 2013). Traditionally, the bacteria comprised in this genus were classified into different species according to 16S or 23S rRNA gene sequences or 16S–23S rRNA spacer regions (Bavykin et al. 2004), DNA hybridization (DDH) (Priest 1981), rep-PCR (repetitive extragenic palindromic) (Cherif et al. 2003), and measures of phenotypic similarity as well as the presence or absence of virulent plasmids (Rasko et al. 2005). In general, these typical bacterial strain differentiation methods mask the real genetic diversity, since a high genetic similarity between strains is observed.

To overcome the lack of well-known features that may easily distinguish the genus *Bacillus* from other aerobic, spore-forming genera of the Bacillaceae family, Bhandary and coworkers, 2013, performed a comparative analysis for the identification of molecular markers in the form of conserved signature indels (i.e., insertions/deletions) or CSIs specific for the genus. CSIs are insertions or deletions within conserved regions of homologous proteins (Gao and Gupta 2012). Since the genus *Bacillus* is considered as being a monophyletic group, it would be expected that

ancestrally derived CSIs would be present in all *Bacillus* species. Nevertheless, no such marker was identified to link the various species as a taxonomic group. However, 11 molecular markers identified as CSIs were then found as specific for *Bacillus subtilis* and phylogenetically related species. Another six CSIs specific for the *B. cereus* clade and its associate species were also identified. Thus, the authors proposed that, in conjunction with phylogenetic studies, the various CSIs determined in their study might provide a more reliable molecular tool for the reorganization of the current *Bacillus* group into a more coherent taxonomic entity.

The *B. subtilis* clade, along with the *B. cereus* clade, represents the largest observed monophyletic groupings comprised of species from the genus *Bacillus*. *B. subtilis* is of great importance in microbial history as a model for Gram-positive bacteria and in the understanding of bacterial stress-resistant endospore formation (Harwood 1992; Logan and De Vos 2009; Zeigler 2011), being the type species of the genus *Bacillus*.

The *B. cereus* clade consists of closely related Gram-positive bacteria widespread in natural environments and that exhibits highly divergent pathogenic properties. Members of this clade have significant impact on human health, agriculture, and food industry (Rasko et al. 2005). The group consists of eight pathogenic and nonpathogenic bacterial species – *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis*. The last two species were classified in recent years, while the six remaining species were identified in the twentieth century. Despite the multiple species names, all these organisms can be considered members of a single species, regarding their low genetic diversity when analyzed by 16S rRNA sequencing (Daffonchio et al. 2003) and multilocus sequence typing (MLST) (Priest et al. 2004), which is a technique used to characterize microbial species using DNA sequences of internal fragments of multiple housekeeping genes.

Considering the importance of the *B. cereus* clade, the identification and taxonomy of the strains within the group have been extensively studied. Although a number of methods have been applied to ultimately differentiate species in the group, many of the techniques have not been able to fully meet this target. Yet, the understanding of the genetic diversity and phylogenetic relationships of the bacteria of the *B. cereus* group is crucial, as it is still under intense and controversial discussion (Helgason et al. 2000; Zwick et al. 2012).

Phylogenetic analysis based on virulence plasmids can play an important role in differentiating different species of this group relative to one another. Nevertheless, the classification based on virulence plasmids is clearly unsuitable, as they can be transferred or lost during evolution. Liu et al. (2015) report, for example, that some strains of *B. cereus*, a common cause of food poisoning, contained the pXO1 plasmid encoding all the primary virulence factors of *B. anthracis*, the causative agent of anthrax (Koehler 2009; Logan 2012). Additionally, insecticidal crystal protein genes (*cry*), which, in general, are naturally harbored by typical *B. thuringiensis* plasmids, were found scattered in the *B. cereus* clade. In terms of phylogenetic evolution, *B. thuringiensis* may become akin to *B. cereus* when its characteristic plasmids are lost (Helgason et al. 2000). On the contrary, a *B. cereus* strain may

present characteristic functional properties of *B. thuringiensis* or *B. anthracis* when it acquires plasmids of these species (Hu et al. 2005; Klee et al. 2010).

The development of next-generation sequencing technologies has largely increased the number of available genome sequences and provoked a shift in the methods used for delimiting bacterial species. Replacing classical DDH, digital DNA:DNA hybridization (dDDH) (Patil and McHardy 2013) is a modern technique based on the reliable Genome Blast Distance Phylogeny method (GBDP) (Auch et al. 2010; Meier-Kolthoff et al. 2013). In comparison with traditional DDH, in which estimated similarity values of 70% are applied as a standard for prokaryotic definition (Moore et al. 1987), dDDH estimates are yielded by Genome-to-Genome Distance Calculator (CGDC) instead of considering the average nucleotide identity. In this manner, nucleotide GBPD provides an opportunity to obtain precise DDH estimates for species delimitation together with phylogenies with statistical branch support in the same integrated approach (Meier-Kolthoff et al. 2013).

After a large-scale study, performed with 224 strains of the *B. cereus* group using whole genome sequences, Liu et al. (2015), proposed the division of the *cereus* group into 30 clusters, each representing independent species including 19–20 putative novel species. The authors also suggest that toxic gene-harboring plasmids such as pXO1 in *B. anthracis* and *cry* gene plasmids in *B. thuringiensis* cannot be signatures of either species. Indeed, after applying the dDDH approach, the authors concluded that some strains previously identified as *B. cereus* or *B. thuringiensis* were actually *B. anthracis*. These bacteria should, therefore, be paid a lot more attention when evaluating their biosafety, especially in the case of selecting highly toxic *B. thuringiensis* strains carrying *cry* genes for biopesticide production.

7.3 *B. thuringiensis* Pangenome

According to the distributed genome hypothesis (Hogg et al. 2007), a bacterial species has a supragenome or pangenome holding a gene pool for any naturally transformable strains to exchange some of their genes through mechanisms such as horizontal gene transfer (Nelson et al. 1999), to adapt to their dwelling environment. This concept is very helpful and supportive and often leads to a global comprehension about genetic content and variability for the species. The pangenome can be either "open" or "closed," depending on its capability of acquiring new genes (Medini et al. 2005). Theoretically and mathematically, an open pangenome can freely acquire genes into its sequence repository along with the addition of new isolates, while a closed pangenome stops accumulating new genes at a limited pangenomic content (Medini et al. 2005). As demonstrated by Fang et al. (2011), when comparing eight strains, the *B. thuringiensis* pangenome was found to have 4196 core genes. Compared to the pangenomes of its closely related species of the same genus, the *B. thuringiensis* pangenome shows an open characteristic, similar to *B. cereus* but not to *B. anthracis*. In this way, a robust pangenome study of

Bacillus thuringiensis would be of great help to achieve a deeper knowledge of its genes' gene content.

7.4 Genomics Applied to *B. thuringiensis* Toxin Discovery

B. thuringiensis is well known for its ability to produce crystal proteins (encoded by *cry* or *cyt* genes) that have toxicity against several organisms. These proteins are the basis for the development of bioinsecticides based upon *B. thuringiensis* for pest control. However, the emergence of insect resistance and the need to control other pests highlighted the importance of strengthening the search for novel toxins. PCR-based systems are the most widely used for the identification of novel *cry* genes (Berón et al. 2005; Noguera and Ibarra 2010), but high-throughput sequencing technology has been also employed in toxin discovery (Sampson et al. 2009; Ye et al. 2012; Wenfei et al. 2014; Palma et al. 2014; Rusconi et al. 2015).

Using a combination of high-throughput sequencing and bioinformatics analysis, Sampson et al. (2009) identified over 200 novel putative pesticidal proteins from *B. thuringiensis* strains, which were categorized into several protein families like (*i*) three-domain delta endotoxins (103 genes), (*ii*) Mtx-like proteins (27 genes), (*iii*) putative binary proteins (16 genes), (*iv*) Vip-like proteins (11 genes), and (*v*) others (60+ genes). Ye et al. (2012) designed a high-throughput system combining mixed plasmid-enriched genome (21 strains) sequencing and a bioinformatics pipeline (http://bcam.hzaubmb.org/BtToxin_scanner) for the identification of *cry* genes. A total of 113 candidate *cry* sequences were discovered from the 21 strains, and among them three potentially represent novel *cry* gene types. These results confirmed that high-throughput sequencing might accelerate the pace of *cry* gene discovery.

7.5 Concluding Remarks

Considering the studies performed to define the *Bacillus cereus* group relationships, the use of pan- and core genomes has helped scientists to have new insights about the bacteria that form this group. It also raised new questions about the relationships of these microorganisms in phylogenetic and phylogenomic level (genomics, proteomics, etc.). Therefore, it is possible to conclude that more research must be performed in order to comprehend the relationships between the three bacillus species discussed here and the reasons that led this group to present such diverse pathogenicity.

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Chapter 8
Expression of *Bacillus thuringiensis* Toxins in Insect Cells

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Abstract Baculoviruses and *Bacillus thuringiensis* (Bt) are biological control agents used for the control of agricultural insect pests and also insect vectors of human diseases. The expression of Cry proteins in insect cells using recombinant baculoviruses has been shown to be an alternative means of production of these proteins for functional and/or structural studies. The combination of the insecticidal activity of Bt and baculovirus lethal infection also has the potential to improve viral pathogenicity toward their insect hosts. The easy manipulation of baculovirus genomes and the increased number of full baculovirus genome sequences available could facilitate the expression of Cry proteins and, besides improving their pathogenicity, also retard the development of resistant insects to both Cry proteins and virus replication. In this chapter, the construction of recombinant baculoviruses containing different *cry* genes (*cry1, cry2, cry4, cry10*, and *cry11*) and the expression of the corresponding Cry proteins in insect cells and insect larvae are described.

Keywords Baculovirus • *Bacillus thuringiensis* • Cry protein • Insect cells

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8.1 Baculovirus

Baculoviruses are large dsDNA insect viruses which have been highly studied due to their insecticidal activity toward agricultural insect pests (Ribeiro and Crook 1998; Moscardi 1999) and also due to their use as expression vectors for heterologous proteins in insect cells and insects (Miller 1997). These viruses belong to the Baculoviridae family of insect viruses and are divided into four genera (alpha-, beta-, gamma-, and deltabaculoviruses) depending on the similarity of various conserved proteins (Jehle et al. 2006; Rohrmann 2013). These viruses are also known as nucleopolyhedroviruses (NPVs) and granuloviruses (GV), and their main characteristics are the production of large amounts of occlusion bodies (OBs) in insect cells at late postinfection times. The nucleopolyhedroviruses (alpha-, gamma-, and deltabaculoviruses) produce OBs in the nuclei of infected cells, and granuloviruses (betabaculoviruses) produce smaller OBs inside the cell's nucleus and cytoplasm (Rohrmann 2013).

The most studied baculovirus to date is the *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) which is the type species of NPVs and was isolated from the alfalfa looper, *Autographa californica* (Rohrmann 2013). Baculoviruses are named by the initials of the first insect where they were isolated. However, these viruses can infect more than one host (Rohrmann 2013).

During the replication of AcMNPV in insect cells, two viral phenotypes are produced: the extracellular (ECV) or budded (BV) virus is formed in the initial phase of infection and is responsible for the spread of infection inside the insect host and the occlusion-derived virus (ODV) is responsible for the spread of infection among insects and is formed at late postinfection times in the nuclei of infected cells. ODVs are surrounded by an occlusion body made mainly of a single polypeptide called polyhedrin, forming the OB (also known as polyhedra) (Smith and Summers 1978).

The regulation of AcMNPV gene expression in susceptible insect cells is mainly divided into genes expressed before viral DNA replication (early phase) and those expressed after the onset of viral DNA replication (late phase). These can be subdivided into immediate early phase, where viral genes are expressed in the first few hours after infection, and in very late phase, where genes are expressed after 18 h of infection (Maruniak 1986). The immediate early phase is characterized by the expression of viral transcriptional factors and genes responsible for preparing the cell for viral DNA replication (Friesen 1997). During the late phase, high amounts of proteins responsible for the production of BV particles are produced (Lu and Miller 1997). In the very late phase of infection, proteins involved in the production of OBs are highly expressed. For instance, polyhedrin, a 30 kDa protein, as cited above, is the main protein of the OBs and is highly produced in this phase of infection (Rohrmann 1986; Jarvis 1997). Due to its high expression, most baculovirus expression vectors were developed to use the polyhedrin promoter in order to express recombinant protein in insect cells (O'Reilly et al. 1992).

OBs are responsible for the transmission of ODVs from insect to insect. When a susceptible insect ingests OBs, the polyhedrin protein is solubilized in the alkaline

environment of the midgut, and the ODV particles are released (Funk et al. 1997). The released ODVs will cross the perithrophic membrane and infect the columnar cells of the midgut (Horton and Burand 1993; Haas-Stapleton et al. 2004). After entering the midgut, the virus can replicate in the midgut cell producing new BV particles or be transported to the basal side of the cell and directly infect tracheal cells or hemocytes spreading the infection to other tissues inside the insect body where the virus will replicate, and in a few days, the infected insect dies full of OBs that are released in the environment (Rohrmann 2013).

8.2 Baculovirus Expression Vectors (BEVs)

Expression of heterologous proteins in insect cells or insects using recombinant baculoviruses has several advantages compared to other expression systems. For instance, insect cells are eukaryotic cells with most of the posttranslational machinery for the correct processing of complex eukaryotic proteins, such as glycosylation (O'Reilly et al. 1992; Jarvis 1997, 2003). Strong promoters like the polyhedrin gene promoter ensure high expression level of most proteins (Datal et al. 2005). This technology is based on plasmid transfer vectors for the transfer of foreign genes to the genome of the baculovirus by homologous recombination or site-specific transposition (Rohrmann 2013), and several vectors are available commercially (Ribeiro et al. 2015).

8.3 *Bacillus thuringiensis*

Biological alternatives to chemical control of insect pests have received more attention in the last few years due to widespread presence of resistant insects, the lack of development of new molecules, and the increase in regulatory restrictions for their use worldwide (https://www.epa.gov/science-and-technology/pesticides-science). There is a high diversity of microorganisms with entomopathogenic activity, and they represent a valuable resource for the development of new biological products (Perlak et al. 1990; Lacey et al. 2015).

Among these microorganisms, the bacterium *Bacillus thuringiensis* (Bt), has been shown to be an excellent choice since it is used efficiently for the control of different insect pests worldwide for decades (Bravo et al. 2011). Bt is known to produce crystalline inclusion bodies composed of insecticidal proteins called δ-endotoxins (Cry and Cyt proteins) and secreted toxins called Vip and Sip (Donovan et al. 2006). These inclusions vary in size and composition and confer distinct entomopathogenic spectra on different strains of this bacterium. When ingested by susceptible insects, the δ-endotoxin crystalline inclusions are, like the occlusion bodies of baculoviruses, solubilized in the alkaline environment of the midgut and proteolytically processed by midgut proteases. The processed proteins attach to membrane

receptors at the midgut columnar cells and induce cell lysis and eventually insect death (Schnepf et al. 1998; Bravo et al. 2007).

Hundreds of δ-endotoxin (*cry* and *cyt*) genes have been sequenced to date, and Cry proteins are classified in 74 groups organized in different subgroups; besides three groups of Cyt proteins based in their amino acid identity and due to the development of new sequencing technologies, the number of novel *cry* genes is still growing (Crickmore et al. 1998, 2016).

Cry proteins have shown toxicity to different insects and have the potential to be used for the control of agricultural insect pests and insect vectors of human and animal disease. However, some Bt strains produce Cry proteins without known insecticidal activity but with toxic activity toward human cancer cells. These proteins were named parasporins, and so far, six classes of parasporin have been identified and are classified as parasporin-1 (PS1), parasporin-2 (PS2), parasporin-3 (PS3), parasporin-4 (PS4), parasporin-5 (PS5), and parasporin-6 (PS6) (Okumura et al. 2016). According to the classification of Cry proteins, parasporins are classified as Cry31A (PS1), Cry41A (PS3), Cry45A (PS4), Cry46A (PS2), Cry63A (PS6), and Cry64A (PS5) (Ohba et al. 2009; Okumura et al. 2016).

Besides Cry proteins, Bt produces and secretes other proteins with insecticidal activity during vegetative growth toxic to coleopteran, hemipteran, and lepidopteran insects that are called vegetative insecticidal proteins (VIP) which do not form crystalline inclusion bodies (Chakroun et al. 2016). Both *vip* and *cry* genes are located on large Bt plasmids (Estruch et al. 1996). Since VIP proteins are produced in the initial Bt growth phase, the mixture of spore, crystals, and supernatant of the culture can be used for the formulation of a bioinsecticide product (Monnerat and Bravo 2000). VIP proteins have shown toxic activity toward coleopteran (Vip1 e Vip2) and lepidopteran (Vip3) insects (Warren et al. 1998). No insecticidal toxic activity has yet been shown for the recently identified Vip4 proteins (Palma et al. 2014).

8.4 Baculovirus and *Bacillus thuringiensis*

8.4.1 Cry1 Proteins

Cry1 proteins are expressed as protoxins during Bt sporulation with a molecular mass of around 130 kDa, forming bipyramidal-shaped crystals. These protoxins are proteolytically processed at their N- and C-terminal ends within the midgut of a susceptible insect to a toxic fragment around half of the size of the protoxin (around 60–65 kDa).

The full-length Cry1Ab and Cry1Ac proteins were the first Cry proteins expressed in insect cells using recombinant baculoviruses (Merryweather et al. 1990; Martens et al. 1990; Ribeiro and Crook 1993, 1998). These proteins were shown to be toxic to lepidopteran larvae and were detected in the cytoplasm of infected cells, but only the Cry1Ab proteins were shown to form inclusion bodies in the cytoplasm of infected cells. However, no improvement of the recombinant

virus insecticidal activity was shown. Truncated versions of the Cry1Ab protein containing the active portion of the protein were also successfully expressed in insect cells using recombinant baculoviruses and shown to be biologically active (Ribeiro and Crook 1993; Martens et al. 1995). However, again no improvement of the viral insecticidal activity was shown. Other Cry1 proteins were also expressed in insect cells using recombinant baculoviruses. For instance, a truncated version of the Cry1C protein (N-terminal 804 amino acids and around 65 kDa) was expressed in insect cells and shown to be toxic to *Spodoptera frugiperda* and *Anticarsia gemmatalis* larvae. This protein also produced cuboid-shaped crystals in the cytoplasm of infected cells. However, the effect on viral pathogenicity was not determined (Aguiar et al. 2006). The full-length Cry1I protein also formed cuboidal-shaped crystals when expressed in insect cells using recombinant baculoviruses (Martins et al. 2008) and shown to be toxic to an important coleopteran pest, the cotton boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae).

8.4.2 Cry2 Proteins

Cry2 proteins form cuboid-shaped crystals in Bt and have a molecular mass between 65 and 71 kDa (Höfte and Whiteley 1989). Cry2Aa and Cry2Ac have known toxic activity to lepidopteran and dipteran insects, and Cry2Ab is known to be toxic only to lepidopteran insects (Höfte and Whiteley 1989; Widner and Whiteley 1989; Dankocsik et al. 1990). Although Cry2Aa and Cry2Ab share high amino acid identity, a few amino acid differences determine their difference in toxicity (Widner and Whiteley 1989). Another aspect of these proteins is the presence of accessory proteins which are necessary for the high level of expression and crystallization of these proteins in Bt (Crickmore and Ellar 1992). Since these proteins bind to different receptors than Cry1 proteins in susceptible lepidopteran insects, they are used to construct transgenic crops expressing two or more Bt toxins that kill the same insect pest in order to delay evolution of pest resistance (Hernandez-Rodriguez et al. 2009; Gouffon et al. 2011; Carrière et al. 2015).

Cry2Aa and Cry2Ab genes from a Brazilian *B. thuringiensis* subsp. *kurstaki* S447 (Btk) strain were separately inserted into the genome of a baculovirus, and the recombinant viruses were used to infect insect cells and larvae (Lima et al. 2008). Both proteins were expressed and shown to have a molecular mass of around 65 kDa in SDS-PAGE of insect cell extracts. No crystals were detected in cultured *S. frugiperda* cells (Sf21) infected by the recombinant viruses. However, cuboidal crystals similar to the ones produced by Bt were seen in larval extracts from *S. frugiperda* infected with the recombinant viruses. These crystals were significantly larger than the ones produced by Bt. This was also seen in other Cry proteins expressed in insect cells (Ribeiro and Crook 1993; Aguiar et al. 2006). The size of the recombinant protein might be dependent on the space for the crystal to grow inside the bacterial or insect cell and/or the presence of host proteins. Since the production of Cry2 crystals in Bt is dependent on the expression of accessory proteins that might

be acting as chaperones, the production of Cry2 crystals in insect larvae cells might be due to the presence of host insect cellular chaperons that are not present in the Sf21 cell line. Recombinant Cry2Aa and Cry2Ab produced in insect cells were tested against *Anticarsia gemmatalis* and *S. frugiperda* and showed similar LC_{50} when compared to the crystals produced by *B. thuringiensis* subsp. *kurstaki* HD-1 (Lima et al. 2008).

8.4.3 Bti *Cry Proteins*

Bacillus thuringiensis subsp. *israelensis* (Bti) is widely used as biological insecticide to control mosquito vectors of human diseases, such as *Culex* spp., *Aedes* spp., and *Anopheles* spp. (Höfte and Whiteley 1989; Schnepf et al. 1998; Beltrão and Silva-Filha 2007; Harwood et al. 2015). At least five Cry proteins can be found in parasporal inclusions in sporulating Bti cells. These proteinaceous crystals are composed of distinguishable classes of δ-endotoxins: Cry11Aa, Cry10Aa, Cry4Aa, Cry4Ba, Cyt1Aa, and Cyt2Ba. The genes responsible for the expression of the crystal-forming proteins are located on a 128 kDa plasmid, pBtoxis (Berry et al. 2002). Genes involved in the expression of auxiliary proteins, P19 and P20, which can function as chaperones and participate in crystal formation, are also present in this megaplasmid (Manasherob et al. 2001). Cry and Cyt proteins have been found to act synergistically in the midgut of mosquito larvae and on cultured mosquito cell lines (Thomas and Ellar 1983; Poncet et al. 1995; Corrêa et al. 2012a, b). Cyt proteins are toxic to mosquito larvae, although the toxicity achieved is usually lower than that observed for Cry proteins (Chang et al. 1993). It has been demonstrated, for example, that Cyt1Aa may act as a primary receptor for Cry11Aa, facilitating the formation of oligomers of this protein (Pérez et al. 2005). Experiments conducted with Cyt1Aa and Cry4Ba also showed interaction between these two proteins (Cantón et al. 2010). Cyt2Ba protein also showed some level of interaction with Cry11Aa and Cry4Aa (Corrêa et al. 2012a, b).

As part of efforts to obtain isolated mosquitocidal Cry toxins for toxicity studies, the *cry4Aa* and *cry4Ba* genes obtained from two Brazilian Bti strains (S1806 and S1989) were separately inserted into the baculovirus AcMNPV genome by homologous recombination or site-specific transposition (Corrêa et al. 2013). Overall, Cry4Aa and Cry4Ba are closely related toxins which exhibit a high level of amino acid sequence identity. However, their proteolytically activated N-terminal portions show only around 55% of identity (Chungiatupornchai et al. 1988). While Cry4Aa is highly toxic to *Culex* and *Aedes*, its activity against *Anopheles* is low. On the other hand, Cry4Ba shows strong toxicity to *Aedes* and *Anopheles*, but *Culex* larvae are less susceptible to this toxin.

The recombinant baculoviruses produced Cry4Aa or Cry4Ba in insect cells (Corrêa et al. 2013). However, cytoplasmic Cry4Aa crystals from the recombinant

virus constructed using the site-specific transposition method seemed to be two- to threefold larger than those produced by homologous recombination. A plausible explanation for this difference in size may reside in the fact that the first recombinant virus lacked the polyhedrin gene. Thus, the synthesis of Cry4Aa crystals may be enhanced as the protein expression machinery of infected cells is not busy producing large amounts of polyhedrin. Morphological differences were observed between Cry4Aa and Cry4Ba crystals expressed in insect or in Bt cells. Cuboidal- or bipyramidal-shaped crystals were formed in insect cells, while spherical crystals are expressed in Bt cells (Schnepf et al. 1998). This structural difference can be related to interactions of heterologous Cry proteins with cellular proteins. Bioassays with Cry4Aa and Cry4Ba expressed in *S. frugiperda* larvae were performed with second instar *A. aegypti* larvae and showed that these proteins were toxic to this insect. Cry4Aa toxins produced by recombinant viruses constructed by either homologous recombination or transposition were equally toxic to *A. aegypti* with no significant difference in LC_{50} (Corrêa et al. 2013).

Cry10Aa is also a toxin produced by *B. thuringiensis* subsp. *israelensis* as a protoxin of around 80 kDa, which is proteolytically cleaved by insect gut proteases to a toxic fragment of around 60 kDa. Among the δ-endotoxins produced by Bti, Cry10Aa seem to be expressed in low levels and show no relevant toxic activity toward different mosquito species (Hughes et al. 2005; Hernández-Soto et al. 2009). The mosquitocidal activity of Bti was shown to be due primarily to the Cry4, Cry11, and Cyt proteins (Martins et al. 2007).

In order to test the toxicity of Cry10Aa against other insect species, Cry10Aa from a Brazilian *B. thuringiensis israelensis* strain (S1804) was expressed in insect cells using a recombinant baculovirus (Aguiar et al. 2012) and tested against *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), an important cotton pest. In contrast to the spherical crystals produced by the S1804 strain, the expressed Cry10A protein was produced as a cuboidal-shaped crystal in insect cells and shown to be highly toxic (LC_{50} of 7.12 μg/mL) to neonate larvae of *A. grandis* when compared to Cry1I (LC_{50} 21.5 μg/mL) or Cry1B (305.32 μg/mL) (Martins et al. 2010). These results indicate the potential of this protein to be used for the construction of transgenic plants for the control of this important insect pest.

Cry11A is another Bti mosquitocidal δ-endotoxin that has been expressed in insect cells by a recombinant baculovirus. Cry11A is the most toxic Bti toxin to *A. aegypti*. It has a molecular mass of 65 kDa, and since it is a "truncated" protein lacking the C-terminal portion found in 130 kDa Cry proteins, Cry11A crystals are formed in Bti cells with the help of two auxiliary proteins, P19 and P20 (Agaisse and Lereclus 1994; Yamagiwa et al. 2002). The *cry11A* gene, obtained from the Brazilian Bti strain S1989, was used to generate a recombinant baculovirus by site-specific transposition (Lima 2009). Crystals of Cry11A were able to form in *S. frugiperda* larvae in the absence of P19 and P20 and shown to be toxic to second instar *A. aegypti* larvae with an estimated LC_{50} of 53.3 ng/ml (Lima 2009).

8.4.4 *Polyhedrin/Cry Fusion*

Chang et al. (2003) constructed a recombinant baculovirus containing a fused gene comprised of the polyhedrin gene, an 1836 bp DNA fragment coding for the N-terminal toxic region of the Cry1Ac protein of Bt subsp. *kurstaki* HD-73 and the green fluorescent protein. This recombinant virus also contained an extra copy of the polyhedrin gene. The fused protein was shown to be expressed and incorporated into the OBs. These OBs were then shown to be highly toxic toward *Plutella xylostella* larvae. This recombinant virus showed a dramatic improvement on the virus pathogenicity since it was able to kill infected larvae by replication of the virus and also by the Cry1Ac toxicity, which should, in theory, reduce the chance of development of resistant insects to both Cry1Ac toxin and the baculovirus.

8.5 Concluding Remarks

The expression of Cry proteins in insect cells using recombinant baculoviruses has been shown to be an alternative to the production of these proteins for functional or structural studies (Table 8.1). The combination of the toxic activity of these proteins and the infectivity of these viruses to different insect species is still not widely used. The genomes of more than 73 baculovirus species have been sequenced to date (Ardisson-Araújo et al. 2016), but only the AcMNPV genome has been genetically

Table 8.1 List of *cry* genes inserted into the genome of a baculovirus and their target insects that showed susceptibility to the expressed toxin

Genes *cry*	Target insect	Reference
cry1Aa	*H. virescens*	Ribeiro and Crook (1993, 1998)
cry1Ab	*H. virescens*	Ribeiro and Crook (1993, 1998)
cry1Ac	*P. xylostella*	Chang et al. (2003)
cry1Ac	*H. virescens*	Ribeiro and Crook (1993, 1998)
cry1Ca	*S. frugiperda and A. gemmatalis*	Aguiar et al. (2006)
cry1Ia	*Anthonomus grandis*	Martins et al. (2008)
cry2Aa	*S. frugiperda and A. gemmatalis*	Lima et al. (2008)
cry2Ab	*S. frugiperda and A. gemmatalis*	Lima et al. (2008)
cry10Aa	*Anthonomus grandis*	Aguiar et al. (2012)
cry4Aa	*A. aegypti and C. quinquefasciatus*	Corrêa et al. (2013)
cry4Ba	*A. aegypti and C. quinquefasciatus*	Corrêa et al. (2013)
cry1Ab	*Pieris brassicae*	Martens et al. (1990)
cry1Ac	*Trichoplusia ni*	Merryweather et al. (1990)
cry1Ab	*Spodoptera exigua*	Martens et al. (1995)
cry11A	*A. aegypti*	Pang et al. (1992), Lima (2009)

manipulated to harbor *cry* genes. Therefore, other baculoviruses might be used for the expression of Cry proteins in order to improve their pathogenicity toward different insect species and also retarding the development of resistant insects to both Cry proteins and virus replication.

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Chapter 9
Bacillus thuringiensis: Different Targets and Interactions

Lidia Mariana Fiuza, Diouneia Lisiane Berlitz, Jaime Vargas de Oliveira, and Neiva Knaak

Abstract In the microbial control of pests, the entomopathogen *Bacillus thuringiensis* offers the best biological alternative to chemical insecticides, either alone or in combination with other methods of field control, and is also a source of genes for the genetic engineering of plants. In this work, aspects related to new targets of this bacterium are described such as: *Acromyrmex* spp.; *Nasutitermes ehrhardt*; *Euschistus heros*; *Oryzophagus oryzae; Blatella germanica*; *Pyricularia grisea*, *Rhizoctonia solani*, *Fusarium oxysporum*; *Fusarium solani* and *Meloidogyne* spp. Also discussed are the interactions of *Bacillus thuringiensis* and *B. subtilis* with other biological control agents: *Purpureocillium lilacinus*; *Campoletis flavicincta*; *Nuclear Polyhedrosis Virus*; plant extracts and essential oils from medicinal plants. Data from our research group of Microbiology and Toxicology in Agroecosystems (MToxAgro/CNPq), as well as collaborating researchers of some public and private institutions of Brazil will be presented.

Keywords Bacteria • Entomopathogens • Target pests • Agroecosystems • Biopesticide interactions • Biological control

Phytosanitary treatments for the control of various pests are routine in agricultural crops. On the other hand, in the last decade awareness about the misuse of chemical pesticides and their problems in agroecosystems and human health has increased. As a result, the development of alternative methods and products for pest control has

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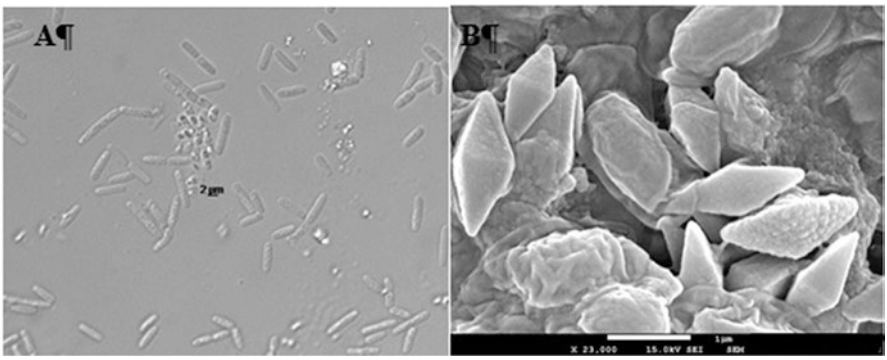

Fig. 9.1 *Bacillus thuringiensis* in differential interference contrast microscopy (**a**) and scanning electron microscopy (**b**)

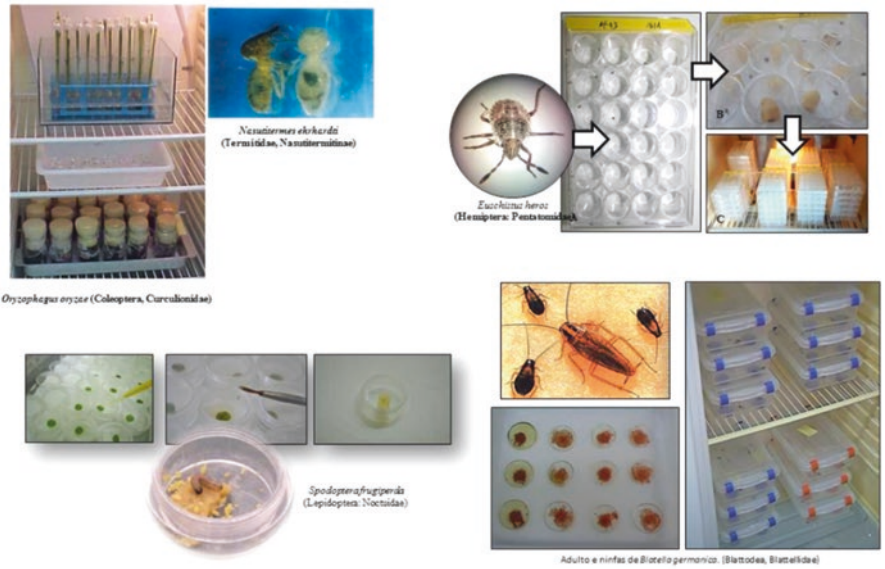

Fig. 9.2 Bioassays performed with target insects: *Nasutitermes ehrhardti*, *Oryzophagus oryzae*, *Euschistus heros*, *Spodoptera frugiperda* and *Blatella germanica*

increased. In integrated pest management, researchers have prioritized the application of natural enemies (parasitoids and predators), plant extracts and entomopathogenic microorganisms, such as the Gram-positive bacterium *Bacillus thuringiensis* (Fig. 9.1).

One way to increase the effectiveness, and the spectrum of action, of entomopathogens such as *B. thuringiensis* is the joint application with other biopesticides, chemical pesticides or natural enemies. These interactions may also act as stressing factors of the insect that cause infectious diseases and increase the susceptibility of the pests to *B. thuringiensis*. In this context, data presented here correspond to the new targets of *Bacillus* spp. (Fig. 9.2), with emphasis on *B. thuringiensis* and interactions with other biological control methods.

9.1 *Bacillus thuringiensis* and Leaf-Cutting Ants of the Genus *Acromyrmex*

The concern with the control of leaf-cutting ants of the genus *Acromyrmex* is constant in many agroecosystems, because they cause great damages in several crop fields. The most used control method to minimize the negative effects of these social insects is the application of insecticides with high residual power, which can cause several environmental problems. This type of management causes contamination throughout the food chain, since leaf-cutting ants are very important for the ecosystem (Spier et al. 2013). Thus, the search for satisfactory alternatives of control and that cause less environmental impact is paramount.

In this case, considering the pesticidal potential of *B. thuringiensis*, characterized by the production of toxins effective for several orders of insects, but with limited information on toxicity to Hymenoptera, we highlight the pioneering research by Pinto et al. (2003) that isolated *B. thuringiensis* from two species of ants that occur in southern Brazil, *Acromyrmex crassispinus* and *A. lundi*.

Native *B. thuringiensis* isolates obtained from leaf-cutting ants (group – MToxAgro/CNPq), by PCR with specific primers for some families of *cry* genes, were evaluated for pathogenicity in *A. lundi*, in laboratory. In this research, 14 isolates of *B. thuringiensis* were obtained from *Acromyrmex* spp. PCR data from *B. thuringiensis* isolates revealed amplification of DNA fragments corresponding to *cry1* genes in 22% of isolates and *cry9* in 67%. The genes *cry2*, *cry3*, *cry7* and *cry8* were not detected in the samples tested and 22% of the isolates did not amplify DNA fragments corresponding to any of the *cry* genes evaluated. In the in vivo assays, with Bt-HA03 (absence of *cry* gene), Bt-HA58 (*cry1* gene) and Bt-HA48 (*cry9* gene) isolates, a mortality exceeding 50% of the target population was observed. In this study, the authors found promising results, both in the identification of the genes present in the new isolates, and in the assays for determination of the LC_{50} of Bt-HA48, which has potential application in the biological control of leaf-cutting ants.

9.2 *Bacillus thuringiensis* Against *Nasutitermes ehrhardti* (Termitidae, Nasutitermitinae)

Termites are also social insects present in almost all warm terrestrial environments which feed on cellulose. Several species play significant ecological roles and participate in the regeneration of disturbed environments. However, some species are responsible for large losses in forests, pastures, crop fields and cities. These insects are difficult to control because of the complexity of their life cycle and behavior.

In this line of research, Castilhos-Fortes et al. (2002) evaluated the potential of 55 serotypes of *B. thuringiensis* (*Bt* serotypes provided by the Institut Pasteur, Paris) against *N. ehrhardti* and among these, seven serotypes were pathogenic: *B. thuringiensis* subsp. *sooncheon* (Bts) and *B. thuringiensis* subsp. *roskildiensis* (Btr) with 100% mortality, followed by *B. thuringiensis* subsp. *yunnanensis* (Bty) with 71.4%, *B. thuringiensis* subsp. *huazhongiensis* (Bth) with 57.1%, *B. thuringiensis* subsp.

brasiliensis (Btb) with 52.3%, *B. thuringiensis* subsp. *colmeri* (Btc) with 42.85% *and B. thuringiensis* subsp. *kurstaki* (Btk) with 28.57% mortality on the seventh day after application of treatments.

The serotypes *B. thuringiensis* subsp. *sooncheon* and *B. thuringiensis* subsp. *roskildiensis*, which caused 100 % mortality during pre-selective trials, were used to determine the LC_{50}. The LC_{50} data, 7 days after application of the treatments (DAT), showed 5.14×10^5 cells/ml for *B. thuringiensis* subsp. *sooncheon* and 4.84×10^7 cells/ml for *B. thuringiensis* subsp. *roskildiensis*. The pathogenicity of *B. thuringiensis* to *N. ehrhardti* workers and soldiers was confirmed by bacterial presence in the intestines of termites submitted to treatments, which were observed under phase contrast microscopy.

9.3 *Bacillus thuringiensis* in *Euschistus heros* (Hemiptera: Pentatomidae)

The nymphs and adults of Hemiptera are sucking pests which attack different parts of plants, this hinders the use of microbial biopesticides, such as the current formulations based on *B. thuringiensis* that act upon ingestion. In Brazil, production losses caused by Pentatomidae in monocultures such as soybeans are difficult to calculate because these pest insects occur locally in certain regions and do not show dispersion throughout the Brazilian territory. On the other hand, the emergence of large populations of stinkbugs has been causing concern to farmers, since they are already considered a complex of pests of critical importance with major impacts on the main world monocultures, particularly on soybeans (Chougule and Bonning 2012).

In the control of sucking pests, the application of *B. thuringiensis* toxins has been investigated through the use of *cry* genes in the genetic transformation of plants, but little is known about the effect of these toxins on pentanomids. Some studies have investigated the effect of Cry toxins on non-target arthropods of *Bt* plants (Bell et al. 2005; Cunha et al. 2012).

In the work of Schünemann (2015), the ingestion of Cry proteins, biopesticides based on *B. thuringiensis* and *Bt* soybean was evaluated in laboratory to observe their possible impacts in the development of the pentatomid *Euschistus heros*. In their bioassays, 12 second-instar nymphs of *E. heros* were used for each experiment, with five replicates. The insects were deprived of food for 16 h and then individualized in mini acrylic plates. The incorporation of the treatments was performed with 2 µl of *B. thuringiensis* toxins per seed, which was offered directly to each nymph in the mini-plate. After 2 h, each insect received a soybean grain without treatment, previously dipped in distilled water for 24 h. The seeds were replaced every 2 days in treatments containing surviving *E. heros* nymphs. Mortality was evaluated until the seventh day after application.

The data show that the *B. thuringiensis* strain MTox144–9, obtained from the research group of MToxAgro/CNPq, which contains the Cry1, Cry2 and Cry9 proteins, was applied at a concentration of 560 µg/seed. The highest corrected mortality obtained with this strain was 40%, not differing significantly from the other treatments with commercial biopesticides.

9.4 *Bacillus thuringiensis* and *Melia azedarach* Against *Oryzophagus oryzae* (Coleoptera, Curculionidae) and *Spodoptera frugiperda* (Lepidoptera, Noctuidae)

In South America, more specifically in southern Brazil, irrigated rice cultivation has great economic value. In the case of economic losses caused by insect pests, the polyphagous caterpillar *Spodoptera frugiperda* stands out in the initial phase and the aquatic weevil (*Oryzophagus oryzae*) infests the rice fields after flooding of plots, whose adults scrape the leaves and the larvae attack the roots, reducing up to 1.5% in early cultivars. In the control of *S. frugiperda* caterpillars, low cost products are usually used in the initial phase, already mixed with the herbicides. In the case of the coleopteran pest, access to the larvae living in the root system is difficult, causing farmers to provide a preventive seed treatment system, according to area history. As these control measures are not in line with sustainable production and environmental conservation practices, researchers and technicians of irrigated rice (EEA/IRGA, EPAGRI and EMBRAPA) are looking for integrated methods of biopesticides applied in the management of pests in irrigated rice.

In biological control, several researchers have focused on the use of *B. thuringiensis* based on the specificity of Cry proteins to the target insects. Also in the line of botanical insecticides, different plants have been studied and applied in pest control. These plants, over time, have developed sophisticated defense mechanisms against herbivores, pathogens and other stressors. Among the toxins produced by plants, there are nitrogenous substances such as non-protein amino acids, cyanogenic glycosides, some peptides and proteins, and various alkaloids. However, the toxicity of a substance is relative to the dose applied per insect, age, mechanism of absorption and mode of excretion (Saito and Lucchini 1998).

Among the plants with insecticidal properties, the chinaberry, *Melia azedarach* (Berlitz and Fiuza 2006), is active against different species of insects, causing behavioral changes, acting as a feed inhibitor, growth retardant, fertility reducer, and causing morphogenetic and feeding changes and death of insects (Carpinella et al. 2003; Breuer et al. 2003). This plant is referenced as an insecticide to Curculionidae such as *Sitophilus oryzae*, *Pantomorus leucoloma* and *Anthonomus grandis* (Fernandes et al. 1996) and to Noctuidae such as *S. frugiperda* and *Anticarsia gemmatalis* (Breuer et al. 2003; Carpinella et al. 2003). In addition, the chinaberry belongs to the family Meliaceae along with neem (*Azadiracta indica*), which produces chemical derivatives that act against insects by contact or ingestion, affecting growth regulating actions and/or provoking repellency, food inhibition and oviposition reduction (Vendramin 2002). Commercialization of different products based on neem oil currently stands out as an effective solution.

Berlitz et al. (2012) evaluated the pathogenicity of two strains of *B. thuringiensis* (Bt-MTox2014-2 and Bt-MTox-1958-2), obtained from the research group of MToxAgro/CNPq, against second-instar larvae of *S. frugiperda* and second and third-instar larvae of *O. oryzae* collected in experimental plots of EEA-IRGA. The same treatments were carried out with aqueous extract of the leaves (10%) of *M. azedarach*. The trials were evaluated 7 days after application of the treatments and

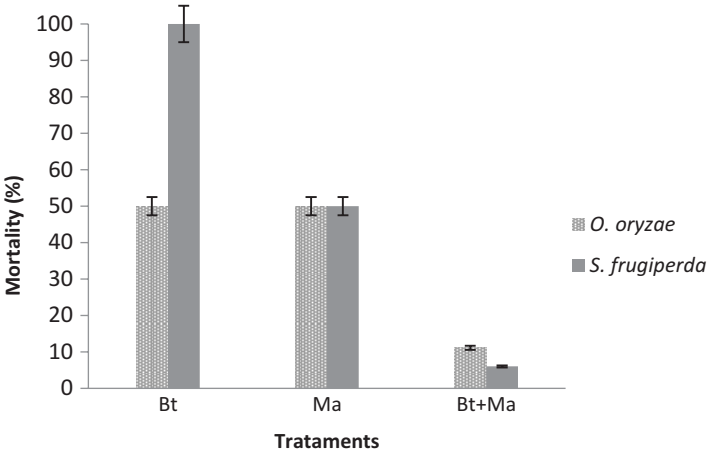

Fig. 9.3 Corrected mortality of *Oryzophagus oryzae* and *Spodoptera frugiperda* larvae treated with *Bacillus thuringiensis* isolates (*Bt*) and *Melia azedarach* aqueous extract (*Ma*)

the corrected mortality of *S. frugiperda* caterpillars was 100% for BtMTox 1958-2 (Cry1 and Cry2 proteins, Fig. 9.3). For *O. oryzae* larvae, the corrected mortality was 50% for the strain Bt-MTox 2014–2 (Cry3 proteins). The extract of *M. azedarach* was found to be toxic to the target pest under study, which may be associated especially in the production of meliacarpine by the leaves of *M. azedarach*. On the other hand, when the *B. thuringiensis* strains were associated with the plant extract under study, a reduction in the mortality of the insects was observed, indicating an antagonism of the biopesticides, as shown in the figure below.

9.5 *Bacillus thuringiensis* Against *Blatella germanica* (Blattodea, Blattellidae)

Among the many urban pests, cockroaches are among the most common insects found in human environs, especially *Blatella germanica*, which spends 75% of the time sheltered next to food. To reduce the application of chemicals with high residual power in the peridomiciliary sites, researchers look for new biological products with action of repellency and lethal effect. Hübner (2004) evaluated, under laboratory conditions, the pathogenicity of *Bacillus thuringiensis* (*Bt*) serotypes, provided by the Institut Pasteur (Paris), on the cockroach *B. germanica*. Five serotypes were used: *Bt colmeri*, *Bt yunnanensis*, *Bt huazhangiensis*, *Bt roskildiensis* and *Bt sooncheon*, at a concentration of 1.10^{10} cells/ml, for acute dose mortality after 7 days of application.

The corrected mortality for *B. germanica* on the seventh day after application of *Bacillus thuringiensis* serotypes were: 6.65% – *Bt colmeri*, 14.85% – *Bt yunnanensis*, 15% – *Bt huazhangiensis*, 16.65% – *Bt roskildiensis* and 30% – *Bt sooncheon*. The authors' data show that the serotypes under study were pathogenic to the target species,

but that high doses and purification of the toxic peptides were required in order to obtain economic viability for the application of *B. thuringiensis* in urban areas infested by these pests, while reducing insect populations and environmental impacts.

9.6 *Bacillus thuringiensis* Against Phytopathogens

In biological control, disease is not only the interaction between pathogen and host, but the result of the interaction between pathogen, host and a series of nonpathogenic microorganisms that also rest in the site of infection. These non-pathogens may limit or increase pathogen activity, or host resistance. The success of biocontrol depends on antagonistic properties, that is, on the interaction of one microorganism with others creating unfavorable conditions for their development (Bettiol 1991). They can be divided into antibiosis, competition, parasitism, predation and induction of host defense. Despite this division, it is considered as a suitable antagonistic characteristic to present more than one mechanism, increasing their chances of success.

There are several diseases caused by phytopathogens, mainly in irrigated rice. Knaak et al. (2007) evaluated the strains *Bacillus thuringiensis thuringiensis* 407 and *B. thuringiensis kurstaki* HD-73 (provided by the Institut Pasteur, Paris), which synthesized Cry1Ab and Cry1Ac, respectively, in phytopathogenic fungi *Pyricularia grisea*, *Rhizoctonia solani*, *Fusarium oxysporum* and *F. solani*, using the Kirby-Bauer method.

These authors verified that the strains of *B. thuringiensis thuringiensis* and *B thuringiensis kurstaki* significantly inhibited the mycelial growth of the phytopathogens evaluated when compared to the control (Fig. 9.4). However, the proteins at the evaluated concentrations had no effect on the control of the tested fungi.

The inhibitory effect of the *B. thuringiensis* strains on phytopathogenic fungi may be associated with the production of enzymes, which act against the fungal cell wall, since some antagonistic bacteria produce chitinases (Mavingui and Heulin 1994; Asaka and Shoda 1996).

9.7 *Bacillus thuringiensis* and *B. subtilis* Against Phytonematoids

In relation to pests, phytonematoids stand out for their economic importance, mainly in legumes (soybean) and grasses (maize). The main pest species are distributed in four genera: *Heterodera*, *Meloidogyne*, *Pratylenchus* and *Globodera* (Moens and Perry 2009; Davis and Curty 2011). In the case of the genus *Meloidogyne*, the main species, *M. incognita* and *M. javanica*, particularly attack legumes and fruit trees, preferring areas of tropical and subtropical climate (Collange et al. 2011).

Several authors (Vovlas et al. 2005; Elling 2013; Castagnone-Sereno et al. 2013) report that *Meloidogyne* species identify host plants through chemical factors, associated with temperature and humidity. Second-stage juveniles (J2), which are in the soil, penetrate the roots of the plants through the region of the root elongation, migrating to the apoplast, reaching the vascular cylinder and forming “giant cells”.

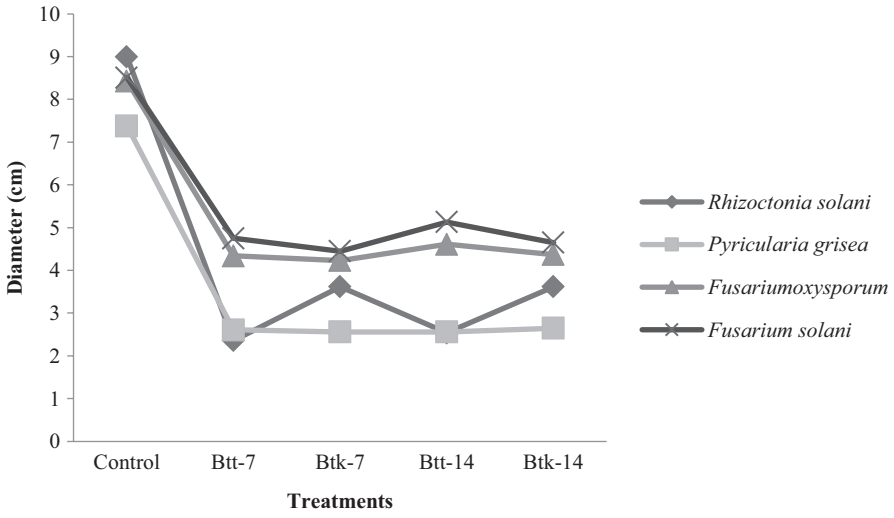

Fig. 9.4 Mycelial growth diameter (cm) of phytopathogenic fungi from the irrigated rice crop in the presence of *Bacillus thuringiensis* strains. *Btt-7 Bacillus thuringiensis thuringiensis* 7 days after the application of the treatments (DAT), *Btk-7 B. thuringiensis kurstaki* – 7 DAT, *Btt-14 B. thuringiensis thuringiensis* – 14 days after the application of the treatments (DAT), *Btk-14 B. thuringiensis kurstaki* – 14 DAT

In this place, the juveniles perform two molts (J3, J4) and in the last, which corresponds to the adult, the females become sedentary and the males can return to the soil. At this point, the females lay a mass of eggs enveloped by a gelatinous matrix. This formation, together with the modification of the root cells, impairs the absorption of water and nutrients from the soil by the plant, reducing the final production. According to Rosso et al. (1999), nematode stylet secretions, produced by the esophageal glands, are crucial factors for the penetration, migration and formation of feeding sites. In addition, secretory enzymes such as pectinases and cellulases are involved in the softening of the root cell wall, facilitating the penetration and migration of nematodes (Rosso et al. 1999; Doyle and Lambert 2002).

Phytonematodes cause losses that vary from the imperceptible to the death of a great number of plants, resulting in the unfeasibility of some crops. These phytopathogens have a wide range of hosts and are difficult to control because they survive for long periods in soil or in cultural remains, which can be easily disseminated by agricultural implements, animals, water (irrigation) and plant material (seedlings and seeds). So far, the main method of controlling these pests is through the use of nematicides with high economic and environmental costs, especially due to accumulation in soil and water.

Currently, research on the biological control of nematodes is focused on *Bacillus*, where *B. thuringiensis* and *B. subtilis* are prominent. In the case of the research developed by Berlitz (2014) with *B. thuringiensis*, strains from the research group of MToxAgro/CNPq were used: Bt-MTox 1886-2; Bt-MTox 3146-3; Bt-MTox 3434-2; Bt-MTox; 2974-11, as well as Bs-MTox 1556-5. The *B. subtilis* strains CCGB LFB 117; CCGB LFB 757, provided by IOC/FIOCRUZ were used as standards. For

bacterial bioassays in laboratory, 10 second-stage juveniles of (J2) *M. incognita*, were put on Elisa plates and five concentrations of *B. thuringiensis* strains (1×10^{10} to 1×10^{6} cells/ml) were added with 3 replicates/treatment, and evaluated 24 h after the application, totaling 180 individuals. Mortality was corrected by Abbott's formula and the Mean Lethal Concentration (LC_{50}) was determined by Probit Analysis. In greenhouses trials were carried out on lettuce plants (*Lactuca sativa*), with 5 ml of *M. javanica* at the concentration of 2000 eggs and J2/ml and 5 ml of each treatment, with 1×10^{9} cells/ml of Bs-MTox 1556-5, Bt-MTox 1886-2, CCGB LFB 117 and CCGB LFB 757, totaling 5 replicates. After 60 days the weight of the aerial part, the root, the number of galls and the number of eggs and J2/root system of the plants were evaluated. Statistical analyzes were performed with SPSS software, using the LSD test at 5% probability. The results of assays with *B. thuringiensis* in the laboratory showed LC_{50} of 0.33×10^{8} cells/ml; 2×10^{8} cells/ml; 2.1×10^{8} cells/ml and 2.6×10^{7} cells/ml for Bt-MTox 3146-3, Bt-MTox 3434-2, Bt-MTox 2974-11 and Bt-MTox 1886-2, respectively. The strain Bt-MTox 1886-2 was also used in greenhouse tests with lettuce plants and reduced by 36% the infestation of *M. javanica* in the roots of the plants when compared to the control (Berlitz et al. 2013). Structural and ultrastructural analyzes of the spore-crystal complex of the *B. thuringiensis* strains revealed the presence of bipyramidal and spherical protein crystals. PCR analysis for Bt-MTox 1886-2 and Bt-MTox 2974-11 identified a *cry1D* like gene.

Other authors also report the toxic action of *B. thuringiensis* on nematodes of the genus *Meloidogyne*, such as Ashoub and Amara (2010), El-Moneim and Massoud (2009), and Joo et al. (2012). More recently, Ravari and Moghaddam (2015) evaluated a *B. thuringiensis* strain with the *cry14* gene on *M. javanica* in greenhouses, which presented a 51% decrease in gall formation in tomato plants treated with the bacterium. Likewise, Yu et al. (2015) identified the inhibition of egg hatching, motility, and decreased plant penetration capacity of *M. hapla* treated with the mixture of spores and crystals of *B. thuringiensis* containing the *cry6Aa2* gene. As for the mode of action of *B. thuringiensis* proteins in phytonematoids, there is a comparison with the action in the midgut of insects, as the authors Wei et al. (2003) present data indicative of nematode intoxication such as constriction and thinning of intestinal cells, wall retraction and degenerative appearance of cells.

9.8 Interaction of *Bacillus subtilis* and *Purpureocilium lilacinus* Against *Meloidogyne javanica* (Tylenchida, Meloidogynidae)

Different bacterial species have nematicidal properties that affect phytonutrient development through the production of enzymes (Siddiqui and Mahmood 1999; Tian et al. 2007). A bacterial species with importance for both the biological control of nematode, and the promotion of plant growth is *B. subtilis* (Cohn 1872). This bacterium produces different compounds such as zwittermicin-A and kanosamine antibiotics, lipopeptides, antifungal proteins, and about 70 types of antibiotics

(Leifert et al. 1995; Pal-Bais et al. 2004; Todorova and Kozhuharova 2010). These compounds, in the soil, interfere in the reproductive cycle of the nematodes, acting on the orientation of the larvae towards the host plant (Sharma and Gomes 1996). *B. subtilis* is also indicated as a plant growth promoter and in the control of other phytopathogenic microorganisms, such as the fungus *Gauemannomyces graminis* var. *tritici* in wheat (Mariano et al. 2004), and *Rhizoctonia solani* and *Colletotrichum truncatum* in soybean (Araújo et al. 2005).

In addition to bacteria, some strains of fungi are producers of substances that inhibit the hatching of nematode eggs or lead to the death of their juvenile stage (Khan and Saxena 1997; Nitao et al. 1999). The fungus *Purpureocillium lilacinus* produces enzymes such as serine proteases and chitinases, acting on the eggs and juvenile stages of nematodes of the genus *Meloidogyne* (Khan et al. 2004; Lamovsek et al. 2013). Currently two commercial products based on *P. lilacinus* are indicated for *Meloidogyne* sp.: Bioact® WG and PL Gold®, from the companies Bayer and BASF respectively (Lamovsek et al. 2013).

In the context of recent researches, Berlitz et al. (2016), evaluated the interaction of *B. subtilis* and *P. lilacinus* on *M. javanica* on infected lettuce plants under greenhouse conditions. The simultaneous use of the two biocontrol agents resulted in a reduction of 90% in the number of eggs and juveniles (J2) and, consequently, a decrease in the number of galls formed in the roots of the plants, compared to the control. The authors infer that the mode of action of the microorganisms is related to the egg mass formation and the fertility of the nematodes.

Throughout evolution the phytonematoids have developed advanced mechanisms of colonization of the host plants. Mitchum et al. (2012) indicate that *Meloidogyne* species alter the cells inside the roots of the plants, so as to form complex feeding structures, followed by phytohormone modifications and the expression of the genes associated with the growth and development of the plants. In addition, the evolutionary success of phytonematoids may be related to the need for survival during long periods in the absence of the host, resulting in behavioral and physiological adaptations of great importance.

9.9 Interaction of *Bacillus thuringiensis* with *Campoletis flavicincta (Hymenoptera: Ichneumonidae)* in *Spodoptera frugiperda*

Ecosystems, in general, constitute a complex of trophic interactions between living beings. In agroecosystems, tri-trophic interactions have been evaluated for their feasibility of control methods that can be used simultaneously in Integrated Pest Management in the field, as well as the simultaneous application of host plants/insecticides (*Bt* plants) and parasitoids of caterpillars. In this sense, the research group of MToxAgro/CNPq has as main target for the screening of new strains of *B. thuringiensis* against the polyphagous insect *Spodoptera frugiperda*, which presents a natural resistance to said entomopathogen. In this way, in vitro and in vivo studies

on the tritrophic interactions with the caterpillar parasitoid, *Campoletis flavicincta*, were performed.

The in vivo study performed in laboratory by Dequesh et al. (2005) evaluated the interactions of *S. frugiperda* parasitized by *C. flavicincta* and fed with *B. thuringiensis aizawai*. This interaction caused mortality higher than 95% of the caterpillars, besides reducing the leaf consumption of this insect-pest. In addition, the biological characteristics of the parasitoid were not affected as a result of feeding the caterpillars with the bacteria, showing a safe and efficient method for simultaneous application.

In another study, in vitro, Dequesh et al. (2007) performed histopathological evaluations of the gut of *S. frugiperda* with the same interactions described previously, showing that there were no morphological changes in the eggs of *C. flavicincta* due to feeding with *B. thuringiensis*. This factor may be related to the specific mode of action of the bacterium, which acts on the midgut cells of susceptible insects, requiring predominantly alkaline pH and specific receptors that may be absent in the parasitoids.

9.10 Application of *Bacillus thuringiensis* with *Nuclear Polyhedrosis Virus* in *Anticarsia gemmatalis* (Lepidoptera: Erebidae)

Brazil is the world's second largest producer of soybeans, behind only the US. In the 2015/2016 harvest, this crop occupied an area of 33.17 million hectares, which totaled a production of 95.63 million tons (Embrapa 2016). *Anticarsia gemmatalis* (Hübner 1818) (Lepidoptera: Erebidae) is among the main pests that occur in soybeans, which is responsible for the use of chemical insecticides in the soybean crop, causing a significant increase in crop costs and imbalance in the ecosystem. The use of microbial agents, such as *Bacillus thuringiensis* serotype *kurstaki* (*Btk*) and *Anticarsia gemmatalis* Nuclear Polyhedrosis Virus (*AgVPNV*), are commercially viable alternatives to chemical insecticides of low selectivity to natural enemies.

Knaak and Fiuza (2005), in the research group of MToxAgro/CNPq, evaluated the digestive system of *A. gemmatalis* caterpillars after in vivo interaction of the entomopathogens *Btk* and *AgVPNV*, represented in the formulations Dipel® (Abbott) and *VPNAg* (Embrapa-CNPSo), respectively. Results from the in vivo assays revealed that the treatment for the association *AgVPNV*/*Btk* (98.68% corrected mortality) was more efficient than *AgVPNV* (81.28% mortality), but *Btk* alone caused 100% mortality. In vitro analyzes of *AgVPNV* and *Btk* in *A. gemmatalis* caterpillars suggest that Dipel® and *VPNAg* products were more efficient when used simultaneously, since the action of *AgVPNV* was enhanced when used in association with *Btk*, causing disturbances in the midgut of caterpillars from 6 h after application of treatments. When the entomopathogens were used alone, changes in intestinal cells were observed only 12 h after application of the treatments.

9.11 *Bacillus thuringiensis* with Plant Extracts and Essential Oils Against *Spodoptera frugiperda*

The interaction of entomopathogens, such as *B. thuringiensis*, with other active ingredients applied to pest control, such as plant extracts or essential oils, arises from the need for new molecules with greater biopesticidal potential. In addition, the growing interest in the use of phytotoxins obtained from raw materials of plants results from the availability of a wide variety of molecules, with great diversity in their structure and biological activity, offering a range of new sites of action in the target organisms.

Lucho (2004), jointly with the research group of MToxAgro/CNPq, evaluated the aqueous extract (10%) of *Melia azedarach* and the *B. thuringiensis* proteins present in the biopesticide XenTari® in second-instar caterpillars of *S. frugiperda*. According to the results obtained, it was verified that the Cry proteins of *B. thuringiensis aizawai* were highly efficient in the control of the target pest, causing 100% of corrected mortality of the caterpillars, at the 6 days after the application of the treatments (DAT).

The values of the 50% lethal concentration for *S. frugiperda* caterpillars were observed in 2 DAT using a concentration of 2.22 μg/ml of the purified Cry1Aa, Cry1Ab, Cry1C and Cry1D proteins from the commercial XenTari® product. The 90% control levels were obtained up to 4 DAT using concentrations between 5.32 and 57.7 μg/ml, were also considered ideal for an insecticide.

In the evaluation of the interaction of *B. thuringiensis* with *M. azedarach* extract, Lucho (2004) verified that this association was lethal in the first DAT. The accumulated mortality was higher in the association of biopesticide and botanical extract. The percentages of mortality of *S. frugiperda* observed at 7 DAT were: 55%, 70% and 90% for the treatments with extract, *B. thuringiensis aizawai*, and association of *B. thuringiensis aizawai* with extract of *M. azedarach*, respectively.

Comparing the survival curves by the Log-Rank test (Fig. 9.5), the association of *B. thuringiensis aizawai* and extract differed from the other treatments. Thus, a synergistic effect of the active ingredients of *B. thuringiensis aizawai* and *M. azedarach* extract against *S. frugiperda* caterpillars was observed.

Other works have also evaluated and confirmed the efficiency of the interaction of plant extracts with *Bacillus thuringiensis*. Knaak et al. (2010) evaluated the hystopathological effects in the midgut of *S. frugiperda* after ingestion of extracts of *Petiveria alliacea*, *Zingiber officinale*, *Cymbopogon citratus*, *Malva silvestris*, *Baccharis genistelloides* and *Ruta graveolens* obtained by maceration and infusion (10%), as well as the association of these extracts with *Bacillus thuringiensis aizawai*.

Their results showed changes in the tissues and intestinal cells of the *S. frugiperda* caterpillars 3 h after application of the treatments (AAT) based on *P. alliacea*, *Z. officinale, C. citratus* and *M. silvestris*, whereas for *B. genistelloides* and *R. graveolens* only disorders after 6 h AAT were observed. In the interactions of extracts with *B. thuringiensis*, changes in microvilli, disorganization of the midgut and hypertrophy of the epithelial cells projecting into the lumen were observed. The results of this work show that the histopathological effect of *Z. officinale*, *M. silvestris*, *R. graveolens* and *B. genistelloides*, were stronger when compared to the extracts of *P. alliacea* and *C. citratus*, which had a positive interaction with *B. thuringiensis*.

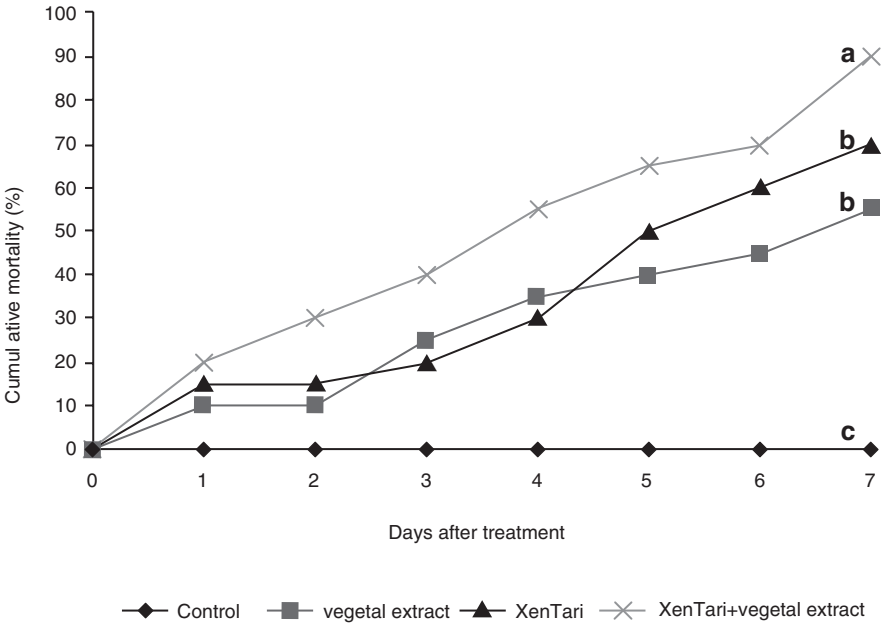

Fig. 9.5 Cumulative mortality of *Spodoptera frugiperda* caterpillars treated with biological insecticide and vegetal extract used in association or alone. *Curves* marked with the *same letter* do not differ from one another by the Log-Rank test at 5%

In all treatments performed by Knaak et al. (2010), it was observed that the macerated form was more active than the infusion, since the microvilli degradation occurred in shorter periods than the infusion. The microvilli are considered very important, since they are involved in digestion, nutrient absorption, water and secretion of liquids.

Knaak et al. (2015) performed bioassays to evaluate the lethal effect on first-instar caterpillars of *S. frugiperda* after interaction of the essential oils (2%) of *Tanacetum vulgare*, *Zingiber officinale*, *Cymbopogon citratus*, *Malva* sp., *Artemisia absinthium*, *Mentha* sp. and *Ruta graveolens* with the entomopathogens *B. thuringiensis aizawai* and *B. thuringiensis kurstaki*. The treatments of the interaction of *Bta* x *T. vulgare*, *A. absinthium*, *Z. officinale* and *C. citratus*, as well as *Btk* x *Malva* sp. and *T. vulgare* were antagonistic. The inhibition of the action of *B. thuringiensis* may be due to the decrease of the treatment intake or the competition between the substances present in the essential oils and the microorganism by the host. In the other associations, there was synergistic effect.

In general terms, the selection of plants with insecticidal activity is based almost exclusively on lethal effects. However, it should be considered that insect mortality should not always be the main objective, since it requires a higher dose, consequently a greater quantity of vegetal raw material. On the other hand, the objective may be to reduce the population growth of the pest, either by sublethal, physiological effects, behavioral changes or any change in the insect's biological cycle.

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Chapter 10
Specificity and Cross-order Activity of *Bacillus thuringiensis* Pesticidal Proteins

Kees van Frankenhuyzen

Abstract Published data on insecticidal activity of crystal proteins from *B. thuringiensis* were incorporated into a database on Bt toxin specificity. At the end of 2013, 158 of the 329 known holotypes were tested against 252 species distributed across 95 families in 25 orders, 8 classes, and 5 phyla. Thirty of the 158 proteins were reported to have no pesticidal activity, 59 were active against Lepidoptera, 42 against Diptera, 40 against Coleoptera, 10 against Hemiptera, 4 against Hymenoptera, and 1 against Orthoptera. Reports of toxicity to Trichoptera, Neuroptera, and Siphonaptera were not substantiated. Twelve proteins were reported to have activity against non-Arthropod species in the phyla Platyhelminthes and Nematoda. Activity outside orders of primary specificity was reported for 28 proteins affecting 75 taxa and was substantiated by reasonable evidence (mortality estimates) for 21 proteins and 51 taxa. Substantiated cross-activity occurred in 14 primary rank families across three classes of pesticidal proteins (Cry, Cyt, and Vip). Within the phylum Arthropoda, cross-activities were substantiated for 12 proteins (Cry1Ca, Cry1Ia, Cry4Aa, Cry8Da, Cry10Aa, Cry11Aa, Cry30Fa, Cry30Ga, Cry51Aa, Cry54Aa, Cry56Aa, Vip1A/Vip2A) affecting species across two orders, five proteins (Cry1Ac, Cry1Ba, Cry3Aa, Cry2Aa, Cyt1Aa) affecting three orders, and one protein (Cyt1Ba) affecting four orders, all within the class Insecta. Testing of insecticidal proteins against species in other Arthropod classes has not produced conclusive evidence of lethal activity outside the class Insecta. Cross-phylum activity was substantiated only for three insecticidal proteins (Cry1Ab, Cry2Ab, Cry3Bb), which affected nematode growth and reproduction at high dose levels. Target toxicity of *B. thuringiensis* pesticidal proteins can be broadly classified as being high when proteins are active in the 0.01–0.10 μg/ml range (e.g., Diptera-active proteins), medium when active in the 0.10–10 μg/ml range (Lepidoptera-, some Diptera-, and most Coleoptera-active proteins), and low when active in the 10–1000 μg/ml range (Coleoptera- and Nematode-active proteins). These categories are based on 25%, (0.1 μg/ml), 50% (1 μg/ml), and 75% (10 μg/ml) percentiles of nonparametric distribution of 50% lethal concentration estimates (LC_{50}s, $n = 256$) when pooled across all tested proteins and taxa. Toxicity (LC_{50}s) outside

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the order of primary specificity was quantified for 16 proteins and 24 taxa. Compared to toxicity ranges established for Diptera-, Coleoptera-, Lepidoptera-, and Nematoda-active proteins, 15 cross-activities were in the low- (10–1000 μg/ml), seven in the medium- (0.10–10), and two in the high-toxicity range (0.01–0.10 μg/ml). Activities outside a protein's suite of orders that is normally affected were mostly in the low-toxicity range. This was the case for toxicity of Cry1Ab, Cry1Ac, Cry2Aa, Cry3Aa, Cry4Aa, Cry11Aa, and Cyt1Aa to Hemiptera (aphids), of Cry51Aa to Hemiptera (*Lygus* spp.), and of Cyt1Ba to Hymenoptera (sawflies). The exception is high toxicity of Cry3Aa to fire ants (Hymenoptera). Activities that are within the suite of orders normally affected but outside a protein's primary-order affinity were often in the medium-toxicity range of corresponding reference proteins. This group includes dipteran toxicity of Cry1Ac, lepidopteran toxicity of Cry8Da, and coleopteran toxicity of Cry1Ba, Cry1Ia, Cry10Aa, Cyt1Aa, and Cyt1Ba. Dipteran toxicity of Cry1Ba, Cry1Ca, and Cyt1Ba occurs at high dose levels compared to Diptera-active reference proteins. Cross-activities of *B. thuringiensis* pesticidal proteins need to be viewed with caution until they are confirmed through independent testing. Nevertheless, current evidence suggests that cross-activities are not uncommon, having been substantiated for ~13% of the 158 proteins tested to date, and may be more prevalent considering that one-third of proteins that were tested against species in two or more orders were confirmed to be cross-active. One-third of reported cross-activities fall within the range of toxicities exhibited by order-specific proteins that are commonly used in pest control applications. Cross-activities therefore should be considered when designing or approving the use of *B. thuringiensis* pesticidal proteins in pest control applications.

Keywords • *Bacillus thuringiensis* • Artropod • Pesticidal-proteins • Toxicity • Cross-activities • Pest-control

Pesticidal proteins produced by *Bacillus thuringiensis* Berliner are widely used in pest control applications. Products containing naturally occurring strains have displaced synthetic insecticides in forestry (van Frankenhuyzen 1993) and public health (Guillet et al. 1990), while worldwide use of insect-resistant transgenic crops expressing *B. thuringiensis* proteins has reduced reliance on agricultural pesticides (Phipps and Park 2002). Of particular interest for pest control applications, and subject of this chapter, are the crystalline δ-endotoxins, or Cry and Cyt proteins (Crickmore et al. 1998), and the soluble vegetative insecticidal (Vip) proteins (Estruch et al. 1996).

A key feature that makes *B. thuringiensis* proteins attractive for pest management is their high degree of specificity. Specificity of a toxin protein is defined as the range of species or taxa that it affects (its activity spectrum). Host range specificity was initially recognized as toxicity of a subspecies or strain that was restricted to a specific insect order, in particular Lepidoptera (subsp. *kurstaki*, *aizawai*), Coleoptera (*tenebrionis*), or Diptera (*israelensis*). The link between host range and the presence of specific crystal proteins was established during the

1980s as more and more toxin protein genes were cloned, expressed, and tested. This leads to recognition of toxin families with strong affinity for Lepidoptera (Cry1), Coleoptera (Cry3), Diptera (Cry4), or Lepidoptera and Diptera (Cry2) (Höfte and Whiteley 1989). Within this primary-order affinity, individual *B. thuringiensis* proteins display a unique spectrum of insecticidal activities, which is manifested in the degree of toxicity toward different species, genera, and even families (Estruch et al. 1996; Warren 1997; van Frankenhuyzen 2009). However, order-specific proteins can display activity outside their primary-order specificity. For example, various Lepidoptera-, Coleoptera-, and Diptera-active proteins were reported to affect species in other orders (e.g., Haider et al. 1986; Tailor et al. 1992; Bradley et al. 1995; Walters and English 1995; Omolo et al. 1997; Zhong et al. 2000; Bulla and Canda 2004; Porcar et al. 2009; de Souza Aguiar et al. 2012) and even other phyla (Höss et al. 2008, 2011, 2013).

The purpose of this chapter is to examine current knowledge on the specificity of *B. thuringiensis* proteins and the extent of their cross order, cross-class, and cross-phylum activity (hereafter referred to as cross-activity). This chapter is an updated compilation of two reviews that were previously published (van Frankenhuyzen 2009, 2013).

10.1 General Considerations and Restrictions

This chapter is limited to toxin proteins that are listed in the Bt Toxin Nomenclature database (Crickmore et al. http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/; December 2013) for which bioassay data are available. At the time of compilation, 158 of the 329 listed proteins had been tested for pesticidal activity against a total of 252 species distributed across 95 families in 25 orders, 8 classes, and 5 phyla. Bioassay data for the remaining 171 proteins are either not available or were missed.

Data on toxicity to target pests were obtained from the Bt Toxin Specificity Database (van Frankenhuyzen and Nystrom http://www.glfc.cfs.nrcan.gc.ca/bacillus; December 2013). The database summarizes published toxicity data by protein gene and target species and is restricted to spore-free preparations of crystals or (pro)toxins, obtained through expression of cloned genes or purification from single-gene strains, that were tested individually (with the exception of binary toxins). Extracted data were supplemented with data from publications post 2010, when the database was last updated. Data on toxicity to nontarget species were obtained from the Nontarget Effects of Bt Crops Database (Marvier et al.; http://delphi.nceas.ucsb.edu/btcrops; December 2013), which is limited to proteins used in transgenic crops (Cry1Ab, Cry1Ac, Cry1F, Cry2Ab, Cry3Aa, Cry3Bb, and Cry9Ca). Extracted data were supplemented with information from relevant papers published after 2006.

For the purpose of this review, activity of a particular protein was inferred from mortality, with the exception of nematode activity of insecticidal proteins, which was scored on the basis of sublethal effects (inhibited growth or reproduction). This

exception was made in order to highlight cross-phylum activities. Data were obtained from laboratory bioassays with toxin proteins, as well as from laboratory and greenhouse tests with insect-resistant transgenic plants expressing single *B. thuringiensis* proteins. Laboratory bioassays were included only when they were conducted with individual toxin proteins purified from parental strains or produced in recombinant systems. Two types of transgenic plant studies were included, those involving exposure of the test species to toxin protein expressed in leaves, pollen, or other plant tissues and those involving exposure via prey (in the case of predators) or hosts (in the case of parasitoids) that had been reared on transgenic plant tissues.

Reported activities were summarized by test species for each tertiary-rank holotype protein, using a binary response (active or not active). Activity was considered regardless of life stage, bioassay method, or crystal protein preparation (crystal, protoxin, or activated toxin). Proteins were scored as "not active" when they did not evoke a mortality response at the highest concentration tested and "possibly active" in the case of conflicting data or when mortality was reported but not supported by actual data. Specificity was evaluated across families and orders by rolling up the species into higher ranking taxa. A toxin was considered active against a family or order when it affected at least one species in that family or order and not active when none of the species tested were affected.

10.2 Specificity

10.2.1 Activity Profiles

Current knowledge of specificity is restricted by the range of toxins tested and the range of species used in those tests. Of the 329 known toxin protein holotypes, 158 were tested against 252 species distributed across 95 families in 25 orders, 8 classes, and 5 phyla. However, the majority (~80%) of the 158 proteins were tested against ten species or less (Fig. 10.1 top) that were distributed across five or fewer families (Fig. 10.1 middle) restricted to one or two orders (Fig. 10.1 bottom). Only 14 proteins, 13 Lepidoptera-active (Cry1, Cry2, Cry9, and Vip3) and 1 Coleoptera-active (Cry3A), were tested against 25 species or more (Fig. 10.1, top). The two proteins with the most thoroughly characterized activity profiles (Cry1Ab, Cry1Ac) are commonly used in transgenic crops and were tested against ~100 species.

Of the 86 proteins tested against Lepidoptera, 59 affected at least one species in at least one of 20 families (Table 10.1).

Lepidopteran activity has been reported in 13 Cry families (Cry1, Cry2, Cry7, Cry8, Cry9, Cry15, Cry22, Cry30, Cry32, Cry51, Cry54, Cry56, and Cry59), as well as in the Cyt1 and Vip3 families. The Noctuidae, Plutellidae, and Pyralidae are the most commonly tested families (Fig. 10.2, top). Of the 72 proteins tested against Diptera, 42 were active across 7 families (Table 10.1). Dipteran activity has been reported for 21 Cry (Cry1, Cry2, Cry4, Cry10, Cry11, Cry16, Cry19, Cry20, Cry24,

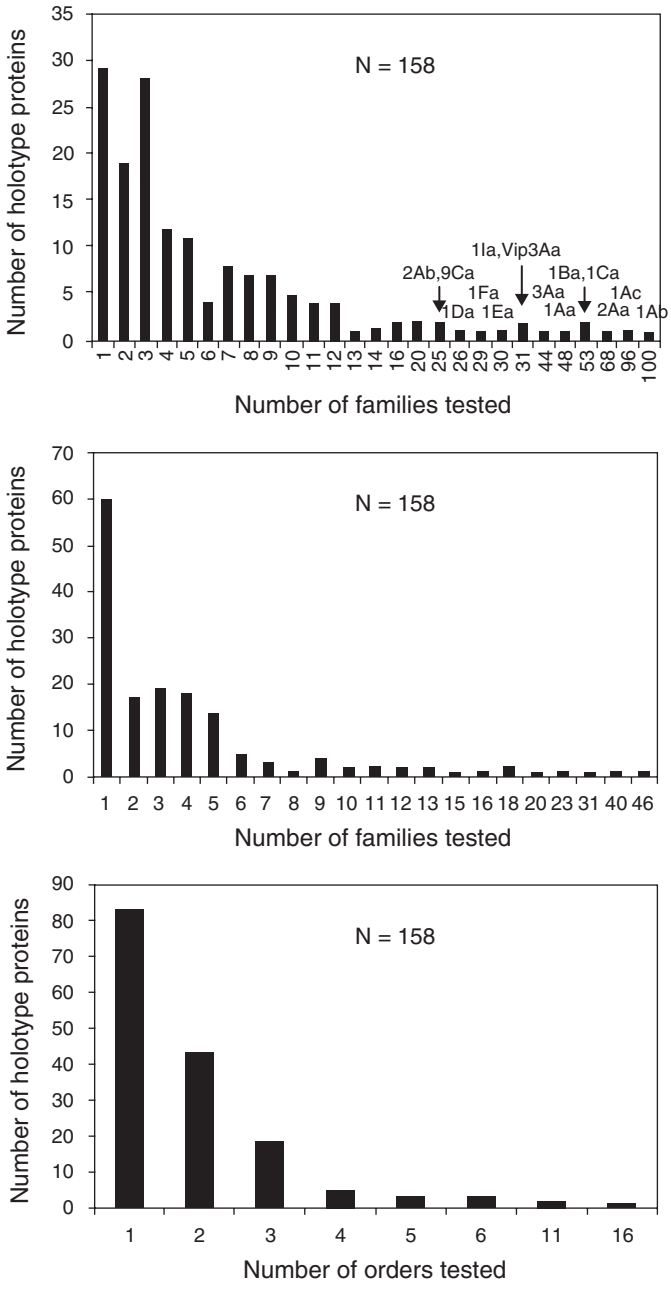

Fig. 10.1 Distribution of the number of *B. thuringiensis* pesticidal proteins (Cry, Cyt, and Vip) as a function of the number of species (*top*), families (*middle*), and orders (*bottom*) they were tested against. The number of orders was tallied across phyla (Arthropoda, Platyhelminthes, and Nematoda); the arthropod subclass of Acari was counted as one order

Table 10.1 Distribution of pesticidal activities of *Bacillus thuringiensis* proteins by families

Hymenoptera				1 Ab	1 Ac	1 Ad	2 Aa	3 Aa	3 Bb	5 Ac	22 Aa	Cyt1 Ba
ART	INS	HYM	Apinae	0	0	0	0		0			
			Braconidae	0	0		0					
			Encyrtidae		0							
			Dipriopnidae						1		1	
			Formicidae					1			2	
			Pamphilidae									1
			Pteromalidae		0		0					
			Tenthredinidae		0							1

Other Arthropods				1 Aa	1 Ab	1 Ac	1 Bf	1 Ia	2 Aa	2 Ab	2 Ac	7 Aa	300a	31 Aa	41 Aa	4 54a	Cyt2Ca
ART	INS	BLA	Blattellidae			0			0	2				0	0	0	
ART	INS	ISO	Rhinotermitidae						0					0	0	0	
ART	INS	NEU	Chrysopidae	0	2	0	0		2	0							
ART	INS	ORT	Acrididae									1		0	0	0	
			Gryllidae						0								
			Pyrgomorphidae											0	0	0	
ART	INS	SIP	Pulicidae														2
			Thripidae			0											
ART	INS	TRI	Helicopsychidae			2											
			Lepidostomatida			0											
			Limnephilidae			0											
ART	HEX	CO	Hypogastruridae			0	0		0		0						
			Isotomidae			0	0		0		0						
			Daphnidae			0											
ART	CRU	AMP	Hyalellidae			0											
ART	CRU	IS	Asellidae			0											
			Porcellionidae			0			0								
ART	ARA	AR	Araneidae			0											
			Theridiidae			0											
			Lycosidae			0											
ART	ARA	ACA	Orbatidae			0	0							0			
			Phytoseiidae			0	0										
			Scheloribatidae				0		0								
			Tetranychidae			0											

Non-Arthropods				1 Ab	1 Ac	1 Ad	3 Bb	5 Aa	5 Ab	5 Ad	5 Ba	6 Aa	6 Ba	1 2 Aa	1 4 Aa	21 Aa	5 54a
PLA	TRE	ECH	Fasciolidae														
NEM	CHR	ASC	Ascarididae									1					
NEM	CHR	DIP	Neodiplogasteridae					0				1	0	0	0	0	0
NEM	CHR	RHA	Ancylostomatida									1					
			Cephalobidae					0				1	1	0	0	0	1
			Haemonchidae								0						
			Heligmonellidae									1	0			1	1
			Heligmosomatid									1					
			Panagrolaimidae					0				1	1	0	0	1	
			Rhabditidae	1	0	1	1	2	1			1	1	0	0	1	1
NEM	CHR	TYL	Maloidogynidae									1	1				1
			Pratylenchidae					1	1			1	1	1			
MOL	GAS		Planorbidae	0													
ANN	CLIT	HAP	Lumbricidae	0													

Phyla*: PLA* Platyhelminthes, *ART* Arthropoda, *NEM* Nematoda, *MOL* Mollusca, *ANN* Annelida. **Classes**: *INS* Insecta, *Hex* Hexapoda, *CRU* Crustacea, *ARA* Arachnida, *TRE* Trematoda, *CHR* Chromadorea, *GAS* Gastropoda, *CLIT* Clitellata. **Orders:** *COL* Coleoptera, *LEP* Lepidoptera, *DIP* Diptera, *HEM* Hemiptera, *HYM* Hymenoptera, *ISO* Isoptera, *NEU* Neuroptera, *SIP* Siphonaptera, *ORT* Orthoptera, *BLA* Blattodea, *THY* Thysanoptera, *TRI* Trichoptera, *CO* Collembola, *AMP* Amphipoda, *IS* Isopoda, *CLA* Cladocera, *ACA* Acari, *AR* Araneae, *ECH* Echinostomida, *ASC* Ascaridida, *RHA* Rhabditida, *DIP* Diplogasterida, *TYL* Tylenchida, *HAP* Haplotaxida 0 = none of the species tested were affected, 1 = at least one species was affected, 2 = conflicting or unsubstantiated data

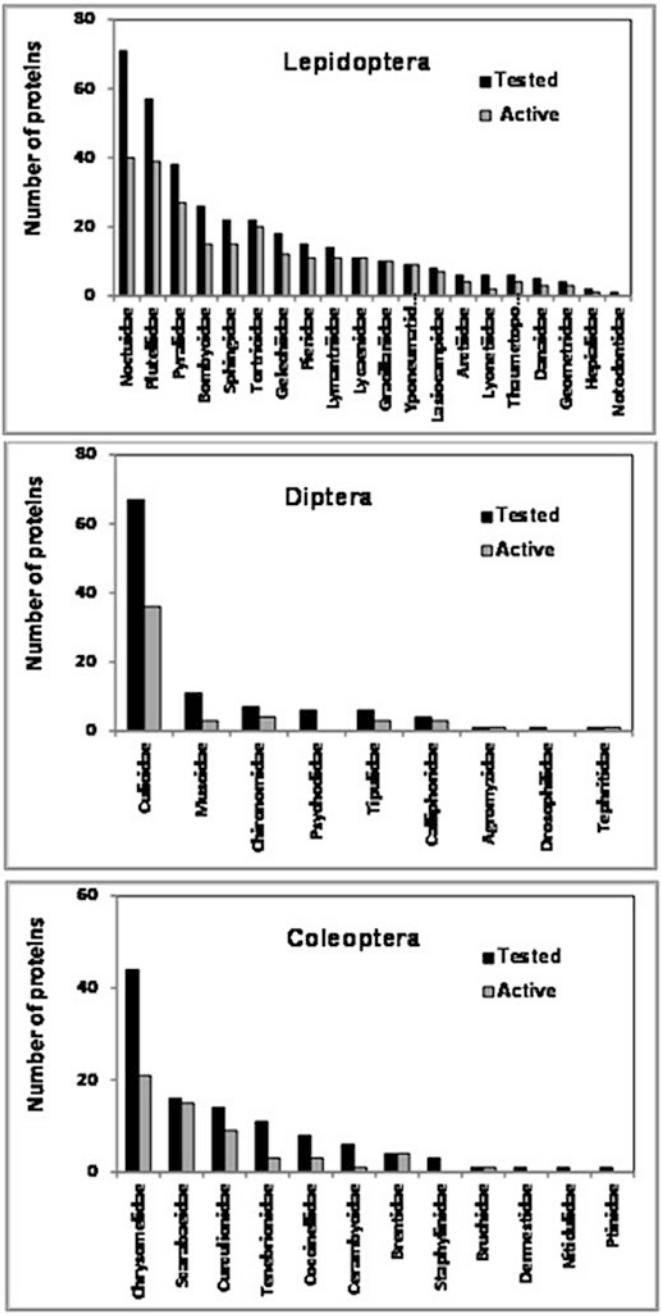

Fig. 10.2 Distribution of the number of *Bacillus thuringiensis* pesticidal proteins (Cry, Cyt, and Vip) that were tested and the number that was found active across families of Lepidoptera (*top*), Diptera (*middle*), and Coleoptera (*bottom*)

Cry27, Cry30, Cry32, Cry39, Cry44, Cry47, Cry48/Cry49, Cry52, Cry54, Cry56, Cry60, and Cry69) and two Cyt (Cyt1 and Cyt2) families, with Culicidae being the most frequently tested taxon (Fig.10.2, middle). Of the 65 proteins tested against Coleoptera, 40 had activity across 8 families (Table 10.1). Coleopteran activity has been reported to occur in 17 Cry families (Cry1, Cry3, Cry7, Cry8, Cry9, Cry10, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry51, Cry55) and in the Cyt1, Cyt2, and Vip1/Vip2 families. Coleopteran toxicity has been most commonly demonstrated in the Chrysomelidae and Scarabaeidae (Fig. 10.2, bottom). Hemipteran toxicity has been reported for 10 (Cry1Ab, Cry1Ac, Cry2Aa, Cry3Aa, Cry4Aa, Cry11Aa, Cry51Aa, Cyt1Aa, Cyt1Ba, and Vip1A/Vip2A) of the 14 tested proteins (Table 10.1).

Most reports of hemipteran toxicity pertain to aphids (Aphididae) (Fig. 10.3, top). Nine proteins have been tested for hymenopteran activity (Table 10.1), and activity has been reported for Cry3Aa, Cry5Ac, Cry22Aa, and Cyt1Ba against sawflies or ants (Fig. 10.3, middle). Toxicity outside these major insect orders has been reported for only four proteins (Cry1Ab, Cry2Aa, Cry7a, and Cyt2Ca) in four insect orders (Trichoptera, Neuroptera, Orthoptera, and Siphonaptera, respectively), despite testing of 14 proteins against species distributed across 24 families spanning 11 Arthropod orders (Table 10.1; Fig.10.3, bottom). Activity outside the phylum Arthropoda was tested with 14 proteins against species across 14 families, 6 orders, and 4 phyla (Table 10.1).

Activity was reported for nine of those (Cry5Aa, Cry5Ab, Cry5Ba, Cry6Aa, Cry6Ba, Cry12Aa, Cry14AA, Cry21AA, Cry55Aa) against a total of five orders in the phyla Platyhelminthes and Nematoda (Fig. 10.4). Nematode activity was reported for three insecticidal proteins (Cry1Ab, Cry2Ab, Cry3Bb) but only in terms of inhibiting growth and reproduction and not mortality (Table 10.1).

10.2.2 No Pesticidal Activity

Of the 158 proteins for which bioassay data are readily available, 30 were reported to have no pesticidal activity (Fig. 10.5). Eleven of those are individually tested proteins of binary toxin pairs belonging to the Vip1/Vip2, Cry34/Cry35, and Cry48/Cry49 families (Warren 1997; Baum et al. 2004; Jones et al. 2008). Fourteen of the 30 proteins (Cry1Ga, Cry1Ha, Cry5Ad, Cry17Aa, Cry24Ba, Cry29Aa, Cry30Aa, Cry30BA, Cry30Ca, Cry33Aa, Cry38Aa, Cry40Aa, Cry40Ba, and Vip3Ad) were tested against four species or less, which means that lack of their toxicity is likely attributable to limited testing. The remaining five proteins are so-called parasporins belonging to the Cry31A, Cry41A, Cry45A, Cry46A, and Cry63A families, which have no known insecticidal activity but are toxic to human cancer cells. None of the parasporins were tested as purified or recombinant proteins. Rather, their lack of insecticidal activity was inferred from screening of parental strains against 5 (Cry31Ad, Cry63Aa), 11 (Cry 41Aa, Cry45Aa), or 13 (Cry31Aa) species from as many as 6 arthropod orders (Lepidoptera, Diptera, Isoptera, Orthoptera, Blattodea,

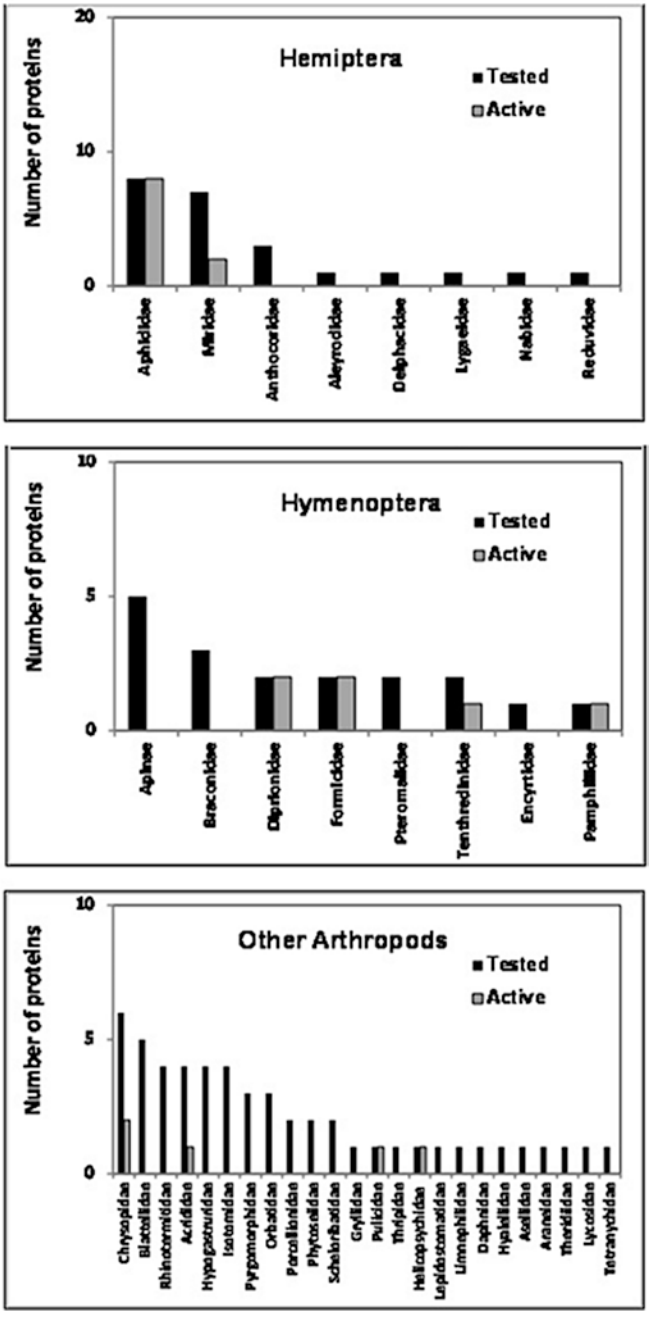

Fig. 10.3 Distribution of the number of *Bacillus thuringiensis* pesticidal proteins (Cry, Cyt, and Vip) that was tested and the number that was found active across families of Hemiptera (*top*), Hymenoptera (*middle*), and other Arthropoda (*bottom*)

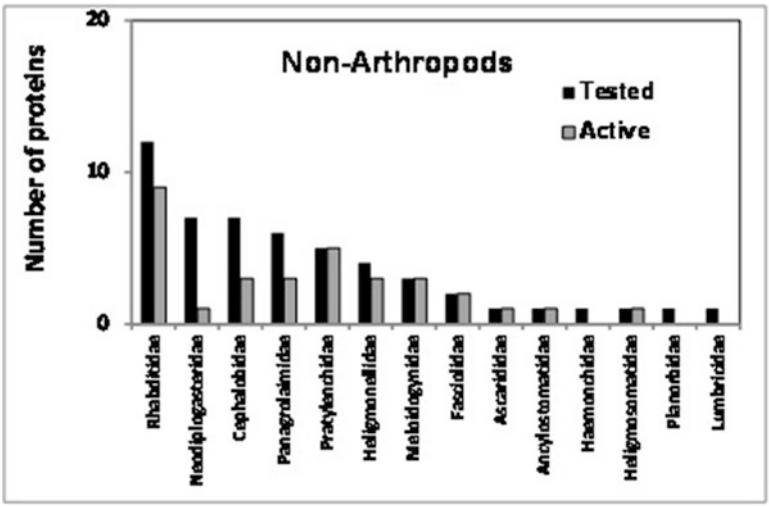

Fig. 10.4 Distribution of the number of *Bacillus thuringiensis* pesticidal proteins (Cry, Cyt, and Vip) that was tested and the number that was found active across families outside the phylum Arthropoda (*bottom*)

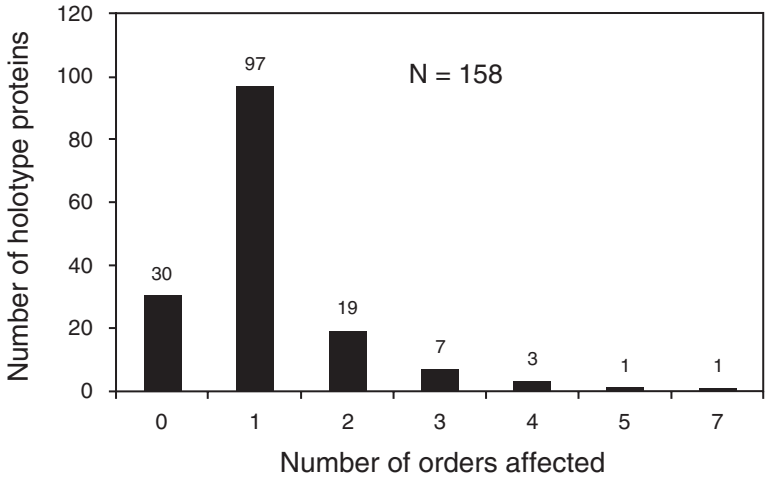

Fig. 10.5 Distribution of the number of *Bacillus thuringiensis* pesticidal proteins (Cry, Cyt, and Vip) as a function of the number of orders (*bottom*) that was affected

Acari) (Mizuki et al. 1999; Okumaru et al. 2005; Hayakawa et al. 2007, Jung et al. 2007, Nagamatsu et al. 2010). It should be noted that none of the parasporin-carrying strains were tested against other orders that are known to be affected by *B. thuringiensis* proteins, most notably Coleoptera and Rhabditida. It is therefore likely that expanded screening will eventually reveal pesticidal activity of parasporins.

10.2.3 Single-Order Proteins

The majority (97% or 61%) of the tested toxin proteins were reported to affect species within one order only (Fig. 10.5). Of those, 44 are active within the order of Lepidoptera, 23 within Coleoptera, and 25 within Diptera. Other insect orders that were reported to be affected by single-activity proteins are Hymenoptera (Cry5Ac) and Orthoptera (Cry7Ca). Unequivocal proof of hymenopteran toxicity of Cry5Ac is lacking, however. Its toxicity to the sawfly *Diprion pini* was inferred from bioassays with a 75-kDa protein that was "highly probable to correspond to the Cry5Ac toxin" (Garcia-Robles et al. 2001), while bioassay data against the pharaoh ant *Monomorium pharaonis* were shown only for the parental strain and unspecified toxin proteins from that strain (see Tables 6 and 7 in Payne et al. 1997). Recombinant Cry7Ca was toxic to *Locusta migratoria* (Orthoptera) with an LC_{50} of 10 μg/ml (Wu et al. 2011), but pesticidal activity outside that order has not been tested. The remaining three proteins were reported to have activity restricted to one order within the phylum Nematoda (Cry6Ba, Cry12Aa, and Cry21Aa). Activity of another putative nematode toxin, Cry13Aa, has not been established unequivocally: the gene is known from nematode-active strains (Narva et al. 1995; Kotze et al. 2005), but tests of purified protein or cloned gene product against nematodes have not been published to date.

10.2.4 Toxicity Profiles

Levels of primary-order toxicity are shown in more detail in Fig. 10.6 for the most commonly tested toxin families and taxa within the three insect orders that are mainly targeted by *B. thuringiensis* pesticidal proteins. Median LC_{50} values for Cry1 and Vip3 proteins across the four most commonly tested lepidopteran families range between 0.007 and 0.04 μg/cm^2 in diet-surface assays and between 0.8 and 10 μg/ml in diet-incorporation assays (Fig. 10.6, top). Average toxicity of Diptera-active proteins varies from 0.07 μg/ml for the most active (Cry11) against the most susceptible genus to 7 μg/ml for the least active protein (Cyt1A) against the most refractory genus (Fig. 10.6, middle). The most frequently tested coleopteran protein, Cry3Aa, ranges in toxicity between 0.04 μg/cm^2 against *Leptinotarsa* and 6.5 μg/cm^2 against *Diabrotica* (Fig. 10.6, bottom).

Reference toxicity ranges were constructed for proteins with well-established primary-order affinities. Nonparametric distributions of published LC_{50} values for Diptera-, Coleoptera-, Lepidoptera-, and Nematoda-active proteins were obtained by creating box plots, and the 25–75% percentile of those distributions were used to define toxicity ranges (Table 10.2). Published LC_{50} estimates were pooled within protein families (as indicated in Table 10.2) and across species as follows: Cry4, Cry11, and Cyt1, all Culicidae (*Aedes aegypti*, *Anopheles stephensi*, *A. gambiae*, *A. albimanus*, *Culex pipiens*, *C. quinquefasciatus*); Cry3, all Chrysomelidae

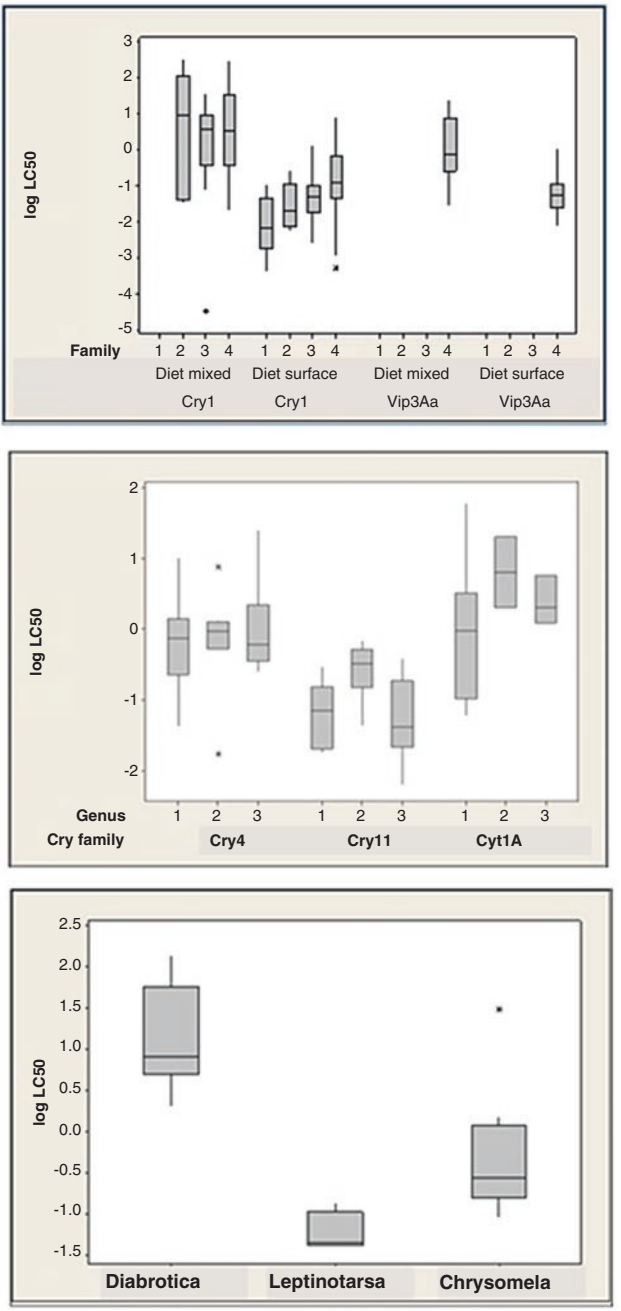

Fig. 10.6 Distribution of 50% lethal concentration (LC_{50}) estimates for Lepidoptera-active Cry1 and Vip3A proteins averaged across families (*1* Plutellidae; *2* Sphingidae; *3* Pyralidae; *4* Noctuidae) (*top*); for Diptera-active Cry4, Cry11, and Cyt1A proteins averaged across genera (*1 Aedes*; *2 Anopheles*; *3 Culex*) (*middle*); and for the Coleoptera-active Cry3Aa averaged across three genera (*bottom*)

Table 10.2 Toxicity ranges (median values and 25% and 75% percentiles of distributions of published LC_{50}s) of Cry4, Cry11, and Cyt1 proteins to Diptera (DIP); various proteins to Nematoda (NEM); Cry3 proteins to Coleoptera (COL) and Cry1 and Vip3 proteins to Lepidoptera (LEP) when layered on diet surface (Surface) or incorporated into diet (Mixed)

Protein[a]	Order	Number of tests	Method	Median	25%	75%	Unit
Cry4	DIP	31		0.90	0.40	1.30	µg/ml
Cry11	DIP	31		0.06	0.02	0.25	µg/ml
Cyt1	DIP	17		1.20	0.70	6.00	µg/ml
	NEM	9		25	15	125.0	µg/ml
Cry3	COL	13	Surface	0.18	0.06	20.0	µg/cm^2
Cry1	LEP	237	Surface	0.07	0.01	0.25	µg/cm^2
Cry1	LEP	160	Mixed	2.00	0.20	15.0	µg/ml
Vip3	LEP	17	Surface	0.08	0.03	0.30	µg/cm^2
Vip3	LEP	8	Mixed	2.5	0.35	20	µg/ml
All	**All**	**256**		**1.2**	**0.12**	**10.0**	**µg/ml**

[a]Proteins were pooled by primary rank; data included in the pooling pertain to the following proteins: DIP: Cry4Aa, Cry4Ba; Cry11Aa, Cry11Ba, Cry11Bb; Cyt1Aa, Cyt1Ab; NEM: Cry5Aa, Cry5Ab, Cry5Ba, Cry6Aa, Cry55Aa; COL: Cry3Aa, Cry3Ba, Cry3Bb; LEP: Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ad, Cry1Ae, Cry1Ba, Cry1Bb, Cry1Ca, Cry1Cb, Cry1Da, Cry1Db, Cry1Ea, Cry1Eb, Cry1Fa, Cry1Ia, Cry1Ie, Cry1Ja, Cry1Jb, Cry1Ka; Vip3Aa; All: above listed proteins combined, excluding diet-surface assays

(*Diabrotica*, *Leptinotarsa*, *Pyrrhalta*, *Chrysomela* spp.); Cry 1, 46 Lepidoptera species belonging to 12 families; and Vip3A, 10 Lepidoptera species belonging to 3 families. Lepidopteran and coleopteran assays were constricted by assay type (diet-incorporation and diet-surface layering). Both methods are commonly used in lepidopteran assays, but the majority of coleopteran toxicity values are derived from diet-surface assays.

In general, dipteran toxins are the most active, having median LC50 values of 1 µg/ml or less and a 75% percentile below 10 µg/ml. Lepidopteran toxins are active in the range of a few µg/ml with a 75% percentile of 15–20 µg/ml, while nematicidal proteins are active above that range. Coleopteran toxins are on average less active than lepidopteran toxins and have a wider toxicity range. By applying the consistent 1:30 ratio of lepidopteran median LC50 values in diet-surface/diet-incorporation assays (Table 10.2) to coleopteran diet-surface LC50s, coleopteran toxins are expected to be active in the 5 µg/ml range if they were tested using diet-incorporation methods.

Based on the 25–75% percentile of the distribution of µg/ml LC_{50} values pooled across all proteins and all taxa (n = 256 assays, Table 10.2), *B. thuringiensis* pesticidal proteins can be classified as having high toxicity when LC50s are in the 0.01–0.10 µg/ml range, medium toxicity in the 0.10–10 µg/ml range, and low toxicity in the 10–1000 µg/ml range.

10.3 Cross-Activity

Reports of cross-activity range in quality from unpublished data to LC_{50} estimates derived from multiple-dose bioassays. Cross-activity was therefore evaluated at three levels. The first level consisted of a comprehensive overview of all possible cross-activities, which was obtained by compiling reported activities regardless of data quality. At the second level, a distinction was made between qualitative and quantitative data to separate cross-activities that were substantiated from those that were not. Reports of a protein affecting a species without supporting data were considered unsubstantiated, whereas estimates of mortality in response to single or multiple doses were viewed as reasonable evidence of cross-activity. At the third level, toxicities of cross-active proteins for which LC_{50} estimates were reported were compared to toxicity levels of major protein families within orders of their primary affinity.

Considering both qualitative and quantitative reports, there is evidence of cross-activity in 31 of the 158 *B. thuringiensis* proteins tested to date. That number includes the dually active (Lepidoptera, Diptera) Cry2Ag (Liang et al. 2011) and nematode-active proteins affecting species across three (Cry6Aa; Guo et al. 2008) or four orders (Cry5Ba; Guo et al. 2008; Hu et al. 2010; Urban et al. 2013; Wang et al. 2012b). Because those cross order activities are within the families' primary specificity, they were not included here under the definition of cross-activity. Of the 28 remaining cross-active proteins, 17 affected species across two orders, 7 across three orders, 2 across four orders, and 1 across five and seven orders (Fig. 10.5).

Which orders were reported as being affected is summarized in a compilation of both qualitative and quantitative reports in Fig. 10.7, which is discussed in more detail in the sections that follow.

Lack of quantitative data is indicated throughout the text by the comment "bioassay data not reported." Species outside the order of primary affinity for which quantitative toxicity data are published are referred to in the text without further comment because the data are presented in Table 10.3.

Studies showing no toxicity to species outside the order of primary affinity are summarized in Table 10.4. Figure 10.7 also reports for each of the cross-active proteins the number of species that were tested in the various taxa; the species are listed in Tables 10.3 and 10.4.

10.3.1 Across Two Orders

Seventeen of the 28 cross-active proteins were reported to affect species across two orders (Fig. 10.7, top). Fourteen of those were active across two insect orders, and three had activity in both Insecta and Nematoda.

Two of the proteins affecting two insect orders are well-known lepidopteran-active proteins: Cry1Ca affects several Diptera (*Aedes aegypti*, *Anopheles gambiae*,

PHYLUM	ART							NEM		
CLASS	INS						ARA	CHR		
ORDER	COL	LEP	DIP	HEM	HYM	SIP	ACA	RHA	TYL	DIP
Cry1Ca		50	3							
Cry1Ia	7	21	2				1			
Cry3Bb	8				1			1		
Cry4Aa			7	1						
Cry6Aa								5	3	1
Cry8Da	4	3								
Cry10Aa	1	2	5							
Cry11Aa		1	9	2						
Cry14Aa	1							5		1
Cry22Aa	2				1					
Cry22Ab	4	8								
Cry30Fa		2	1							
Cry30Ga		1	1							
Cry55Aa	1								2	
Cry54Aa		2	1							
Cry56Aa		2	1							
Cyt2Ca	3					1				
Vip1A/2A	5	7	1	1						

PHYLUM	ART								MOL	PLA	NEM		
CLASS	INS							HEX	ARA	GAS	TRE	CHR	
ORDER	COL	LEP	DIP	HEM	HYM	NEU	BLA	CO	ACA		ECH	RHA	DIP	TYL
Cry1Ac	7	61	2	8	8	2	1	2	3	1		1		
Cry1Ba	7	42	3											
Cry2Ab	1	17	4	1		1						1		
Cry3Aa	33	3	2	3	1			2						
Cry5Aa											1	4	1	1
Cry5Ab											1	1		1
Cry51Aa	3	6		2										

PHYLUM	ART														ANN	NEM		
CLASS	INS										HEX	CRU			CLIT	CHR		
ORDER	COL	LEP	DIP	HEM	HYM	ISO	NEU	ORT	BLA	THY	TRI	CO	AMP	IS	CLA	ACA	AR	HAP	ASC	RHA	DIP
Cry1Ab	7	56	2	9	3		1			3	4	2	1	2	1	3	3	2		1	
Cry2Aa	5	37	9	5	4	1	2	1	1			2		1							
Cry5Ba																			1	7	1
Cyt1Aa	1	3	10	1																	
Cyt1Ba	4	1	1	1	4																

Fig. 10.7 Distribution of pesticidal activities of *Bacillus thuringiensis* proteins that affect species across two (*top*), three (*middle*), or more (*bottom*) orders. The number of species that was tested within each order is indicated inside the corresponding *box*, and species are identified in Tables 10.3 and 10.4. No shading (*white*) means that none of the species tested was affected. *Gray* shading means that at least one of the species tested was affected. *Light gray* indicates cross-activities that are substantiated (published estimates of LC_{50} or mortality response to a defined dose). *Dark gray* indicates cross-activities that are not unequivocally established (no quantitative data or conflicting data). *Phyla PLA* Platyhelminthes, *ART* Arthropoda, *NEM* Nematoda, *MOL* Mollusca, *ANN* Annelida. Classes: *INS* Insecta, *HEX* Hexapoda, *CRU* Crustacea, *ARA* Arachnida, *GAS* Gastropoda; *TRE* Trematoda, *CHR* Chromadorea, *CLIT* Clitellata. Orders: *COL* Coleoptera, *LEP* Lepidoptera, *DIP* Diptera, *HEM* Hemiptera, *HYM* Hymenoptera, *ISO* Isoptera, *NEU* Neuroptera, *SIP* Siphonaptera, *ORT* Orthoptera, *BLA* Blattodea, *THY* Thysanoptera, *TRI* Trichoptera, *CO* Collembola, *AMP* Amphipoda, *IS* Isopoda, *CLA* Cladocera, *ACA* Acari, *AR* Araneae, *HAP* Haplotaxida, *ECH* Echinostomida, *ASC* Ascaridida, *RHA* Rhabditida, *DIP* Diplogasterida, *TYL* Tylenchida

Table 10.3 Toxicity of *Bacillus thuringiensis* proteins outside the order of their primary specificity or with unknown primary-order specificity as shown in Fig. 10.7

Toxin	Order	Genus	Species	Method	EC_{50}	Dose	Units	% Mortality	Reference
Cry1Ab	COL	*Cheilomenes*	*sexmaculatus*	2a		1000	µg/ml	40	Dhillon and Sharma (2009)
		Adalia	*bipunctata*	5c		25	µg/ml	50	Schmidt et al. (2009)
	DIP	*Aedes*	*aegypti*	3	25–50		µg/ml		Haider et al. (1986)
	HEM	*Acyrthosiphon*	*pisum*	2a		500	µg/ml	40	Porcar et al. (2009)
	NEU	*Chrysoperla*	*carnea*	2a		100	µg/ml	57	Hilbeck et al. (1998)
		Chrysoperla	*carnea*	2c		na		66	Hilbeck et al. (1999)
		Chrysoperla	*carnea*	2c		0.72	µg/g	82	Dutton et al. (2002)
	TRI	*Helicopsyche*	*borealis*	2b		na		43	Rosi-Marshall et al. (2007)
	NEM	*Caenorhabditis*	*elegans*	3	55–225*		µg/ml		Hoss et al. (2008)
Cry1Ac	DIP	*Glossina*	*morsitans*	2a	0.7		µg/ml		Omolo et al. (1997)
	HEM	*Acyrthosiphon*	*pisum*	2a		500	µg/ml	71	Li et al. (2011)
Cry1Ba	DIP	*Musca*	*domestica*	2a	20		µg/ml		Zhong et al. (2000)
	COL	*Chrysomela*	*scripta*	4	150		µg/ml		Zhong et al. (2000)
		Chrysomela	*scripta*	5b	0.3		µg/cm^2		Federici and Bauer (1998)
		Chrysomela	*scripta*	5b	0.2		µg/cm^2		Bradley et al. (1995)
		Leptinotarsa	*decemlineata*	4	140		µg/ml		Naimov et al. (2001)
		Hypothenemus	*hampei*	5a		5	µg/cm^2	44	Lopez-Pazos et al. (2009)
Cry1Ca	DIP	*Aedes*	*aegypti*	3	140		µg/ml		Smith et al. (1996)
		Anopheles	*gambiae*	3	280		µg/ml		Smith et al. (1996)
		Culex	*quinquefasciatus*	3	125		µg/ml		Smith et al. (1996)

(continued)

Table 10.3 (continued)

Toxin	Order	Genus	Species	Method	EC_{50}	Dose	Units	% Mortality	Reference
Cry1Ia	COL	*Leptinotarsa*	*decemlineata*	4	35		µg/ml		Naimov et al. (2001)
		Leptinotarsa	*decemlineata*	4	10		µg/ml		Ruiz de Escudero et al. (2006)
		Anthonomus	*grandis*	2a	20		µg/ml		Martins et al. (2008)
Cry2Aa	HEM	*Macrosiphum*	*euphorbiae*	2a		200	µg/ml	95	Walters and English (1995)
	NEU	*Chrysoperla*	*carnea*	2c		na		45	Hilbeck et al. (1999)
Cry2Ab	NEM	*Caenorhabditis*	*elegans*	3	25*		µg/ml		Hoss et al. (2013)
Cry3Aa	HEM	*Acyrthosiphon*	*pisum*	2a		500	µg/ml	40	Porcar et al. (2009)
		Acyrthosiphon	*pisum*	2a		500	µg/ml	60	Porcar et al. (2009)
		Acyrthosiphon	*pisum*	2a		500	µg/ml	70	Li et al. (2011)
		Macrosiphum	*euphorbiae*	2a		360	µg/ml	100	Walters and English (1995)
	HYM	*Solenopsis*	*invicta*	2a	0.07		µg/ml		Bulla and Canda (2004)
Cry3Bb	NEM	*Caenorhabditis*	*elegans*	3	10*		µg/ml		Hoss et al. (2013)
		Caenorhabditis	*elegans*	3	25*		µg/ml		Hoss et al. (2011)
Cry4Aa	HEM	*Acyrthosiphon*	*pisum*	2a		125	µg/ml	40–90	Porcar et al. (2009)
Cry5Aa	PLA	*Fasciola*	*hepaticus*	2a		100	µg/ml	95	Narva et al. (1991)
Cry8Da	LEP	*Plutella*	*xylostella*	4		2	µg/ml	40	Asano et al. (2003)
Cry10Aa	COL	*Anthonomus*	*grandis*	2a	5		µg/ml		de Souza Aguiar et al. (2012)
Cry11Aa	HEM	*Acyrthosiphon*	*pisum*	2a	125–500		µg/ml		Porcar et al. (2009)

		Macrosiphum	*euphorbiae*	2a		350	µg/ml	100	Walters and English (1995)
Cry30Fa	LEP	*Plutella*	*xylostella*	2a	155		µg/ml		Tan et al. (2010)
	DIP	*Aedes*	*aegypti*	3	7		µg/ml		Tan et al. (2010)
Cry30Ga	DIP	*Aedes*	*aegypti*	3	7		µg/ml		Zhu et al. (2010a)
	LEP	*Plutella*	*xylostella*	4	57		µg/ml		Zhu et al. (2010a)
Cry51Aa	HEM	*Lygus*	*hesperus*	2a	75–250		µg/ml		Baum et al. (2012)
		Lygus	*lineolaris*	2a		200	µg/ml	52	Baum et al. (2012)
	COL	*Leptinotarsa*	*decemlineata*	5a	25		µg/cm^2		Baum et al. (2012)
Cry54Aa	DIP	*Aedes*	*aegypti*	3	10		µg/ml		Tan et al. (2009)
	LEP	*Spodoptera*	*exigua*	2a	5		µg/ml		Tan et al. (2009)
		Helicoverpa	*armigera*	2a	14		µg/ml		Tan et al. (2009)
Cry56Aa	DIP	*Aedes*	*aegypti*	3	0.2		µg/ml		Zhu et al. (2010b)
	LEP	*Plutella*	*xylostella*	2a	17		µg/ml		Zhu et al. (2010b)
		Helicoverpa	*armigera*	2a	44		µg/ml		Zhu et al. (2010b)
Cyt1Aa	LEP	*Plutella*	*xylostella*	4	2		µg/ml		Sayyed et al. (2001)
	COL	*Chrysomela*	*scripta*	5b	0.3		µg/cm^2		Federici and Bauer (1998)
	HEM	*Acyrthosiphon*	*pisum*	2a		125	µg/ml	10	Porcar et al. (2009)
Cyt1Ba	COL	*Hypera*	*postica*	5a		0.5	µg/cm^2	55–90	Bradfisch et al. (1998)
		Diabrotica	*virgifera*	5a		5	µg/cm^2	90	Payne et al. (1995)
	LEP	*Choristoneura*	*fumiferana*	1		0.1	µg/larva	30	van Frankenhuyzen and Tonon (2013)

(continued)

Table 10.3 (continued)

Toxin	Order	Genus	Species	Method	EC_{50}	Dose	Units	% Mortality	Reference
	DIP	*Liriomyza*	*trifolii*	2a	10		µg/ml		Payne et al. (1995)
	HEM	*Lygus*	*hesperus*	2a		5	µg/ml	82	Stockhoff and Conlan (1998)
	HYM	*Diprion*	*similis*	1	500		µg/ml		van Frankenhuyzen and Tonon (2013)
		Pikonema	*alaskensis*	1		500	µg/ml	70	van Frankenhuyzen and Tonon (2013)
		Acantholyda	*erythrocephala*	1		500	µg/ml	55	van Frankenhuyzen and Tonon (2013)
		Neodiprion	*sertifer*	1		500	µg/ml	20	van Frankenhuyzen and Tonon (2013)
Vip1A/Vip2A	COL	*Diabrotica*	*virgifera*	2a	0.03		µg/ml		Warren (1997)
	HEM	*Aphis*	*gossypii*	2a	0.6		µg/ml		Sampurna and Maiti (2011)

Order: *COL* Coleoptera, *DIP* Diptera, *HEM* Hemiptera, *NEU* Neuroptera, *TRI* Trichoptera, *NEM* Nematoda, *HYM* Hymenoptera, *PLA* Platyhelminthes (Phylum), *LEP* Lepidoptera. Method: bioassay methods are indicated by numbers (*1* forced ingestion; *2a* diet incorporation; *2b* transgenic plant tissue; *2c* prey or hosts reared on transgenic plant tissue; *3* free ingestion; *4* leaf dip; *5a* diet surface; *5b* leaf surface; *5c* toxin-coated prey). EC_{50}: 50% effective concentration determined in multiple-dose tests; response variable is mortality, except in cases marked with * which indicates inhibition of reproduction or growth. Dose: dose administered in single dose tests; *na* dose not available

Table 10.4 Tests that indicate lack of cross-activity of *B. thuringiensis* pesticidal proteins shown in Fig. 10.7

Toxin	Phylum	Class	Order	Family	Genus	Species	Method	Dose	Unit	Reference
Cry1Ab	ART	INS	COL	Coccinellidae	*Coleomegilla*	*maculata*	2b	3	µg/g	Pilcher et al. (1997)
					Coleomegilla	*maculata*	2b	na		Lundgren and Wiedenmann (2004)
					Propylea	*japonica*	2c	na		Bai et al. (2006)
					Oulema	*melanopus*	2b	40	µg/g	Meissle et al. (2012)
					Adalia	*bipunctata*	2c	5	µg/g	Alvarez-Alfageme et al. (2011)
					Adalia	*bipunctata*	2a	45	µg/ml	Alvarez-Alfageme et al. (2011)
					Adalia	*bipunctata*	2a	50	µg/ml	Porcar et al. (2010)
					Stethorus	*punctillum*	2c	na		Alvarez-Alfageme et al. (2011)
					Cryptolaemus	*montrouzieri*	2a	50	µg/ml	Porcar et al. (2010)
				Staphylinidae	*Atheta*	*coriaria*	2a	50	µg/ml	Garcia et al. (2010)
	ART	INS	DIP	Tipulidae	*Tipula*	*abdominalis*	2b	na		Jensen et al. (2010)
	ART	INS	HEM	Aphididae	*Rhopalosiphum*	*padi*	2b	na		Lumbierres et al. (2004)

(continued)

Table 10.4 (continued)

Toxin	Phylum	Class	Order	Family	Genus	Species	Method	Dose	Unit	Reference
					Rhopalosiphum	*padi*	2b	na		Lozzia et al. (1998)
					Rhopalosiphum	*padi*	2b	3	µg/g	Dutton et al. (2002)
					Sitobion	*avenae*	2b	0.2	µg/g	Ramirez-Romero et al. (2008)
				Delphacidae	*Nilaparvata*	*lugens*	2b	na		Bernal et al. (2002)
				Miridae	*Cyrtorhinus*	*lividipennis*	2c	na		Bernal et al. (2002)
				Anthocoridae	*Orius*	*majusculus*	2c	na		Zwahlen et al. (2000)
					Orius	*majusculus*	2b	na		Pons et al. (2004)
					Orius	*majusculus*	2b	6	µg/g	Lumbierres et al. (2012)
					Orius	*majusculus*	2c	3	µg/g	Lumbierres et al. (2012)
					Orius	*insidiosus*	2b	3	µg/g	Pilcher et al. (1997)
					Orius	*insidiosus*	2b	5	µg/g	Al-Deeb et al. (2001)
					Orius	*albidipennis*	2c	na		Gonzales-Zomara et al. (2007)
	ART	INS	HYM	Braconidae	*Cotesia*	*marginiventris*	2c	0.6	µg/g	Vojtech et al. (2005)

					Cotesia	*flavipes*	2c	na		Prutz and Dettner (2004)
				Apidae	*Apis*	*mellifera*	2b	na		Novartis (2000)
					Apis	*mellifera*	2b	7	µg/g	Hanley et al. (2003)
	ART	INS	NEU	Chrysopidae	*Chrysoperla*	*carnea*	2b	3	µg/g	Pilcher et al. (1997)
					Chrysoperla	*carnea*	2c	na		Lozzia et al. (1998)
					Chrysoperla	*carnea*	2a	1000	µg/ml	Romeis et al. (2004)
					Chrysoperla	*carnea*	2c	3	µg/g	Dutton et al. (2002)
					Chrysoperla	*carnea*	2c	na		Rodrigo-Simon et al. (2006)
	ART	INS	THY	Thripidae	*Frankliniella*	*tenuicornis*	2b	4	µg/g	Obrist et al. (2005)
					Stenchaetothrips	*biformis*	2b	1	µg/g	Akhtar et al. (2010)
	ART	INS	TRI	Lepidostomatidae	*Lepidostoma*	spp.	2b	na		Jensen et al. (2010)
					Lepidostoma	*liba*	2b	na		Chambers et al. (2007)
				Limnephilidae	*Pycnopsyche*	*scabripennis*	2b	na		Jensen et al. (2010)
	ART	HEX	CO	Isotomidae	*Folsomia*	*candida*	2a	na		Sims and Martin (1997)
					Folsomia	*candida*	2b	2	µg/g	Clark and Coats (2006)

(continued)

Table 10.4 (continued)

Toxin	Phylum	Class	Order	Family	Genus	Species	Method	Dose	Unit	Reference
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin (1997)
	ART	CRU	AMP	Hyalellidae	*Hyalella*	*azteca*	2b	na		Chambers et al. (2007)
			IS	Asellidae	*Caecidotea*	*communis*	2b	na		Jensen et al. (2010)
				Porcellanidae	*Porcellio*	*scaber*	2b	na		Escher et al. (2000)
	ART	CRU	CLAD	Daphniidae	*Daphnia*	*magna*	2b	na		MRID
	ART	ARA	ACA	Tetranychidae	*Tetranychus*	*urticae*	2b	1	µg/g	Lozzia et al. (2000)
					Tetranychus	*urticae*	2b	3	µg/g	Dutton et al. (2002)
				Orbatidae	*Oppia*	*nitens*	2b	na		Yu et al. (1997)
				Phytoseiidae	*Neoseiulus*	*cucumeris*	2c	5	µg/g	Obrist et al. (2006)
	ART	ARA	AR	Araneidae	*Araneus*	*diadematus*	2b	3	µg/g	Ludy and Lang (2006)
				Lycosidae	*Pirata*	*subpiraticus*	2c	1	µg/g	Chen et al. (2009)
	MOL	GAS		Planorbidae	*Gryllus*	sp.	2b	na		Chambers et al. (2007)
	ANN	CLIT	HAP	Lumbricidae	*Eisenia*	*fetida*	2a	2	µg/g	Clark and Coats (2006)
					Lumbricus	*terrestris*	2a	na		Saxena and Stotsky (2001)

					Lumbricus	*terrestris*	2a	15	µg/g	Zwahlen et al. (2003)
Cry1Ac	ART	INS	COL	Coccinellidae	*Coleomegilla*	*maculata*	2a	100	µg/ml	Li et al. (2011)
					Cheilomenes	*sexmaculatus*	2a	1000	µg/ml	Dhillon and Sharma (2009)
					Propylea	*japonica*	2c	0.01	µg/g	Zhang et al. (2006)
					Hippodamia	*convergens*	2a	20	µg/ml	Sims (1995)
				Curculionidae	*Anthonomus*	*grandis*	2a	100	µg/ml	Sims (1995)
				Chrysomelidae	*Leptinotarsa*	*decemlineata*	2a	100	µg/ml	Sims (1995)
					Diabrotica	*undecimpunctata*	2a	100	µg/ml	Sims (1995)
	ART	INS	DIP	Culicidae	*Aedes*	*aegypti*	2a	100	µg/ml	Sims (1995)
	ART	INS	HEM	Aphididae	*Myzus*	*persicae*	2b	na		Schuler et al. (2001)
					Myzus	*persicae*	2a	100	µg/ml	Sims (1995)
					Aphis	*gossypii*	2b	na		Zhang et al. (2008)
					Macrosiphum	*euphorbiae*	2a	400	µg/ml	Walters and English (1995)
				Nabidae	*Nabis*	sp.	2c	na		Ponsard et al. (2002)
				Reduviidae	*Zelus*	*renardii*	2c	na		Ponsard et al. (2002)
				Anthocoridae	*Orius*	*sauteri*	2c	na		Zhang et al. (2008)
					Orius	*albidipennis*	2a	1000	µg/ml	Gonzales-Zomara et al. (2007)

(continued)

Table 10.4 (continued)

Toxin	Phylum	Class	Order	Family	Genus	Species	Method	Dose	Unit	Reference
	ART	INS	HYM	Braconidae	*Cotesia*	*plutellae*	2c	na		Schuler et al. (2003, 2004)
					Diaeretiella	*rapae*	2c	na		Schuler et al. (2001)
					Microplitis	*mediator*	2a	500	µg/ml	Liu et al. (2005)
				Encyrtidae	*Copidosoma*	*floridanum*	2c	na		Baur and Boethel (2003)
				Pteromalidae	*Nasonia*	*vitripennis*	2a	20	µg/ml	Sims (1995)
				Tenthredinidae	*Athalia*	*rosea*	2b	8	µg/g	Howald et al. (2003)
				Apidae	*Apis*	*mellifera*	2a	20	µg/ml	Sims (1995)
					Bombus	*occidentalis*	2b	0.01	µg/g	Morandin and Winston (2003)
	ART	INS	NEU	Chrysopidae	*Chrysoperla*	*carnea*	2c	0.3	µg/g	Lawo et al. (2010)
					Chrysoperla	*carnea*	1	4	µg/ larva	Rodrigo-Simon et al. (2006)
					Chrysoperla	*carnea*	2c	na		Rodrigo-Simon et al. (2006)
					Chrysoperla	*carnea*	2a	20	µg/ml	Sims (1995)
					Chrysoperla	*pallens*	2c	na		Guo et al. (2008)
	ART	INS	BLA	Blatellidae	*Blatella*	*germanica*	2a	100	µg/ml	Sims (1995)
	ART	HEX	CO	Isotomidae	*Folsomia*	*candida*	2a	na		Sims and Martin (1997)
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin (1997)

	ART	ARA	ACA	Orbatidae	*Oppia*	*nitens*	2b	na		Yu et al. (1997)
				Scheloribatidae	*Scheloribates*	*praeincisus*	2b	na		Oliveira et al. (2007)
				Phytoseiidae	*Euseius*	*californicus*	2b	na		de Castro et al. (2013)
Cry1Ba	ART	INS	COL	Chrysomelidae	*Phaedon*	*cochleariae*	4	2000	μg/ml	Zhong et al. (2000)
				Cerambycidae	*Anoplophora*	*glabripennis*	1	na		D'Amico et al. (2004)
	ART	INS	DIP	Culicidae	*Aedes*	*aegypti*	3	2000	μg/ml	Zhong et al. (2000)
Cry1Ia	ART	INS	COL	Chrysomelidae	*Agelastica*	*coerulea*	4	1900	μg/ml	Shin et al. (1995)
					Phaedon	*brassicae*	4	1900	μg/ml	Shin et al. (1995)
					Diabrotica	sp.	5a	na		Kostichka et al. (1996)
				Tenebrionidae	*Tenebrio*	*molitor*	2a	na		Gleave et al. (1993)
	ART	INS	DIP	Tipulidae	*Tipula*	*oleracea*	4	100	μg/ml	Ruiz de Escudero et al. (2006)
				Culicidae	*Culex*	*pervigalans*	3	2000	μg/ml	Gleave et al. (1993)
	ART	ARA	ACA	Phytoseiidae	*Euseius*	*concordis*	2a	6	μg/ml	de Castro et al. (2013)
Cry2Aa	ART	INS	COL	Chrysomelidae	*Leptinotarsa*	*decemlineata*	5a	9	$\mu g/cm^2$	Sims (1997)

(continued)

Table 10.4 (continued)

Toxin	Phylum	Class	Order	Family	Genus	Species	Method	Dose	Unit	Reference
					Diabrotica	*undecimpunctata*	5a	9	µg/cm^2	Sims (1997)
					Diabrotica	*virgifera*	5a	15	µg/cm^2	Sims (1997)
				Coccinellidae	*Hippodamia*	*convergens*	2a	50	µg/ml	Sims (1997)
				Curculionidae	*Anthonomus*	*grandis*	2a	100	µg/ml	Sims (1997)
	ART	INS	HEM	Aphididae	*Rhopalosiphum*	*padi*	2a	200	µg/ml	Sims (1997)
				Aleyrodidae	*Bemisia*	*tabaci*	2a	200	µg/ml	Sims (1997)
				Lygaeidae	*Oncopeltus*	*fasciatus*	2a	200	µg/ml	Sims (1997)
				Miridae	*Lygus*	*hesperus*	2a	500	µg/ml	Sims (1997)
	ART	INS	HYM	Apidae	*Apis*	*mellifera*	2a	50	µg/ml	Sims (1997)
				Braconidae	*Macrocentrus*	*ancylivorus*	2a	200	µg/ml	Sims (1997)
					Meteorus	*pulchricornis*	2a	200	µg/ml	Sims (1997)
				Pteromalidae	*Nasonia*	*vitripennis*	2a	50	µg/ml	Sims (1997)
	ART	INS	ISO	Rhinotermitidae	*Reticulitermes*	*flavipes*	2a	200	µg/ml	Sims (1997)
	ART	INS	NEU	Chrysopidae	*Chrysoperla*	*sinica*	2b	34	µg/g	Wang et al. (2013)
					Chrysoperla	*sinica*	2a	300	µg/ml	Wang et al. (2013)
					Chrysoperla	*carnea*	2a	50	µg/ml	Sims (1997)
	ART	INS	ORT	Gryllidae	*Acheta*	*domesticus*	2a	100	µg/ml	Sims (1997)
	ART	INS	BLA	Blatellidae	*Blatella*	*germanica*	2a	100	µg/ml	Sims (1997)
	ART	HEX	CO	Isotomidae	*Folmosia*	*candida*	2a	na		Sims and Martin (1997)
					Folmosia	*candida*	2a	200	µg/ml	Sims (1997)
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin (1997)
					Xenylla	*grisea*	2a	200	µg/ml	Sims (1997)

	ART	CRU	IS	Porcellanidae	*Porcellio*	*scaber*	2a	200	µg/ml	Sims (1997)
Cry2Ab	ART	INS	COL	Coccinellidae	*Coleomegilla*	*maculata*	2a	400	µg/ml	Li et al. (2011)
	ART	INS	HEM	Anthocoridae	*Orius*	*albidipennis*	2c	na		Gonzales-Zomara et al. (2007)
	ART	INS	NEU	Chrysopidae	*Chrysoperla*	*carnea*	2c	na		Rodrigo-Simon et al. (2006)
Cry3Aa	ART	INS	LEP	Notodontidae	*Clostera*	*anachoreta*	2b	13	µg/g	Zhang et al. (2011)
				Noctuidae	*Spodoptera*	*exigua*	2a	na		Hernstadt et al. (1986)
					Trichoplusia	*ni*	2a	na		Hernstadt et al. (1986)
	ART	INS	DIP	Culicidae	*Aedes*	*aegypti*	2a	na		Hernstadt et al. (1986)
				Muscidae	*Musca*	*domestica*	2a	na		Hernstadt et al. (1986)
	ART	INS	HEM	Aphididae	*Macrosiphum*	*euphorbiae*	2b	na		Ashouri et al. (2001)
					Myzus	*persicae*	2b	na		Kalushkov and Nedved (2005)
	ART	HEX	CO	Isotomidae	*Folsomia*	*candida*	2a	na		Sims and Martin (1997)
				Hypogastruridae	*Xenylla*	*grisea*	2a	na		Sims and Martin (1997)
Cry8Da	ART	INS	LEP	Noctuidae	*Spodoptera*	*litura*	4	2	µg/ml	Asano et al. (2003)

(continued)

Table 10.4 (continued)

Toxin	Phylum	Class	Order	Family	Genus	Species	Method	Dose	Unit	Reference
				Bombycidae	*Bombyx*	*mori*	4	2	µg/ml	Asano et al. (2003)
Cry11Aa	ART	INS	LEP	Sphingidae	*Manduca*	*sexta*	5a	1	µg/cm^2	Feldmann et al. (1995)
Cry22Aa	ART	INS	COL	Chrysomelidae	*Diabrotica*	*virgifera*	5a	na		Mettus and Baum (2000)
Cry22Ab	ART	INS	LEP	Noctuidae	*Heliothis*	*virescens*	na			Mettus and Baum (2000)
					Helicoverpa	*zea*	na			Mettus and Baum (2000)
					Spodoptera	*exigua*	na			Mettus and Baum (2000)
					Spodoptera	*frugiperda*	na			Mettus and Baum (2000)
				Pyralidae	*Ostrina*	*nubilalis*	na			Mettus and Baum (2000)
	ART	INS	COL	Chryso	*Leptinotarsa*	*decemlineata*	na			Mettus and Baum (2000)
				melidae	*Diabrotica*	*undecimpunctata*	na			Mettus and Baum (2000)
Cry51Aa	ART	INS	LEP	Noctuidae	*Heliothis*	*virescens*	5a	26	µg/cm^2	Baum et al. (2012)
					Helicoverpa	*zea*	5a	26	µg/cm^2	Baum et al. (2012)
					Agrotis	*ipsilon*	5a	26	µg/cm^2	Baum et al. (2012)
					Spodoptera	*frugiperda*	5a	26	µg/cm^2	Baum et al. (2012)

				Pyralidae	*Ostrina*	*nubilalis*	5a	26	µg/cm^2	Baum et al. (2012)
	ART	INS	COL	Chrysomelidae	*Diabrotica*	*virgifera*	5a	26	µg/cm^2	Baum et al. (2012)
Vip1A/Vip2A	ART	INS	COL	Chrysomelidae	*Leptinotarsa*	*decemlineata*	2a	na		Warren (1997)
				Tenebrionidae	*Tenebrio*	*molitor*	2a	na		Warren (1997)
	ART	INS	LEP	Pyralidae	*Ostrina*	*nubilalis*	2a	na		Warren (1997)
				Noctuidae	*Agrotis*	*ipsilon*	2a	na		Warren (1997)
					Spodoptera	*exigua*	2a	na		Warren (1997)
					Spodoptera	*frugiperda*	2a	na		Warren (1997)
					Heliothis	*virescens*	2a	na		Warren (1997)
					Helicoverpa	*zea*	2a	na		Warren (1997)
				Sphingidae	*Manduca*	*sexta*	2a	na		Warren (1997)
	ART	INS	DIP	Culicidae	*Culex*	*pipiens*	3	na		Warren (1997)
Cyt1Aa	ART	INS	LEP	Sphingidae	*Manduca*	*sexta*	1	na		Koni et al. (1994)
				Gelechiidae	*Pectinophora*	*gossypiella*	2a	1	µg/ml	Meyer et al. (2001)
				Plutellidae	*Plutella*	*xylostella*	4	40	µg/ml	Meyer et al. (2001)

Phylum: *ART* Arthropoda, *ANN* Annelida, *MOL* Mollusca. Class: *INS* Insecta, *HEX* Hexapoda, *CRU* Crustacea, *ARA* Arachnida, *GAS* Gastropoda, *CLIT* Clitellata. Order: Abbreviations as in Table 10.2; *ISO* Isoptera, *ORT* Orthoptera, *BLA* Blattodea, *THY* Thysanoptera, *CO* Collembola, *AMP* Amphipoda, *IS* Isopoda, *CLAD* Cladocera, *ACA* Acari, *AR* Araneae, *HAP* Haplotaxida. Methods: numbers indicating various bioassay methods are as in Table 10.2; *na* not available; Dose: *na* not available. References: citations not listed in Reference section can be found in Marvier et al. (http://delphi.nceas.ucsb.edu/btcrops)

Culex quinquefasciatus; Smith et al. 1996), while Cry1Ia is toxic to two Coleoptera (*Leptinotarsa decemlineata*, *Anthonomus grandis*; Tailor et al. 1992; Naimov et al. 2001; Ruiz de Escudero et al. 2006; Martins et al. 2008). Two other proteins are active against Coleoptera and Lepidoptera: the Coleoptera-active Cry8Da affects *Plutella xylostella (*Lepidoptera; Asano et al. 2003), while Cry22Ab affects some Lepidoptera (*Trichoplusia ni*, *P. xylostella*; Mettus and Baum 2000, bioassay data not reported). Coleoptera-active proteins can affect other orders as well: Cry22Aa is toxic to *A. grandis* (Isaac et al. 2001; bioassay data not reported) and pharaoh ants (Hymenoptera: *M. pharaonis*; bioassay data not reported; Payne et al. 1997), while Cyt2Ca was reported to affect the cat flea (Siphonaptera: *Ctenocephalides feli*; bioassay data not reported; Rupar et al. 2000). Two Diptera toxins, Cry4Aa and Cry11Aa, affect aphids (Hemiptera: *Acyrthosiphon pisum*, Porcar et al. 2009; *Macrosiphum euphorbiae*, Walters and English 1995), while a third, Cry10Aa, was recently demonstrated to be toxic to cotton boll weevil (*A. grandis*; de Souza Aguiar et al. 2012). Proteins with dual toxicity to Lepidoptera and Diptera include Cry30Fa (*A. aegypti* and *P. xylostella*; Tan et al. 2010), Cry30Ga (*A. aegypti* and *P. xylostella*; Zhu et al. 2010a), Cry54Aa (*A. aegypti* and *Spodoptera exigua*, *Helicoverpa armigera*; Tan et al. 2009), and Cry56Aa (*A. aegypti* and *P. xylostella*, *H. armigera*; Zhu et al. 2010b). The last of the 14 proteins affecting two insect orders is the binary Vip1A/Vip2A toxin, which is toxic to corn rootworms (Coleoptera: *D. virgifera*, *D. longicornis*, *D. undecimpunctata*; Warren 1997) and cotton aphid (Hemiptera: *Aphis gossypii*; Sampurna and Maiti 2011).

The remaining three proteins were reported to affect species in both Insecta and Nematoda. More specifically, coleopteran activity has been cited for Cry14Aa (*Diabrotica* spp.; Payne and Narva 1994; bioassay data not reported) and Cry55Aa (flea beetles; Bradfisch et al. 1999; bioassay data not reported), proteins which are known to affect nematodes (Payne et al. 1996; Wei et al. 2003; Guo et al. 2008; Peng et al. 2011). In addition, recent studies have revealed activity of the well-known coleopteran Cry3Bb toxin against a free-living soil nematode (Höss et al. 2011, 2013).

10.3.2 Across Three Orders

Seven of the 28 cross-active proteins were reported to have activity in three orders (Fig. 10.7, middle). The Lepidoptera-active Cry1Ac is toxic to tsetse flies (Omolo et al. 1997) and an aphid (Li et al. 2011). The Lepidoptera-active Cry1Ba affects several Coleoptera, including *L. decemlineata*, *Chrysomela scripta*, *Hypothenemus hampei* (Bradley et al. 1995; Federici and Bauer 1998; Zhong et al. 2000; Naimov et al. 2001; Lopez-Pazos et al. 2009), and *A. grandis* (Martins et al. 2006; bioassay data not reported), as well as some Diptera (*Lucilia cuprina*; Heath et al. 2004), bioassay data not reported; *Musca domestica* (Zhong et al. 2000). Cry2Ab has well-documented toxicity to many species of Lepidoptera, is toxic to *A. aegypti* (Ahmad et al. 1989) and *A. gambiae* (McNeil and Dean 2011), and affects nematodes

(Höss et al. 2013). Besides its well-established toxicity to Coleoptera, Cry3Aa was reported to have activity to Hemiptera (*M. euphorbiae* and *A. pisum*; Walters and English 1995; Porcar et al. 2009; Li et al. 2011) and Hymenoptera (*Solenopsis invicta*; Bulla and Canda 2004). Cry5Aa and Cry5Ab are toxic to nematodes (Phylum: Nematoda) in two orders (Narva et al. 1991; Sick et al. 1994) and were reported to affect the liver fluke *Fasciola hepatica* (Phylum: Platyhelminthes; Narva et al. 1991), but validity of those tests was compromised by high control mortality. Cry51Aa is toxic to plant bugs *Lygus hesperus* and *L. lineolaris* (Hemiptera), as well as to *L. decemlineata* (Coleoptera) (Baum et al. 2012). Lepidopteran activity needs to be confirmed: Cry51Aa2 was not toxic to several Lepidoptera (*Heliothis virescens*, *Agrotis ipsilon*, *S. frugiperda*, *Ostrinia nubilalis*, *H. zea*) (Baum et al. 2012), whereas Cry51Aa1, which differs by seven amino acids, was toxic to *Bombyx mori* (Huang et al. 2007; bioassay data not reported).

10.3.3 Across Four or More Orders

Cry2Aa and Cyt1Aa were reported to affect species in four orders (Fig. 10.7, bottom). In addition to its known dipteran and lepidopteran activities, Cry2Aa is toxic to *M. euphorbia* (Hemiptera; Walters and English 1995). Hilbeck et al. (1999) reported low levels of mortality in the lacewing *Chrysoperla carnea* (Neuroptera) when reared on Cry2Aa-fed *S. littoralis* larvae, but other studies showed no effect of high Cry2Aa concentrations on either *C. carnea* (Sims 1997) or *C. sinica* (Wang et al. 2012a). The Diptera-active Cyt1Aa is toxic to Coleoptera (*C. scripta*; Federici and Bauer 1998), Hemiptera (*A. pisum*; Porcar et al. 2009), and Lepidoptera (*P. xylostella*) in one study (Sayyed et al. 2001) but not in another (Meyer et al. 2001).

The two remaining proteins (Cry1Ab, Cyt1Ba) were reported to be active across more than four orders. Because of its widespread use in transgenic crops, Cry1Ab has been tested against the broadest range of species, spanning three phyla and six classes, resulting in reports of possible activity in one nematode order (Höss et al. 2008) and six insect orders. The only cross order activity of Cry1Ab within the class Insecta that has been substantiated, however, is its toxicity to Hemiptera (*A. pisum*; Porcar et al. 2009). Its dipteran activity was inferred from Haider et al. (1986), who reported *A. aegypti* toxicity of a purified *aizawai* crystal protein but did not demonstrate that the activity was due to Cry1Ab. Subsequent work with the Cry1Ab7 gene cloned from that strain demonstrating toxicity to lepidopteran and dipteran cell lines did not include in vivo tests (Haider and Ellar 1987). Survival of the coccinellids (Coleoptera) *Cheilomenes sexmaculatus* and *Adalia bipunctata* were affected by high doses of Cry1Ab (Dhillon and Sharma 2009; Schmidt et al. 2009), but subsequent studies did not confirm toxicity to *A. bipunctata* (Porcar et al. 2010; Alvarez-Alfageme et al. 2011) or other coccinellids (Pilcher et al. 1997; Lundgren and Wiedemann 2004; Bai et al. 2006; Alvarez-Alfageme et al. 2008). Likewise, reports of Cry1Ab toxicity to *C. carnea* (Neuroptera; Hilbeck et al. 1998, 1999; Dutton et al. 2002) are at odds with other studies showing no effects (Pilcher et al. 1997;

Rodrigo-Simon et al. 2006), even at very high dose levels (Romeis et al. 2004). Effects of Cry1Ab-corn material on survival of the caddisfly *Helicopsyche borealis* (Trichoptera) reported by Rosi-Marshall et al. (2007) cannot be unequivocally attributed to Cry1Ab toxicity because isogenic corn was not included as a control for effects of other plant-derived factors (Beachy et al. 2008).

Cyt1Ba has possibly the most diverse insecticidal activity spectrum of all *B. thuringiensis* proteins known to date. It was reported to be toxic to the leaf-mining fly *Liriomyza trifolii* (Diptera; Payne et al. 1995); the tarnished plant bug *L. hesperus* (Hemiptera; Stockhoff and Conlan 1998); several Coleoptera including *D. virgifera* and *Hypera postica*, as well as *A. grandis* and *Lissorhoptrus oryzophilus* (bioassay data not reported) (Payne et al. 1995; Bradfisch et al. 1998); at least one Lepidoptera (*Choristoneura fumiferana*; Van Frankenhuyzen and Tonon 2013); and several species of sawflies (Hymenoptera; Van Frankenhuyzen and Tonon 2013). Hemipteran toxicity needs to be confirmed, as Wellman-Desbiens and Côté (2005) reported no toxicity of Cyt1Ba against *Lygus* bugs and provided evidence that solubilization buffer may have contributed to the toxicity reported by Stockhoff and Conlan (1998).

10.3.4 Reported Versus Substantiated

Using mortality estimates as reasonable but minimum evidence for cross-activity, there is insufficient evidence (i.e., qualitative data only) in the following cases: toxicity of Cry5Aa and Cry5Ab to Platyhelminthes, Cry14Aa to Coleoptera, Cry22Aa to Coleoptera and Hymenoptera, Cry22Ab to Lepidoptera, Cry51Aa to Lepidoptera, Cry55Aa to Coleoptera, and Cyt2Ca to Siphonaptera. In addition, quantitative data contradicted by other studies present enough uncertainty to conclude lack of evidence for unequivocal cross-activity. This is the case for Cry1Ab and Coleoptera, Diptera, Neuroptera, and Trichoptera; Cry2Aa and Neuroptera; Cyt1Aa and Lepidoptera; and Cyt1Ba and Hemiptera. Excluding those cases reduces the number of reported cross-activities for 28 proteins affecting 75 high-ranking taxa to substantiated cross-activities for 21 proteins affecting 51 taxa. Substantiated cross-activities are indicated in Fig. 10.7 by light-gray shading.

10.4 Biological Significance of Cross-Activities

Quantitative estimates of toxicity (LC_{50}s) to species outside orders of primary affinity are available for 16 proteins and 24 taxa (excluding known or novel dual-order activities of Cry2Aa, Cry2Ag, Cry30Fa, Cry30Ga, Cry54Aa, and Cry56Aa) (Table 10.3). Plotting those toxicity values with toxicity ranges of the reference proteins from Table 10.2 shows how cross-activities vary widely across proteins and taxa (Fig. 10.8). Fifteen of the reported activities (LC_{50}s; but EC_{50}s for nematode activity of Cry1Ab, Cry2Ab, Cry3Bb) group within or above those of

nematode-active proteins in the low-toxicity range, seven group with the majority of insect-active proteins in the medium-toxicity range, and two fall in the high-toxicity category bench-marked by Diptera-active Cry11 proteins.

The first type of cross-activity that is pertinent to the issue of ecological safety of *B. thuringiensis* pesticidal proteins are activities in orders outside the suite of orders that is normally affected, such as toxicity of Lepidoptera-, Diptera-, and Coleoptera-active proteins to Hemiptera, Hymenoptera, and Nematoda. Hemipteran toxicity was substantiated for nine proteins (Cry1Ab, Cry1Ac, Cry2Aa, Cry3Aa, Cry4Aa, Cry11Aa, Cry51Aa, Cyt1Aa, Vip1A/Vip2A) and quantified for four of those (Table 10.3). Cry4Aa and Cry11Aa have low toxicity to aphids with LC_{50} estimates ranging between 125 and 500 μg/ml. Qualitative data indicate similar toxicity of Cry1Ab, Cry1Ac, Cry2Aa, Cry3Aa, and Cyt1Aa, with doses of 100–500 μg/ml resulting in mortalities between 10% and 90%. Toxicity of Cry51Aa to *Lygus* bugs is up to sixfold higher than aphid toxicity of other Cry proteins but still falls in the low-toxicity range (Fig. 10.8). In contrast, the Vip1A/Vip2A binary toxin is two to three orders of magnitude more toxic to aphids than most aphid-active crystal proteins and falls well within the range of medium toxicity. Hymenopteran activity is confined to two proteins: Cyt1Ba has low toxicity to sawflies, and Cry3Aa has high toxicity to fire ants. Three insect-active proteins (Cry1Ab, Cry2Ab, Cry3Bb) affected growth or reproduction of a soil-dwelling nematode but at dose levels that were two orders of magnitude higher than is typical for nematode-active proteins (Table 10.5).

The second type of cross-activity that is of interest from an ecological safety perspective includes activities that are within the suite of orders normally affected but outside a protein's primary-order affinity, especially when that activity occurs at levels that are within the toxicity range of corresponding reference proteins. This group includes dipteran toxicity of Cry1Ac, which is within the expected toxicity range of Diptera-active proteins, lepidopteran toxicity of Cry8Da within the range of Lepidoptera-active reference proteins, and coleopteran toxicities of lepidopteran (Cry1Ba, Cry1Ia) and dipteran (Cry10Aa, Cyt1Aa, Cyt1Ba) toxins that are within the range of Coleoptera-active proteins. The high toxicity of Vip1A/Vip2A to corn rootworm makes it the most active coleopteran toxin known to date, having an LC_{50} that is ~200-fold lower than the (inferred) median value of Coleoptera-active proteins in diet-incorporation assays. Dipteran toxicity of Cry1Ba, Cry1Ca, and Cyt1Ba are of less interest from a nontarget safety perspective because those toxicities occur at high dose levels compared to Diptera-active reference proteins.

10.5 Discussion

The past few decades have revealed an astounding diversity of *B. thuringiensis* crystal protein genes encoding proteins that are pesticidal to a broad array of taxa. Available data undoubtedly underestimate actual diversity because characterization of biological activity lags far behind gene discovery. The specificity picture is fragmentary at best, considering that (i) more than half (171) of the 329 proteins have

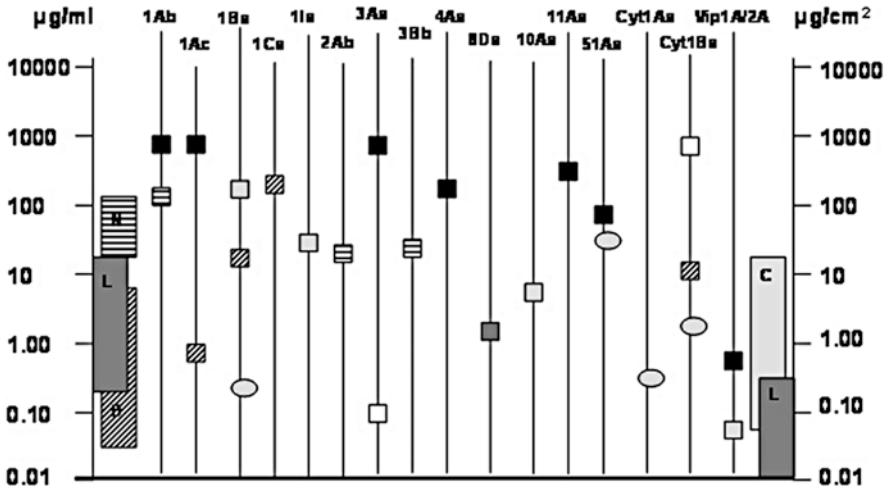

Fig. 10.8 Comparison of toxicities outside orders of primary specificity of 16 cross-active proteins with reference toxicity ranges of predominantly Lepidoptera (*L*)-, Coleoptera- (*C*), Diptera- (*D*), and Nematoda- (*N*) active proteins (from Table 10.2). Reported LC_{50} values are presented as *squares* (estimates expressed in µg/ml, left *Y*-axis) or *ovals* (estimates expressed in µg/cm^2, right *Y*-axis). Hatching inside the symbols corresponds to orders indicated by hatching of the *bars* representing toxicity ranges of the reference proteins. Additional order activities: Hemiptera (*black squares*) and Hymenoptera (*open squares*). Positions of symbols are approximate; exact values can be found in Table 10.3

not been tested at all, (ii) the majority of the 158 tested proteins were bioassayed against a limited number of species (10 or less), and (iii) species and toxins tested are not equally distributed across protein families and taxa (Fig. 10.1).

Host specificity of *B. thuringiensis* is a much treasured feature that makes it attractive for environmentally acceptable pest control applications. Specificity was initially recognized as biological activity of strains that was limited to specific insect orders, in particular Lepidoptera, Coleoptera, and Diptera. The first classification of crystal proteins (Höfte and Whiteley 1989) reflected that order specificity and included only one family with dual specificity. Since then, testing has revealed cross order activity in at least 21 toxin proteins distributed over 14 primary rank families across all three protein classes (Cry, Cyt, and Vip). Cross-toxicities supported by reasonable evidence are so far limited to the class Insecta in the phylum Arthropoda. Testing of insecticidal proteins against species in other Arthropod classes and other phyla (Table 10.4, Fig. 10.7) has not produced conclusive evidence of lethal activity outside the class Insecta.

The lack of cross-activity in the remaining protein family's needs to be interpreted with caution, because the lack of presence is not proof of absence. Although it is unknown to what extent the absence of cross-activity is the result of insufficient testing, the majority of proteins have not been tested extensively across high-ranking taxa (Fig. 10.1). Only 36% of 97 proteins with reported "single-order" activity (Fig. 10.5) were actually tested against species outside their primary order (data not

Table 10.5 Concentrations (μg/ml) of various proteins causing 50% inhibition of reproduction, growth or survival in three species of Nematoda (citation in parentheses)

	C. elegans			*M. hapla*	*M. incognita*
Protein	Reproduction	Growth	Survival	Survival	Survival
Cry1Ab	54 (2)	225 (2)			
Cry2Ab	23 (4)				
Cry3Bb	8 (3,4)	22 (3)			
Cry5Aa			10 (5)		
Cry5Ab			32 (5)		
Cry5Ba	0.066 (7)[a]	0.042 (8)	6.7 (4)[b]	18 (1)	146 (6)
Cry6Aa	0.230 (7)[a]			24 (6)	383 (6)
Cry14Aa	0.016 (7)[a]				
Cry21Aa	0.047 (7)[a]				
Cry55Aa				23 (1)	103 (6)

(1) Guo et al. (2008); (2) Höss et al. (2008); (3) Höss et al. (2011); (4) Höss et al. (2013); (5) Narva et al. (1991); (6) Peng et al. (2011); (7) Wei et al. (2003); (8) Wang et al. (2012a)

[a]Wei et al. reported EC_{50} values in ng/μl in the text but in ng/ml in their Fig. 10.3c; the correct unit is ng/ml (see Höss et al. (2013)) [2]

[b]Reported in that reference as a personal communication by R. Aroian

shown), illustrating how selection of test species is biased toward the order for which activity was initially reported. Such bias obviously limits the evaluation of cross-activities, and it is likely that more will be uncovered as testing is expanded. This notion is supported by the fact that 37% of the 75 proteins that were tested against species in two orders or more (Fig. 10.1, bottom) were reported to be cross-active (Fig. 10.5). This high proportion suggests that cross-activities may be more common than indicated by the number of substantiated cases. For example, many cross-activities were reported in patents without supporting data but are presumably based on evidence locked up in proprietary company files. Notable examples are the frequently cited but unsubstantiated coleopteran activity of several nematode-active proteins (Cry6A, Cry14A, and Cry 55A).

Positive evidence of cross-activity needs to be interpreted with caution as well. Although supported by actual mortality data for 21 proteins (Table 10.3), the evidence is not strong in most cases, considering that (i) only a little more than half (58%) of the mortality data reported in Table 10.3 involve actual LC_{50} estimates, (ii) very few of those include statistics that allow rigorous evaluation of dose-mortality regressions, and (iii) most reports are based on single studies that have yet to be confirmed by work in other laboratories. The only cross-activities that have been validated by independent studies are toxicity of Cry1B, Cry1Ia, and Cyt1Ba to Coleoptera and toxicity of Cry3Aa and Cry11Aa to Hemiptera (Table 10.3). All other cross-activities need to be viewed with caution until they are confirmed through additional testing, in particular the high toxicity of Cry3Aa to fire ants, which has not been confirmed nor followed up since its discovery more than 10 years ago.

Cross-activities of *B. thuringiensis* pesticidal proteins have potential implications for ecological (nontarget) safety of transgenic crops, an issue that has become

the focus for intense scientific, pubic, and regulatory debate (Wolfenbarger and Phifer 2000). The degree of concern depends on the level of toxicity relative to toxicities of proteins within their orders of primary specificity. This was evaluated by plotting quantitative toxicity estimates (LC_{50}s) for cross-active proteins with their normal (target) toxicity range (Fig. 10.8). Almost two-thirds of the quantified cross-activities are presumably of limited biological significance because they involve toxicities that are low (occurring at >10 mg/ml) compared to toxicity ranges of the so-called order-specific reference proteins (Fig. 10.8). For example, low inherent toxicity of Cry proteins to aphids in laboratory assays is congruent with numerous studies showing no adverse effects of Cry proteins on aphids in transgenic crops (e.g., Kalushkov and Nedved 2005; Ramirez-Romero et al. 2008; Zhang et al. 2008; Digilio et al. 2012). However, low-toxicity proteins can affect (target) pests when expressed in transgenic plants, as was the case for Cry5Ba expressed in tomato roots against root-knot nematodes (Li et al. 2008) and Cry51Aa expressed in cotton against *Lygus* bugs (Baum et al. 2012). This means that cross-activities in the low-toxicity category could have implications for susceptible nontarget species that may require closer scrutiny.

Effects of Cry1Ab, Cry2Ab, and Cry3Bb on *C. elegans* (Höss et al. 2008, 2011, 2013) are the first substantiated cases of cross-phylum activity and merit closer scrutiny. Although such reports are readily cited as proof of the lack of specificity of proteins used in transgenic crops, it is important to note the dose levels at which nematodes were affected. Although activities of Cry1Ab, Cry2Ab, and Cry3Bb plot within the range of Nematode-active proteins in Fig. 10.8, they are based on inhibition of growth and reproduction, a response that typically requires a 100-fold higher dose than mortality (Table 10.5, LC_{50} vs EC_{50} of Cry5Ba). Because estimates of the EC_{50}s for inhibition of reproduction were about two orders of magnitude greater than such EC_{50}s of known Nematode-active proteins (Table 10.5, EC_{50} of Cry1Ab, Cry2Ab, and Cry3Bb vs EC_{50} of Cry5B, Cry6A, Cry14A, and Cry21A), reported activities are not likely to be of ecological significance. This is supported by studies showing no effects of transgenic crops on nematode abundance, growth, reproduction, or community structure (Saxena and Stotzky 2001; Griffiths et al. 2007; Höss et al. 2011).

One-third of reported cross-activities fall within the range of toxicities exhibited by order-specific proteins commonly used in pest control applications. These activities are of potential biological interest, meaning that their development for pest control applications (such as Cry10Aa for control of boll weevil) would benefit from additional nontarget effect studies. Cross-activities in the high-toxicity category flag a need to address possible ecological concerns. For example, if Cry3Aa is confirmed to be highly toxic to fire ants, it may be desirable to further investigate direct and indirect effects of Cry3A crops on ants. Although transgene applications of Vip1A/Vip2A to combat corn rootworm are not being considered, such applications certainly need to take into account possible effects on plant-sucking insects like aphids.

In conclusion, expanded testing of *B. thuringiensis* pesticidal proteins has revealed activities outside their ranges of primary specificity. Cross-activities are not uncommon, having been substantiated for ~13% of the 158 proteins tested to

date, and may be more prevalent considering that at least one-third of proteins that were tested against species in two or more orders were reported to be cross-active. Substantiated cross-activities are to date primarily limited to the class Insecta, with 12 proteins affecting species across two orders, 5 proteins affecting three orders, and 1 protein affecting four orders. Cross-phylum activity is known for three insecticidal proteins that also affect nematodes, albeit at a sublethal level. One-third of quantified cross-activities occurred at toxicity levels that are well within or even below the toxicity range of order-specific proteins, indicating a potential for effects on (nontarget) species outside their normal host range. Most cross-activities have yet to be confirmed through independent testing, but the few that have been validated firmly establish the notion that *B. thuringiensis* proteins are not as order-specific as was conventionally believed to be the case. This requires attention in the design and regulatory approval of their use in pest control applications.

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Chapter 11
The American *Bacillus thuringiensis* Based Biopesticides Market

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Abstract Bt-based biopesticides are among the main tactics for agricultural pest control in many production systems, mainly due to high efficiency and the ability to preserve natural enemies and the environment. North America (Canada and USA) and Brazil stand out as the two largest world markets for these products. The main targets are larvae of the spruce budworm (*Choristoneura fumiferana*) responsible for forest defoliation in Canada and the gypsy moth (*Lymantria dispar*) in the United States. Bt biopesticides have been used against these pests since the 1980s and represent the oldest IPM system using these products worldwide. The Brazilian example is more recent and involves two species of noctuids, *Helicoverpa armigera* and *Chrysodeixis includens* which became very serious problems in all agricultural areas. Bt biopesticides provided a satisfactory control efficiency, making Brazil a world reference. The total Bt biopesticide usage reached over 4.5 million liters/kilograms in the 2013/2014 crop seasons, which corresponds to a sprayed area of approximately 9 million ha. Reasons for this increase, and subsequent decrease, in the Brazilian Bt biopesticides market are discussed in this chapter.

Keywords Biological control • Bt • Lepidoptera • IPM systems

Bacillus thuringiensis (Bt) based biopesticides, available worldwide since the 1970s, have received special attention as a tool to reach a more sustainable agriculture aiming to preserve the environment and to ensure production. Moreover, due to the increasing costs of production of new pesticides (Glare et al. 2012) and the

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increasing number of resistance reports (Tabashnik et al. 2014), these biopesticides are an important alternative to be increasingly exploited in integrated pest management systems.

The formulation of Bt based biopesticides was favored by the effectiveness and specificity of Bt strains and their toxins and in 2010, 300 products accounted for 53% of bioinsecticide worldwide market, representing an annual turnover of 210 million dollars. The share of Bt based biopesticides in the worldwide market has been declining since 2000. In that year, the share was 90%, decreasing to 60% in 2005 and to 53% in 2010. This reduction was due to large increases in the use of entomopathogenic viruses (+100%) and entomopathogenic fungi (+52%), while the market for Bt products increased by only 36%. The American continent is responsible for 50% of this market, mainly the United States and Canada while Latin America represents only 8–10% of the total (CAB International Center 2010).

This chapter describes Bt biopesticide use in two very typical cases: North America (Canada and USA) and Brazil. These two cases represent the major consumers of Bt-based bio-insecticides; the first with a longer history and the second the most recent case, where pest outbreaks have resulted in the explosion of the Bt biopesticides market, making Brazil, for a moment, the world's largest Bt biopesticide market.

11.1 Brazil

In Brazil, the potential for *Bacillus thuringiensis* based bioinsecticides in the control of agricultural pests was first reported by Figueiredo et al. (1960) and Pigatti et al. (1960), however, the first big research project was only started in 1993 by EMBRAPA, with the objective of controlling the fall armyworm of corn, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Until the 1990s only three commercial *B. thuringiensis* based products were available in the Brazilian market, all of which were based on *B. thuringiensis kurstaki* (Dipel, Thuricide and Bactospeine) (Habib and Andrade 1991). The reduced use of these products was related to incorrect use, lack of advertising and with competition with conventional and low-cost insecticides with a quick action and broad spectrum (Alves et al. 1998). Until the beginning of this century, even though the number of products available in the market did not increase significantly, 6 *B. thuringiensis* based bioinsecticides were recommended for the control of 26 pests in forests, vegetables and row crops (Polanczyk et al. 2008).

This time defined a change in the concept of pest control, with the companies that sold these products highlighting some important advantages in the use of Bt bioinsecticides, such as, for example, the preservation of populations of parasitoids, predators and pollinators and the absence of insecticide residues in the food (Alves et al. 1998). However, in spite of said advantages, the area treated with Bt biopesticides was only around 150,000 ha at that time (Souza 2001). Some factors, in addition to those mentioned before, such as reduced spectrum and low field persistence contributed for such small market expansion.

The citrus fruit borer, *Ecdytolopha aurantiana* (Lepidoptera: Tortricidae) was one of the most significant pests in citrus orchards in Brazil at the end of the 1990s with losses of up to 60% in the production (Fundecitros 2000). The Bt based biopesticides are effective in the control of this pest when the first application is made, with over six females per trap and the second application is made 20–30 days thereafter. These products were used on around 50,000 ha of citrus orchards (Alves et al. 2001), mainly in the State of São Paulo. A great limitation for the use of Bt bioinsecticides is due to problems synchronizing the short period of exposure of the caterpillars and the application of the product, since the residual period is of around 2 days.

At that same time, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) was one of the most relevant defoliators of soybean and despite the high efficiency of the Bt bioinsecticides against such pest, its use was not significant at that time (Habib and Amaral 1985; Bobrowski et al. 2002), mainly due to the broad use of *Baculovirus anticarsia* (Nuclear Polyhedrosis Virus) in the control of said pest (Moscardi 1999).

One of the main pasture pests in Brazil is *Mocis latipes* (Striped Grass Looper) which appears in a cyclic manner throughout the years; high populations may significantly reduce the amount of forage available, completely devouring the leaf blade of the plants. Bt bioinsecticides can be used against small caterpillars at the initial outbreaks of the infestation (Fragoso and Alcantara 2014).

Before the use of the Bt plants in the control of *S. frugiperda*, the potential of Bt bioinsecticides in the control of this pest was reported by Correia (1986) and Habbib et al. (1987) and, later, some products were registered for its control. However, the need for periodic monitoring, low persistence and difficulty reaching the target inside the corn husk limited the efficiency of these products.

The Brazilian market for Bt bioinsecticides until 2012 was characterized by 9 registered products (Fig. 11.1), recommended for 24 species of pests in 12 crops,

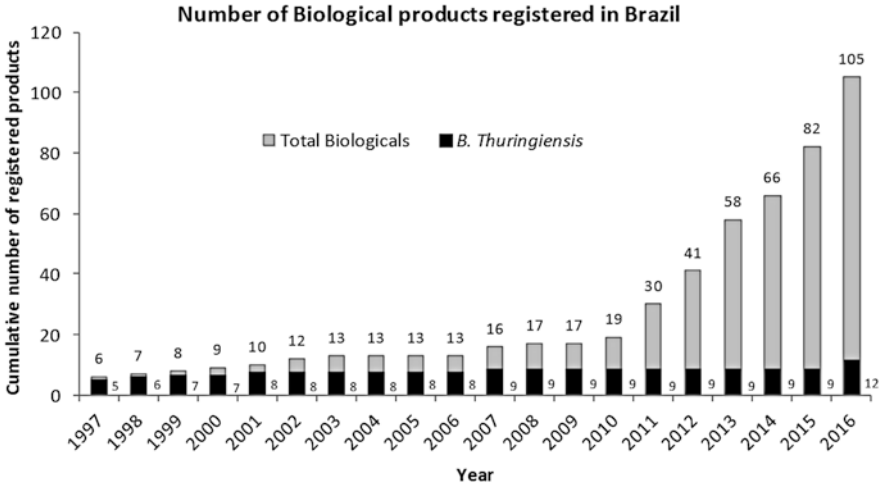

Fig. 11.1 Number of biological products and *Bacillus thuringiensis* based products registered in Brazil

but mostly for the control of *A. gemmatalis*, *S. frugiperda*, *E. aurantiana* and *P. xylostella* in crops such as soybean, cotton, corn and citrus. The volume of 300 tons traded annually generated sales results of USD 13 million (CAB International Center 2010).

During 2013/2014 crop seasons, the Brazilian market for Bt bioinsecticides had a significant increase, reaching over 4.5 million liters/kilograms traded per year, which corresponds to a sprayed area of approximately 9 million ha, distributed mainly among Soybean, Cotton, Corn and Citrus crops. These numbers make Brazil the largest market for Bt bioinsecticides in the world and, consequently, Bt as the most used biorational control agent, however, these estimates included the sales of registered and unregistered products, which makes it difficult to obtain exact data regarding this market.

The transformation of this market, with the explosion of sales of Bt based products, occurred due to the entry and rapid dissemination of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Brazil and the increase of *Chrysodeixis includens* (Lepidoptera: Noctuidae) in soybean areas. The expected losses caused by *H. armigera* for the 2013/2014 crop season was initially estimated at US$ 300 million for cotton and US$ 3 billion for soybean throughout Brazil. Subsequently, the losses were confirmed as US$ 600 million for cotton, only for the Bahia state, and US$ 300 million for the soybeans, only for Mato Grosso state. In Brazil, until the 1990s, *C. includens* was considered a secondary pest of various agricultural crops. Currently, large populations of this pest are frequently detected in soybeans in western Bahia, Goiás, Mato Grosso, Mato Grosso do Sul, São Paulo, Paraná and Rio Grande do Sul, causing severe defoliation and damage in formed pods (Tomquelski et al. 2015).

Another reason that drove the use of biological agents was the noted inefficiency of traditional pesticides for *H. armigera*, demanding integrated management strategies and, many times, the joint application of chemical and biological insecticides by the farmers. The integration of different controls is crucial for successfully implement the pest management. Therefore, even in the era of biotechnology, biological control, pesticides, and other pest control tactics will continue to be of great importance in controlling *H. armigera* (Pomari-Fernandes et al. 2015).

The populations of *H. armigera* have developed resistance to several traditional insecticides, as well as products that were recently released in the market (Ahmad et al. 2003; Patil et al. 2006; Wu 2007). Wyckhuys et al. (2013) highlight that 640 cases of resistance to insecticides of populations of said pest were reported in the world, surpassing *P. xylostella*. The incorrect identification of this species when the first outbreaks occurred in Brazil led to the option for inadequate pesticides and, consequently, to the inefficiency of this control strategy. Often, the farmer initially chooses to increase the number of applications and the dosage of the insecticides used, which may have facilitated the evolution of the resistance of populations of this pest to pesticides.

Furthermore, according to Sosa-Gomez et al. (2016) the introduction of *H. armigera* in Brazil occurred before October 2008. The authors also register that in

Table 11.1 Agrochemical sales by class 2011–2015. Brazilian Market

Class	Value – US$ MM					% Variation			
	2011	2012	2013	2014	2015	15/11	15/12	15/13	15/14
Total	8.488	9.710	11.454	12.249	9.608	13.20	−1.05	−16.12	−21.56
Insecticides	2.945	3.607	4.554	4.893	3.171	7.67	−12.09	−30.37	−35.19
Herbicides	2.743	3.135	3.739	3.903	3.086	12.50	−1.56	−17.46	−20.93
Acaricides	110	101	119	117	103	−6.36	1.98	−13.45	−11.97
Fungicides	2.315	2.469	2.592	2.907	2.901	25.31	17.50	11.92	−0.21
Others	375	398	450	429	347	−7.47	−12.81	−22.89	−19.11

Adapted from Sindiveg (2015)

August 2012 *H. armigera* was already present from southern to the extreme northern Brazil. Tay et al. (2013) pointed out that it is possible that the founders of the invasive populations include individuals with genetic make-ups that predisposes them to insecticide adaptation. There would be strong selection on these individuals to enable them to rapidly exploit agricultural systems where the endemic pests remain susceptible to conventional pesticides.

The possibility of using Bt based bioinsecticides, or even Bt plants, in the control of *H. armigera* is not new. In countries where this pest is broadly disseminated, there is a management system that involves monitoring with sexual pheromones, plants conveying Bt toxins, nuclear polyhedrosis virus (NPV), *Trichogramma* spp. and pesticides (Downes and Mahon 2012). Within this context, in addition to Bt products, Baculovirus based bioinsecticides were quickly accepted in the Brazilian market and, after the emergency registration of some formulas, also became part of the management of *H. armigera* in the main soybean and cotton production areas of Brazil.

The efficacy of Bt for this pest drew the attention of the companies, which accelerated the registration of Bt based bioinsecticides, such as Best®, Costar® and BtControl®, all of which were registered in 2016. The amount of Bt bioinsecticides available in the Brazilian market almost doubled (Fig. 11.1), with 12 products currently registered with MAPA and over 10 under registration or emergency registration for *H. armigera* (Agrofit 2017).

The soybean crop with incidences of *H. armigera* and *C. includens* was the great propeller for the use of Bt based products in Brazil during 2012/2013/2014 crop seasons. However, a Bt soybean expressing the protein Cry1Ac was launched in 2013/2014 crop season. The Intacta® soybean was extensively used in 2015, mostly due to the efficiency and quick acceptance in Brazil (Dourado et al. 2016).

A rapid decrease in the incidence of larvae in soybean crops and, consequently, a steep drop in the demand for chemical and biological insecticides were observed. The annual sale of insecticides in 2015 was $3.17 billion (Table 11.1), representing a retraction of 35.19% compared to 2014 when sales reached $4.89 billion (Sindiveg 2015). Considering only the Bt biopesticide market, it is estimated that during the 2015 season, a retraction of over 70% in the sales of Bt based products was observed, with said reduction being even steeper for baculovirus based products.

Despite the decline observed in those last two crop seasons, the Brazilian market continues to be very promising for Bt products. Bt formulations have been consoli-

dated in the integrated pest management in different crops, with emphasis on cotton, citrus, soy and forest, and new opportunities will appear in the medium and long term that will make the use of these control agents grow again in Brazilian agriculture. In the crop season 2016/2017 *H. armigera* outbreaks were reported in some Brazilian states leading to the used of wide broad spectrum pesticides although Bt biopesticides are available on the market. It is essential that the rural extension service be strengthened to make possible the implementation of an IPM system to this pest.

11.2 North America (Canada and USA)

Bacillus thuringiensis var. *kurstaki* (Btk) is currently the most successful microbial pest control product used around the world (Entwistle et al. 1993). Its first large-scale development as a commercial product was for suppression of defoliating forest insects in North America during the second half of the previous century. Forests in North America are home to various defoliators that periodically erupt in wide-spread outbreaks, which provided a market that was easy to access (single users) and economically attractive (large scale, high product volume). Furthermore, only a proportion of the current year's foliage needs to be protected from insect feeding to prevent tree mortality and minimize impacts on tree growth and yield, which meant that a considerable degree of uncertainty in efficacy could be tolerated. How Btk became the mainstay in forest protection programs is briefly summarized below (see also Reardon et al. 1994; van Frankenhuyzen 1995; van Frankenhuyzen et al. 2015).

The development of Btk for forest insect control occurred in two main testing arenas, provided by massive outbreaks of spruce budworm (*Choristoneura fumiferana;* Lepidoptera: Tortricidae) in Canada and of gypsy moth (*Lymantria dispar*; Lepidoptera: Lymantriidae) in the United States. Outbreaks of both species became targets for large-scale aerial spray programs in the late-1940s throughout northeastern North America to protect threatened timber resources, using DDT for the first decade and various organophosphates after that. Research was initiated to develop environmentally safe alternatives when negative environmental impacts of those operations started to accumulate. The bacterium *B. thuringiensis* quickly became a key focus for that research as it had been known as an entomopathogen since the start of the century, and had already been tested for insect control during the late-1920s and early-1930s. Initial field tests in the early-1960s yielded poor results but test results improved during the 1970s after adoption of HD-1 *kurstaki* as the production strain. The advent of high-potency formulations that could be applied undiluted in ultra-low spray volumes with concurrent improvements in aircraft-based application technologies reduced initial constraints of high cost and unreliable efficacy. Changing public and social attitudes towards large-scale use of synthetic insecticides pushed Btk into operational use in the early-1980s before parity with synthetic insecticides in terms of cost and effectiveness had been achieved. This provided the practical experience that was needed to further improve field efficacies, and necessitated competitive bidding by suppliers, which forced

reductions in product cost. By the mid-1980s, Btk was generally accepted as a viable and cost effective alternative to synthetic insecticides, and between 1983 and 1990 it became the primary agent for control of budworms and gypsy moths (Table 11.2). During the 35 years following its first operational use in 1980, Btk has been applied to suppress outbreak populations of native forest defoliators and eradicate invasive forest pests on a cumulative total of 11.3 million ha in Canada, and 6.2 million ha in the United States.

The eastern spruce budworm is a native defoliator of spruce (*Picea* spp.) and fir (*Abies* spp.) in boreal and mixed hardwood forests across North America and is the most destructive pest of these forests. Outbreaks are cyclical and every 30–40 years populations increase to outbreak levels, where they remain for 10 years or longer (Royama 1984). Severe defoliation sustained for 5–7 years causes extensive mortality of fir and spruce hosts. For protection against spruce budworm, and coniferous defoliators in general, most commonly used Btk products containing 12.7–16.9 Billion International Units (BIU) per liter are usually applied undiluted at 30 BIU in 1.6–2.4 L per ha, using rotary atomizers to produce a finely dispersed spray cloud with a median droplet diameter of 50–80 μm (Bauce et al. 2004; van Frankenhuyzen et al. 2007). Btk is applied after buds have flushed and shoots are starting to elongate in order to maximize foliage protection. Between 1980 and 2014, Btk was applied to a cumulative total of 8.0 million ha of spruce budworm-infested forests, mostly in Canada (Table 11.2). Its use has declined since the early-1990s and shifted towards western Canada as spruce budworm populations in eastern provinces declined. Recurrence of epidemic spruce budworm populations in Québec caused the increase in spraying after 2017 and may signal the onset of a new outbreak in eastern North America.

Outbreaks of the jack pine budworm, *Choristoneura pinus*. Occur throughout the range of its primary host (jack pine; *Pinus banksiana*) at intervals of 10–15 years and can cause severe defoliation over millions of ha (McCullough 2000; van Frankenhuyzen et al. 2011). Foliage protection programs with Btk have been successfully conducted in Ontario during three outbreak episodes since the mid-1980s, on a cumulative total of ~1.2 million ha (Table 11.2). Outbreaks of the western spruce budworm, *Choristoneura freemani* (formerly *C. occidentalis*), occur west of the Continental Divide and are intermediate to those of the previous two species in terms of frequency, duration and severity. Outbreaks typically last 10–15 years and can cause severe defoliation of Douglas fir (*Pseudotsuga menziesii*) on hundreds of thousands of ha every 20–35 years (Axelson et al. 2015). Outbreak populations have been suppressed with Btk since the mid-1980s on a cumulative total of ~0.54 million ha in the western United States and ~0.88 million ha in western Canada (Table 11.2).

Another native coniferous defoliator in eastern North America that is targeted with Btk sprays is the eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Lepidoptera: Geometridae). Outbreaks occur predominantly in stands of balsam fir (*Abies balsamea*) and vary in size over time and space from a few thousand to a few hundred thousand ha (van Frankenhuyzen et al. 2002). Larger scale outbreaks cycle at 10–15 year intervals and last from 3 to 6 years. Since larvae feed on both new and old foliage, severe defoliation can kill trees within 1 or 2 years. Foliage protection

Table 11.2 Ha[a] (in thousands) treated with commercial *Bacillus thuringiensis* var. *kurstaki* products for suppression of major forest defoliators[b] in North America between 1980 and 2014[c]

Year	ESBW Canada	ESBW USA	WSBW Canada	WSBW USA	JPBW	EHL	GM Canada	GM USA[d]	Total
1980	61.0	56.6	0.0	0.0	0.0	0.0	0.0	6.8	124.4
1981	53.5	50.6	0.0	0.0	0.0	0.0	0.0	8.9	113.0
1982	46.1	35.8	0.0	0.0	0.0	0.0	0.0	26.9	108.8
1983	59.5	47.7	0.0	0.0	0.0	0.0	0.0	190.3	297.5
1984	360.6	88.4	0.0	0.0	0.0	0.0	0.0	87.3	536.3
1985	675.7	133.1	0.0	16.5	220.0	2.4	0.17	108.1	1155.8
1986	356.8	0.0	0.0	0.0	482.0	5.4	216.27	87.7	1148.2
1987	404.8	0.0	0.0	64.3	105.4	5.1	80.50	127.5	787.6
1988	434.5	0.0	1.8	251.3	0.0	25.6	27.57	110.4	851.3
1989	432.7	0.0	0.5	4.9	14.3	9.1	25.90	165.3	652.8
1990	1061.9	0.0	0.0	29.0	0.0	44.9	67.91	340.6	1544.4
1991	526.7	0.0	3.0	46.1	0.0	16.9	73.15	295.3	961.2
1992	261.1	0.0	35.6	74.5	0.0	0.9	80.00	263.8	716.0
1993	195.2	0.0	34.2	25.9	0.1	45.2	2.62	148.9	453.4
1994	47.9	0.0	21.0	0.0	21.5	17.7	2.08	138.3	260.2
1995	204.4	0.0	0.0	11.3	51.0	89.6	1.06	108.8	479.3
1996	213.5	0.0	0.0	8.1	25.6	145.5	0.36	80.6	481.3
1997	112.8	0.0	16.1	0.0	0.0	14.4	0.00	18.7	169.6
1998	201.5	0.0	21.2	6.5	0.0	7.2	0.00	36.7	286.9
1999	283.0	0.0	21.7	0.0	0.0	16.3	32.42	61.3	417.7
2000	102.3	0.0	8.4	0.0	0.0	107.2	0.00	91.1	342.7
2001	103.6	0.0	26.8	0.0	0.0	68.2	0.92	109.6	334.1
2002	160.3	0.0	30.9	0.0	0.0	141.2	0.00	59.9	403.9
2003	79.1	0.0	22.1	0.0	0.0	42.0	0.00	27.2	198.5
2004	0.00	0.0	25.5	0.0	0.0	0.0	0.60	29.4	108.0
2005	60.4	0.0	30.4	0.0	0.0	0.0	0.00	2.9	137.2
2006	52.6	0.0	43.9	0.0	104.2	0.0	0.00	58.0	297.1
2007	0.2	0.0	57.3	0.0	173.0	0.0	0.63	64.8	319.0
2008	21.2	0.0	61.9	0.0	0.0	0.0	0.00	180.2	281.6
2009	63.4	0.0	72.9	0.0	80.8	29.7	2.10	116.6	380.1
2010	105.4	0.0	47.9	0.0	0.0	31.6	0.00	2.3	210.8
2011	161.7	0.0	49.6	0.0	0.0	4.5	0.00	1.0	234.1
2012	178.0	0.0	117.2	0.0	0.0	0.0	0.69	1.1	309.5
2013	241.1	0.0	78.0	0.0	0.0	18.6	0.00	21.7	370.5
2014	289.7	0.0	56.7	0.0	0.0	0.0	0.00	3.0	357.8
Total	**7621.5**	**412.1**	**885.2**	**538.5**	**1278.0**	**889.5**	**614.9**	**3181.2**	**15830.5**

[a]Number of ha sprayed × number of applications, except GM USA data (see[d])

[b]Main target insects: *ESBW* eastern spruce budworm, *WSBW* western spruce budworm, *JPBW* jack pine budworm, *EHL* eastern hemlock looper, *GM* gypsy moth

[c]Sources: WSBW USA: Sheehan (1996); GM USA: Gypsy Moth Digest, USDA Forest Service, Northeastern Area, http://na.fs.fed.us/fhp/gm, accessed May 2015; Other species: Canadian National Forestry Database (http://nfdp.ccfm.org; accessed May 2015) supplemented by Annual Pest Forum reports, available at http://cfs.nrcan.gc.ca/publications, Canadian Forestry Service

[d]Surface area treated regardless of number of applications, not including eradication programs (1980–2014: 1.66 million ha, Asian and European gypsy moth) or Slow-the-Spread program (1993–2014: 0.42 million ha)

programs with Btk have been undertaken since the early-1980s on a cumulative total of ~0.89 million ha (Table 11.2).

The gypsy moth is the most important hardwood defoliator in North America. It was accidentally introduced from France to the Boston area in 1868 and has defoliated more than 37 million ha of hardwood forests since 1924 (USDA Forest Service Gypsy Moth Digest; http://www.na.fs.fed/fhp/gm; accessed May 2015). Microbial control products have featured prominently in gypsy moth suppression programs since the early-1980s, in particular Btk. In the United States, doses of 60–95 BIU per ha are applied undiluted in volumes of 3.0–4.7 L, usually in a single application. In Canada, the recommended prescription is two applications of 30–50 BIU in 2.4–3.9 L per ha. Spray applications are typically conducted when oak leaves are 40–50 % expanded or when the majority of larvae are second instars (van Frankenhuyzen et al. 2007). In Canada, Btk is the only product that has been used for gypsy moth suppression since the mid-1980s on a total of ~0.6 million ha (Table 11.2), whereas in the United States the market is split between Btk and an insect-growth regulator (Dimilin). Since 1980, Btk has been applied to a cumulative total of ~3.2 million ha for suppression of gypsy moth outbreaks (Table 11.2).

Btk is also used extensively in the so-called Slow-the-Spread management program, a multi-agency program that targets new gypsy moth populations along the leading edge in the transition zone between infested and non-infested areas (Tobin and Blackburn 2007). Newly established populations are aggressively targeted for eradication to limit their influence on the rate of spread. Btk doses generally range from 60 to 95 BIU per ha using either one or two applications during the period of early-instar activity. Btk has been used on ~0.42 million ha since the pilot stage of the program in 1993 (USDA Forest Service Gypsy Moth Digest; http://www.na.fs.fed/fhp/gm; accessed May 2015).

A third use of Btk in gypsy moth management is eradication of isolated populations that arise from human-mediated dispersal. Of particular concern are frequent introductions and potential establishment along the Pacific Coast, the main target for annual small-scale eradication programs. Large-scale eradication programs using multiple applications of Btk were conducted in Oregon in 1985–1986, involving treatment of ~168,000 ha of urban area, and on ~13,400 ha in the greater Victoria region on Vancouver Island in 1999 (de Amorim et al. 2001). A special case is eradication of the Asian gypsy moth, *Lymantria dispar asiatica.* It has a broader host range than its European counterpart and its females can fly. Because Asian gypsy moths typically arrive on the continent via cargo ships, eradication programs are most often conducted in urban areas near ports of entry. Notable examples of large-scale eradication programs include aerial treatment of ~20,000 ha in Vancouver (British Columbia) and ~46,600 ha in Tacoma (Washington) in 1992 and treatment of ~55,800 ha in North Carolina in 1994. Those programs have been discussed in detail by Hajek and Tobin (2009). It is estimated that between 1980 and 2014, Btk has been used on ~1.66 million ha to eradicate isolated infestations of both gypsy moths across the United States (USDA Forest Service Gypsy Moth Digest; http://www.na.fs.fed/fhp/gm; accessed May 2015).

11.3 Concluding Remarks

Fifty years on Bt-based bio-insecticides have been an important biological option for pest control and have consolidated this importance over time. *H. armigera*, *C. fumiferana, C. includes* and *L. dispar* are examples that these biopesticides work as well as pesticides and has the advantage of preserving natural enemies and the environment. Glare et al. (2012) emphasized the necessity of further studies on biopesticide field persistence, chemistry of bioactives from microorganisms, more strategic selection of target pests and markets and finally registration and legislative changes to improve the biopesticides market.

Extensive cultivation areas and warm and often dry climate make Brazilian agriculture an excellent place for pest proliferation. Since the implantation of the soybean crop in Brazil in the 1960s until the beginning 2000s, the soybean fauna increased from 10 to about 37 species among insects and other arthropods (Hoffmann-Campo et al. 2003), disregarding *H. armigera* and *Spodoptera* spp. There are currently over 70 species of insects and mites that occur in soybean in other countries, and that, potentially, have conditions to be introduced in Brazil and cause damage to culture (Hirose and Moscardi 2012). *Bemisia tabaci*, one "old introduced pest" in Brazil, is our most famous example that only pesticides are not able to control a recently introduced pest.

Most of Bt's Cry toxins have unknown toxin activity against a wide range of pests (van Frankenhuyzen 2009, 2013). Available Bt biopesticides currently exploit less than 5 % of all toxins available, mainly Cry1A toxins. Less than 10 % of Cry toxins were assayed against lepidopterous pests, including Noctuidae that comprises the main pest group of Brazilian agriculture. Therefore, we have a great Cry arsenal still to be explored with conditions to generate biopesticides efficients and with lower cost of development than the conventional pesticides.

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Chapter 12
Mass Production, Application and Market Development of *Bacillus thuringiensis* Biopesticides in China

Lin Li, Zhenmin Chen, and Ziniu Yu

Abstract *Bacillus thuringiensis* (Bt) biopesticides are the most produced bacterial pesticide in China, accounting for more than 95% of the total market share of microbial pesticides. Bt biopesticides are widely used to control agricultural pests, forest and fruit tree pests, storage pests, and medical insects because of their remarkable economic, social, and ecological benefits. The Bt industry in China currently has an output of approximately 30,000 tons (with a potency of 16,000 IU/μl) each year, and annual sales are approximately RMB 100 million yuan. Research and industrialization of Bt in China can be divided into four major stages: starting, primary mass production and application, intensified basic research and application, and in-depth research and large-scale application. This chapter reviews the major aspects of Bt biopesticide research and development, mass production, application, and marketization in China.

Keywords Bt biopesticide • Mass production • Marketization • Application • Fermentation

In agricultural and forest production, diseases and insect pests cause substantial damage that results in huge economic losses each year. During the mid-1990s in China, the grain and cotton yields reduced each year by 10% and 20%, respectively, and about 120 million mu (1 mu = 1/15 ha) of forest were subjected to damage each year because of diseases and insect pests, leading to an annual direct loss of over RMB 15 billion. Thus, plant diseases and insect pests need to be reduced, and the agricultural output per unit area should increase. Minimizing insect pest damage through effective pest management and promoting the agricultural output per unit area are key to achieving this goal. In the long-term practice of controlling agricultural pests, multiple pest management through chemical and biological approaches is generally recognized as a solution to

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control pest infestation effectively. The use of chemical pesticides is currently the main pest control method. However, biopesticides based on microbial metabolites, or pesticidal microorganisms, have attracted special attention because of their unique functions and insecticidal mechanisms while protecting ecological balance and avoiding environmental pollution. Since the mid-1960s in China, microbial pesticides with different functions and activities have been emerging and gradually applied for insect pest control in various fields, such as agriculture, forestry, animal husbandry, and medical fields (Yu 1993, 1990).

Microbial pesticides are derived from biologically active ingredients produced by microorganisms. These preparations are used to control insect pests, sanitation insects, weeds, and rodent pests, as well as to promote plant growth (Saxena and Pandey 2001; Sudakin 2003). Microorganisms that can be used in the preparation of microbial pesticides include bacteria, fungi, viruses, and protozoa (Roh et al. 2007; Sudakin 2003). Currently, Bt preparations are important microbial pesticides. In 1938, the world's first industrial Bt preparation known as Sporeine was commercially launched in France (Aronson et al. 1986; Yu 1993). Since then, the industrialization of Bt has been promoted continuously, given the increased understanding of the growth and reproduction, physiology, biochemistry, and genetics of this insecticidal bacterium (Aronson et al. 1986; Schnepf et al. 1998). Relative to other types of pesticides (e.g., chemical pesticides), Bt insecticides exhibit strong insecticidal specificity, environmental friendliness, and satisfactory safety performance as evidenced by nontoxicity to humans, livestock, or non-target insects. Bt insecticides consist of several types of insecticidal crystal proteins (ICPs); hence, the development of insecticidal pesticide resistance is difficult or slow. Furthermore, these insecticides are cheaper than conventional ones (Roh et al. 2007; Rosas-Garcia 2009; Sanahuja et al. 2011). As a result, the industrialization of Bt insecticides is developing rapidly in China, and these biopesticides have become widely used. In fact, Bt biopesticides have the highest production and sales, occupying more than 95% of the total market share of microbial pesticides (Guan and Cai 2014).

12.1 Development of Bt Research in China

Since the discovery of Bt in 1901, the international research on this bacterium and its industrialization has been divided into three stages: initial stage (1901–1952), essence-understanding and functionalization stage (1953–1976), and research scope expansion stage (1977-present) (Guan and Cai 2014). Studies on Bt have been conducted in a wide variety of fields. In terms of microbiology, the research fields involve classification, growth, metabolism, formation of parasporal crystals, production of thuringiensin, genetics, ecology, and so on. In terms of toxins, the research fields involve the morphology, structure, and physical and chemical properties of ICPs and thuringiensin, as well as their insecticidal mechanisms. In terms

of industrialization, the research fields involve strain, fermentation technology, formulation technology, product standardization and safety, and product registration. In terms of application, the research fields involve target pests and application scope, application technology, and factors that influence application effects (Yu 1990). In China, the starting research on Bt lags behind about half a century overall relative to the countries that first studied Bt, and the process of research and industrialization generally experienced the following stages:

12.1.1 Starting Stage (1941–1963)

In 1941, Prof. Zhelong Pu first carried out experiments on *Pieris rapae* larval control using Bt in the South China region. Meanwhile, the earliest research on Bt was conducted by Prof. Ji Cao et al. in 1955 by preparing bacterial powder in laboratory trials for *Ostrinia nubilalis* control using strains isolated from a commercial preparation imported from France. Then, in 1959, Prof. Chongle Liu introduced Bt subsp. *thuringiensis* (H_1) from Czechoslovakia and found that laboratory-prepared Bt cultures are highly efficient in controlling *Clostera anachoreta*, *P. rapae*, and *Dendrolimus* sp.. He also explored approaches to improve *Leucania seperatura* control as well as the pathological changes of blood cells. In the same year, Prof. Lvhong Zhang imported Bt subsp. *thuringiensis* (H_{3abc}), Bt subsp. *galleriae* (H_{5ab}), and Bt subsp. *dendrolimus* (H_{4ab}) from the Soviet Union and carried out indoor tests on the control of Lepidoptera pests. In 1961, Prof. Zhongyun Peng imported a Bt biopesticide powder (namely "Chongjun No. 3") from the Soviet Union and isolated strains for a series of indoor and outdoor bioassays. Meanwhile, the Ministry of Forestry and other departments used Bt preparations developed by China to control *Dendrolimus* sp. by aerial application for the first time in Hongan County of Hubei Province. Later, the Institute of Agricultural Sciences in Hubei Province studied liquid-state submerged fermentation technologies of Bt subsp. *galleriae* and Bt subsp. *thuringiensis*. Until the end of 1965, the first commercial Bt biopesticide product "Qingchong" with the registered trademark "SanWu Brand" produced in Wuhan and a subsequent Bt biopesticide named "424 Bt preparation" produced in Changsha were launched in the Chinese market. These early investigations played an important role in promoting in-depth research on Bt in China (Yu 1990, 1993).

12.1.2 First High and Low Tide (1964–1980)

Since 1964, China's Bt production and area of application have undergone rapid growth, displaying an upsurge for the first time. Field tests were extensively carried out in more than 20 provinces and cities, and more than 150 species of agricultural

and forestry insect pests were controlled to a certain extent. In field control tests, various Bt biopesticides exhibit a high mortality ranging from 69.4% to 100% in controlling various main agricultural pests, such as *P. rapae*, *Plutella xylostella*, *O. nubilalis*, *Parnaia guttata*, *Clanis bilineata*, *Hyponomeuta malinella*, *Dendrolimus* spp., *Apocheima cinerarius*, *Euproctis pseudoconspersa*, and *Heliothis assulta* (Yu 1993). In Hubei, Shanxi, and Hunan, a large area control of agricultural and forest pests was conducted by aerial application. In the early 1970s in Yichang City of Hubei Province, 11 planes with 365 flights were launched to control *Dendrolimus* sp. using a pesticide mixture of Bt and Beauveria preparations. The total control area of *Dendrolimus* sp. reached 820,000 mu with an average mortality of more than 71.3%. Consequently, the demands of Bt preparation exceeded the supply for several plants, and factory constructions increased in China. Specifically, more than 60 factories or workshops were engaged in Bt production during that period. Meanwhile, the workshops producing Bt preparations via the indigenous method of semi-solid state fermentation also expanded. However, the production technology of most of these factories did not meet production standards, which resulted in poor-quality Bt biopesticides and in the death of Bt vegetative cells because of phage infestation. A large number of untested "products" from the indigenous method were irresponsibly sold to farmers; these products exerted weak or even no insecticidal effects as a result of phage contamination. The lack of reliable quality standard, the instability of production, and the downstream use of these inefficient products caused the backlog of Bt preparation products. As a result, most factories stopped their operations or switched to manufacturing other products. Until the late 1970s, Bt research and development (R & D) in China had gradually dropped from climax into ebb.

12.1.3 Recovery Stage (1981–1985)

During this stage, biological control technology was listed as a special research project of the Chinese National Science and Technology Commission and the Ministry of Agriculture. New research collaborations were led by the Biological Control Laboratory of the Chinese Academy of Agricultural Sciences. The commercial Bt products manufactured in the 1980s were mainly derived from Bt subsp. *kurstaki* HD-1 imported from the United States. The Institute of Agricultural Sciences in Hubei Province successfully developed a Bt liquid formula agent and Bt emulsion in succession. Meanwhile, the Luoyang Pesticide Plant of the Ministry of Forestry successfully manufactured a Bt powder product and was issued a corresponding registration. The national annual output of Bt biopesticides after recovery was only a hundred or more tons. Given the backward fermentation and post-processing technologies during this period, the products often blocked the nozzles of sprayers in field use, and the control effect was not satisfactory. Thus, Bt quality still did not reach the technical requirements, and the products were unmarketable and backlogged.

12.1.4 Re-emergence Stage (1986–1995)

During this stage, the research on Bt biopesticides was officially listed in the National Key Technology R & D Program of China. Under the organization of the Biological Control Laboratory of the Chinese Academy of Agricultural Sciences, Bt scientific research was coordinated to build up substantial collaboration for Chinese researchers. In the 1980s, China initiated research on the standardization of Bt biopesticides in the aspect of Bt product quality control and standard bioassay technology using *P. xylostella* and *Heliothis armigera* as the indicative bioassay insects in agreement with the international quality testing technology. These parameters and support allowed improvements for key technical problems of product quality standardization for more than 20 years. In terms of technology innovation for commercializing Bt products, which aimed at solving a series of technical problems including the backward fermentation technology, a variety of Bt strains with distinguished potentials were screened or introduced abroad, the fermentation medium components were intensively investigated, the optimized production and post-processing technologies were determined, and a batch of Bt suspension agents and wettable high-content powder products were successfully developed. Technology innovation continuously promoted the overall progress and development of the Bt industry. Until the 1990s, China had isolated tens of thousands of strains from soil and infected insects and also made some breakthroughs in strain breeding, fermentation technology improvement, product standards preparation, and formulation. In 1995, the Institute for the Control of Agrochemicals of the Ministry of Agriculture issued standards for Bt biopesticides. These standards are based on bioassays, which promoted the steady development of Bt in China. By the mid-1990s, the number of formally registered Bt plants had increased to more than 40, and the industrial fermentation level [toxicity potency, referring to the international unit (IU) per μl or mg] had increased from less than 1,000 IU/μl to a steady level of more than 2,000 IU/μl. In addition, some factories with advanced technologies reached 4,000–5,000 IU/μl. In 1990, the output of Bt pesticide preparations was about 1,500 tons, 3,500 tons in 1991, and then 30,000 tons in 1994. In the 2000s, Bt mass production enterprises had become a main base for Bt production. In fact, the products were exported to the United States, Japan, and Southeast Asia. For instance, 60% of the Bt raw powder produced by Wuhan Konuo Biopesticide Co., Ltd. in 2007 (200 tons of raw powder) was used for export (Guan and Cai 2014; Wang et al. 2011).

To date, the main achievements in terms of the commercial production of Bt biopesticides and the control of agricultural and forest pests by Bt biopesticides in China could be summarized in the following aspects: successful commercialized production of high-quality and cost-effective Bt suspensions and highly effective powder biopesticides; the development of a quality-controlled standardization and technical system based on standard bioassay technologies; and intensive investigations on influences of adjuvants and storage conditions that provide a reliable basis for selecting adjuvants and effectively storing Bt products. Bt biopesticides were marketed for cotton, grain, fruit, vegetable, and forest pest controls and achieved

good results. In brief, the toxicity potency and pest-controlling effects of Bt biopesticides produced in China have reached the advanced level of comparable international products. In particular, a set of strict quality control technology using *P. xylostella* and *H. armigera* as the standard bioassay pests was established in the whole Bt industrial system. Such an establishment not only laid a good foundation for establishing industry standards of China′s Bt biopesticides but also guaranteed the quality standards of Bt biopesticides while maintaining international quality standards.

Currently, Bt R & D in China is directed toward improving the technological process and product quality. At the same time, continuous focus should be on the search for new naturally occurring Bt strains and genetically modified engineered strains based on cell engineering and molecular biology technologies. Bt products have been widely used in the control of agricultural, forest, urban gardening, and health pests throughout more than 20 provinces in China. Bt products are applied on an agricultural area of more than 40 million mu each year, and they are also exported in batches. Bt insecticides have developed into the dominant breed of China's green microbial pesticides. This series of progress indicates that the R & D and commercialization of China's Bt insecticides have begun to enter the ranks of advanced countries.

12.2 Target Pests of Bt Preparation Biocontrol in China

Bt products have been successfully applied in the biological control of agricultural, forest, grain storage, and health pests (Fig. 12.1). According to a partial list, Bt products have been tested or used for the prevention and control of more than 40 types of Lepidoptera, Diptera, and Coleoptera pests (Liu et al. 2000; Peng et al. 2015; Zhu et al. 2004).

12.2.1 Agricultural Pests

More than 40 types of Lepidoptera pests in cotton, corn, rice, vegetables, fruits, tea, hemp, tobacco, and forest have been tested for control effectiveness. The main target pests include *H. armigera*, *O. nubilalis*, *P. xylostella*, *Spodoptera exigua*, *Prodenia litura*, *P. rapae*, *Ectropis oblique*, *H. assulta*, *Cnaphalocrocis medinalis*, *Casinaria colacae*, *Macroglossum corythus*, *E. pseudoconspersa*, *Homona magnanima*, *Manduca sexta*, *Mamestra brassicae*, *Pieris brassicae*, *Agrotis segetum*, *Mythimna separata*, *Galleria mellonella*, *Cryptolestes turcicus*, *Tenebroides mauritanicus*, *Sitophilus zeamais*, *Myzus persicae*, and various plant-pathogenic nematodes.

Fig. 12.1 Applications of *B. thuringiensis* biopesticides for biocontrol of cotton, vegetable, forest and orchard pests in China

12.2.2 Greenhouse Pests

Aphids, *Trialeurodes vaporariorum*, mites, thrips, leaf miners, and *P. xylostella* have caused serious damages on vegetables and other economic plants cultivated in the greenhouse. In consideration that the greenhouse or vinyl house has an isolated or semi-closed structure, environmental factors such as temperature, humidity, light, and oxygen are relatively stable and comparable with those on the field, which are more conducive to the occurrence of insect pest damage. In addition, differences in the hemerophyte structure (in general, vegetables with a high economic value, such as cucumber, tomato, and eggplant, are cultivated in the greenhouse) usually lead to great discrepancies of pest species and their occurrence frequency and damage compared with the field crops. Bt products show obvious effects on the biocontrol of these greenhouse pests. With the significant increase in the demand of China's urban and rural residents for green, pollution-free vegetables, Bt preparations have become an important pesticide for the control of greenhouse pests.

12.2.3 Forest Pests

Dendrolimus punctatus, *Parocneria furva*, *Chinolyda flagellicornis*, *Hyphantria cunea*, *Choristoneura fumiferana*, and various leaf miners.

12.2.4 Grain Storage Pests

Grain storage pests are mainly Lepidoptera grain storage pests, such as *Plodia interpunctella*, *Ephestia elutella*, *Sitotroga cerealella*, *Ephestia kuehniella*, and *Aglossa dimidiata*, which can cause surface damage on grains.

12.2.5 Health Pests

Aedes sp., *Culex* sp., and *Musca domestica* larva.

12.2.6 Warehouse Pests

S. zeamais, *T. mauritanicus*, *Callosobruchus chinensis*, *Bruchus pisorum*, *Sitophilus granarius*, *Lasioderma serricorne*, and *E. elutella* are the pests of warehouse and tobacco storage.

12.2.7 Chinese Medicinal Material Pests

Bt preparations mainly exert control effects on *Stegobium paniceum*. This insect pest occurs in many regions, including the provinces of Guangdong, Shanxi, Shandong, and Guizhou, and is also the dominant species of Chinese medicinal material pests during storage.

12.2.8 Flora and Fauna Parasitic Nematodes

Indoor tests have proven that Bt preparations exert obvious insecticidal effects on various nematodes, such as *Bursaphelenchuh xylophilus*, *Meloidogyne incognita*, *Meloidgyne hapla*, *Heterodera glycines*, *Radopholus similis*, *Panagrellus redivivus*, *Haemonchus contortus*, and *Schistosoma japonicum* (Liu et al. 2012; Rao et al.

2005; Xu et al. 2010; Yu et al. 2004; Zhao et al. 2007), while a few preparations have been used for field tests (Chen et al. 2008; Yu et al. 2008).

12.3 Market Development of Bt Biopesticides

Despite various difficulties, the production and sale of Bt biopesticides in China developed rapidly as described above. During 1986–1995, the annual output of Bt insecticides (which are mainly suspending agents) increased from approximately 200 tons to more than 20,000 tons, and Bt insecticides had developed into one of China's most important biopesticide agents. In addition, the annual output of Bt powder reached a production scale of hundreds of tons. China became the production country with the largest output of Bt insecticides in Asia, and products were exported to Southeast Asian countries and Taiwan. Bt insecticides were popularized and applied to about 24,000 mu of crops in more than 20 provinces. These preparations obtained good control effects on various agricultural pests, especially on those resistant to chemical pesticides, such as *P. xylostella* and *H. armigera*. For example, in Shenyang of Liaoning Province, aerial spraying of Bt preparation was carried out to control 1.3 million mu of *O. nubilalis* wherein the control efficiency reached 83.1%. During 1987–1992 in Shijiazhuang of Hebei Province, Bt preparations were used to control *H. armigera*-dominated cotton pests with an application area of 352,000 mu and a control efficiency higher than 75%. In the provinces of Sichuan, Hubei, Liaoning, and Hunan, Bt insecticides were used to control more than 3 million mu of agricultural pests, such as *Tryporyza incertulas* and *C. colacae*. In the provinces of Hebei, Shanghai, Hubei, and Guangdong, Bt suspending agent and Bt variant liquid preparations were used to prevent and control 1.3 million mu of *P. xylostella* wherein the control efficiency reached 80–90% (Chen et al. 2008; Zhu et al. 2004). The target insect pests controlled by Bt insecticides have expanded from primarily vegetable and forest pests to pests of important crops such as cotton, rice, and corn. These Bt insecticides provide new reliable and practical pesticide products for China to develop pollution-free agriculture.

According to incomplete statistics, at present, more than 170 Bt manufacturers have produced an annual output of Bt biopesticides of over 30,000 tons (Fig. 12.2), with an annual average application area of about 80 million mu. Some large-scale production enterprises include Hubei Kangxin Pharmaceutical Co., Ltd., Wuhan Konuo Biotechnology Co., Ltd., Fujian Pucheng Lvan Biopesticide Co., Ltd., Yangzhou Biological Products Factory, and Shandong Lukang Pharmaceutical Group Co., Ltd. Among these Bt manufacturers, about 70 of them are engaged in producing single-formula active compounds and raw powders. In addition, the number of registration certificates issued by the Ministry of Agriculture for single-formula Bt pesticides is more than 100. In addition to these raw material product manufacturers that produce raw powders and single-formula by fermentation, about 100 manufacturers have registration certificates for producing mixed-type Bt pesticides. In the industrialization of Bt, Konuo Biotechnology Co., Ltd. and Kangxin

Fig. 12.2 Representative *B. thuringiensis* biopesticides produced in China

Pharmaceutical Co., Ltd. in Hubei Province and Lvan Biopesticide Co., Ltd. in Fujian Province have been at the head of the R & D of Bt biopesticides in China.

Bt preparations produced here are not only used in the Chinese territory but also exported to many countries. Currently, China's total Bt preparation output is approximately 30,000 tons (equivalent to a toxicity potency of 16,000 IU/μl) each year, the annual sales are estimated at 4,000–5,000 tons or approximately RMB 100 million yuan. Other Bt preparations are exported to the United States, Japan, Southeast Asia, and other countries. The sales market or application scope is mainly divided into the following regions:

1. Vegetable producing area in South China (the south of Fujian Province; the east and the Pearl River Delta of Guangdong Province), especially the vegetable supply bases for Hong Kong, Macao, and Taiwan. Given that pollution-free vegetables have strict requirements for minimal pesticide residues, the pesticide-using habits of local farmers have changed. The market demand of this area is about 2,000 tons.
2. Rice and cotton cultivation areas in regions of Hubei, Hunan, and Sichuan. Due to governmental support and efforts of enterprises in recent years, Bt biopesticides have obtained considerable acceptance among farmers, and the annual market demand has increased to approximately 1,000 tons.

3. Cotton region in Sinkiang. Because of the military-style production management and production requirements for high-quality cotton, Bt preparations are widely popularized in this region, and the annual sales in the market are 500 tons or more.
4. Greenhouse vegetable production area in Jiaodong Peninsula of Shandong Province. The application amount in this region presents approximately 500 tons.
5. Corn production bases in North and Northeast China. Bt preparations are mainly used to prevent and control *O. nubilalis*, which has formed a market demand of approximately 500 tons.
6. Other regions in China. The total market demand is approximately 1,000 tons, which includes ~200 tons of Bt biopesticide demand for forest pest control. This market demand is very fragmented but has vast potential, which requires considerable manpower and material resources to continue the market popularization.

The current situation of China's main large-scale Bt manufacturers is described here. Wuhan Konuo Biotechnology Co., Ltd. is committed to R & D, manufacturing, and promotion of Bt series of products. At present, it has 14 Bt strains and 20 types of biopesticide products. These are widely used in crops (mainly rice and corn) for the control of Lepidoptera and Coleoptera pests. Its market share is in the leading position. Wuhan Konuo Biotechnology Co., Ltd. has established eight Bt green ecological prevention and control bases, including Wuhan Shuangliu cowpea-resistant bioassay base, Hunan Jiangyin rice-resistant bioassay base, and Shandong Heze forest bioassay base, which collectively reached 4 million mu application area. Their Bt products have passed the organic product certification and obtained 14 pesticide registration certificates from the Ministry of Agriculture and 14 production approvals/licenses from the Ministry of Industry and Information Technology and the National Bureau of Quality Inspection. Moreover, the products have been sold to more than ten countries and regions, including America, Japan, and Southeast Asia. From 2005 to 2011, the total sales of Bt biopesticides were 4,567 tons, amounting to RMB 916.8 million. Fujian Pucheng Lvan Biopesticide Co., Ltd. has begun to produce the pesticide WG-001 (an efficient genetically-engineered Bt pesticide) in 2006. From 2006 to 2011, the cumulative production of Bt biopesticides was 1,681 tons, and the total sales value was RMB 33.62 million. The Bt pesticide WG-001 have been widely used in Fujian Province and Guangdong Province. Its key target insect pests include *Dendrolimus* sp., *Pantana phyllostachysae*, and Tortricidae larvae in forestry of the north of Fujian Province, *E. pseudoconspersa* and *E. oblique* on tea leaves in the south of Fujian Province, and *P. xylostella* and *P. rapae* on vegetables in the south of Fujian Province and Guangdong Chenghai region. The application area of this Bt pesticide is more than 60 million mu each year.

However, R & D spending in an overwhelming majority of these enterprises only accounts for less than 1% of the sales revenue. Thus, green environment-friendly products are scarce, effective disposal of waste is not ensured, the structure of applied Bt products is not rationally constructed, and products exert weak effects on frequent and refractory pests, underground pests, nematodes, and invasive pests. At present, Bt production enterprises are committed to promoting formulations that

develop toward efficient and safe products, such as water-based, dust-free, and release-controlled forms, and encouraging the development of economical and environment-friendly packing materials. Furthermore, they pay great attention to the development of pesticide intermediates and special adjuvants, as well as to the strict control of the use of poisonous and harmful solvents and adjuvants (toluene, xylene, etc.). The prospects of Bt development are very optimistic, considering that China is a large agricultural country. With the continuous development in various disciplines, researchers are constantly broadening the domains of Bt development focusing on the integration of various disciplines, starting from the research ideas of upstream technologies, such as biochemistry and genetic engineering, and combining with downstream technologies, such as metabolism kinetics, fermentation kinetics, post-fermentation processing chemical technology, and biochemical reaction engineering technology, to study the microbial fermentation process. At the same time, with the continuous development of modern genetic-engineering-dominated biotechnology, wherein new genetically engineered pesticides show good development and application prospects, the search for genetically engineered Bt strains with good performance will become a research hotspot in the future.

12.4 Production and Preparation Technology of Bt Biopesticides in China

12.4.1 Main Production Technologies of Bt Biopesticides in China

In 1965, China's first commercial Bt insecticide product "Qingchong" was used for the effective control of vegetable pests, such as *P. brassicae* and *P. xylostella*. In 1979, the Institute of Microbiology Research in Tianmen County of Hubei Province used a Bt preparation named 7216 Bt suspension to control *M. sexta*. In 1985, Miu et al. compared the control effectiveness of Bt preparation by HD-1 strain and several commercial chemical pesticides in the field test of vegetable pests. They found that the number of natural enemies, such as *Neoscona theisi*, *Pardosa tinsignita*, *Erigonidium graminicolum*, *Chrysopa septempunctata*, *Chrysopa sinica*, *Brachymeria lasus*, *Pteromalus puparum*, and *Diaeretiella rapae*, in the scope of HD-1 treatments, is 15–62 times higher than that of chemical insecticide treatments (Miu et al. 1985). In 1996, Guan et al. reported a high-toxic Bt preparation named 8010 wettable powder to control *S. exigua* and *P. xylostella* (Guan et al. 1996). In 1997, Ma used Bt SB-1 and SB-8 strains to control *P. xylostella* chemical pesticide-resistant strains that were collected from Taiwan, Thailand, and Shenzhen; results showed high toxicity of Bt preparations against these pests (Ma et al. 1997). In the same year, Yang et al. developed a MP-342 preparation by mass production. This product exhibits high toxicity against *Spodoptera frugiperda* and *H. armigera* (Yang and Yue 2001). In 2000, Feng et al. isolated the Coleopteran-active Bt strain HBF-1, which exhibits high insecticidal activities against *Anomala exoleta* and *Plagiodera*

versicolora (Feng et al. 2000). This was the first report of a Coleopteran-active Bt strain in China. Field plot experiments using HBF-1 strain suspending agent for the control of *A. exoleta* and *P. versicolora* showed 71.4–75.4% control efficiency using a 50-fold dilution suspension of HBF-1 (Feng et al. 2000). In 2001, Yu et al. developed the YBT-1520 preparation, which exerts higher toxicity against *H. armigera* and *P. xylostella* by 0.2 to 11-fold compared with the strains from Abbott and Sandoz companies (Unpublished data). In addition to *H. armigera* and *P. xylostella*, the YBT-1520 preparation also shows very high virulence to other Lepidoptera pests. This product has been widely used to control *H. armigera*, *P. xylostella*, and *O. nubilalis*, with a control efficiency reaching 85%. In 2005, various genetically engineered Bt strains were reported in China. For example, a genetically modified Bt strain (i.e., WG-001) developed by Yu et al. had been issued the national security certificate "agricultural genetically modified organisms (production and application)" and had been classified as a new generation of Bt insecticidal genetically engineered strains with intellectual property rights in China. This strain also became China's first genetically engineered Bt biopesticide that was approved for commercial production. It has high ICP yield and high virulence. Its control efficiency to the 2nd-instar larvae of *H. armigera* is approximately 80–91% and 85–90% to that of *P. xylostella*, indicating its good application potential (Zhang et al. 2005, 2011a, b; Zhu et al. 2008). In 2006, Li et al. used the Bt suspending agent to control Coleoptera pests including *Cryptoleses turcicus*, *T. mauritanicus*, and *S. zeamais* in distillers' grains (Ko-ji). They found that a mixture of Bt insecticide and Al_5P_3 in the absence of sunlight controls the main insect pest of Ko-ji, remarkably reducing damage from these pests (Li et al. 2006). In 2014, Hebei Province Academy of Agricultural Sciences developed Bt JQD117, which can effectively control the damage caused by *Bradysia odoriphaga*, with a control efficiency up to 85% (Unpublished data). As an environment-friendly microbial preparation, Bt JQD117 serves as a new technology for the green control of leek pests. In 2016, Qin et al. mixed Bt raw powder with nanomaterials, such as modified nano-SiO_2, and prepared nanometer Bt insecticidal agents (Qin et al. 2016). This preparation significantly increases the ability to resist UV and prolongs the storage of Bt preparations. In field experiments, its overall insecticidal effect on *P. xylostella* increases by 30%. Continuously screening and optimizing yield strains, as well as improving Bt production and post-processing technologies, have facilitated the coexistence of liquid state fermentation and solid state fermentation in China's Bt production industry. At the same time, biological technologies such as cell engineering and genetic engineering were used to construct different Bt strains and further improve the fermentation process. Since the turn of the twenty-first century, the Bt fermentation level has been increased by nearly ten times. Liquid-state submerged fermentation has been increased to about 5,000 IU/μl of toxicity potency, and the canning coefficient has reached 70%; solid-state fermentation has reached 16,000–20,000 IU/μl, and the canning coefficient has reached about 40% (Chen et al. 2002; Li et al. 2001; Zhu et al. 2003).

Overall, the research, development, and application technologies of China's Bt biopesticides have reached the international advanced level. The emergence of new equipped large-tonnage fermenters, the maturity of industrial fermentation technology, the improvement of liquid-state submerged fermentation processing technology,

and the diversification of formulation undoubtedly create favorable conditions for the development of the Bt biopesticide industry in China. The main achievements of Bt fermentation production in China are the following. First, a quality standardization and technicalization system for the determination of product toxicity potency was established based on bioassays using *P. xylostella* and *H. armigera* as the standard indicative insects. Moreover, a quality-control technology system through quality standardization of Bt products in China was established. These progresses not only laid a good foundation for establishing industry standards of Bt insecticides but also provided an effective guarantee for the agreement of quality standards of Bt insecticides with international quality standards. Second, the influences of adjuvants and the storage conditions of Bt were intensively investigated. The results provided a reliable basis for selecting adjuvants and effectively storing Bt products. Third, cheap and high-quality Bt suspending agents and efficient powder products were developed. In addition, the toxicity potency and pest control effects of Bt powder have reached the international advanced level. Fourth, Bt insecticides were effectively popularized for the control of cotton, grain, fruit, vegetable, and forest pests.

Two type of Bt production technologies exist in China: solid-state fermentation and submerged fermentation.

1. Bt solid-state fermentation

Bt solid-state fermentation originated from the traditional Chinese koji manufacturing technology, which uses the adsorbed nutrients on the surface of biomasses or the nutrients provided by the particles themselves to cultivate microorganisms. In a relatively small space, these biomasses can provide considerable gas–liquid interface and thus meet the demands of aerobic microbial growth for water, oxygen, and nutrients. In the 1950s, some researchers outside China started to use this technology for Bt fermentation production. In the 1970s, several Chinese researchers conducted Bt solid-state fermentation. Until the 1980s, its production technology improved and was validated gradually (Chen and Li 2002a). According to the designed scale, traditional solid-state fermentation can be divided into the net-dish thin-layer method, the vessel-box method, the large-pool ventilation method, and the terrace-type method, as shown in Fig. 12.3.

Raw materials that can be used for Bt solid-state fermentation are very extensive. However, both the trophism of materials and the vent ability of carriers should be considered when choosing raw materials. In terms of choosing solid raw materials, new, cheap, and raw materials (carriers) with rich nutrition, good ventilation, strong wetting ability should be observed, and suitable surfactant products that improve the fermentation level and toxicity potency while shortening the wetting time should be selected. Commonly used carriers can be divided into two categories, namely, organic biomasses and inorganic carriers. Organic biomasses, such as wheat bran, rice bran, yellow bean cake powder, and peanut meal, can be used as both nutrient sources and carriers. Inorganic carriers, such as expanded perlite and fine sand, have good ventilation performance but need additional nutrients. The air permeability of the culture medium is mainly controlled by adjusting the moisture content. Appropriate initial moisture content can guarantee a suitable looseness of

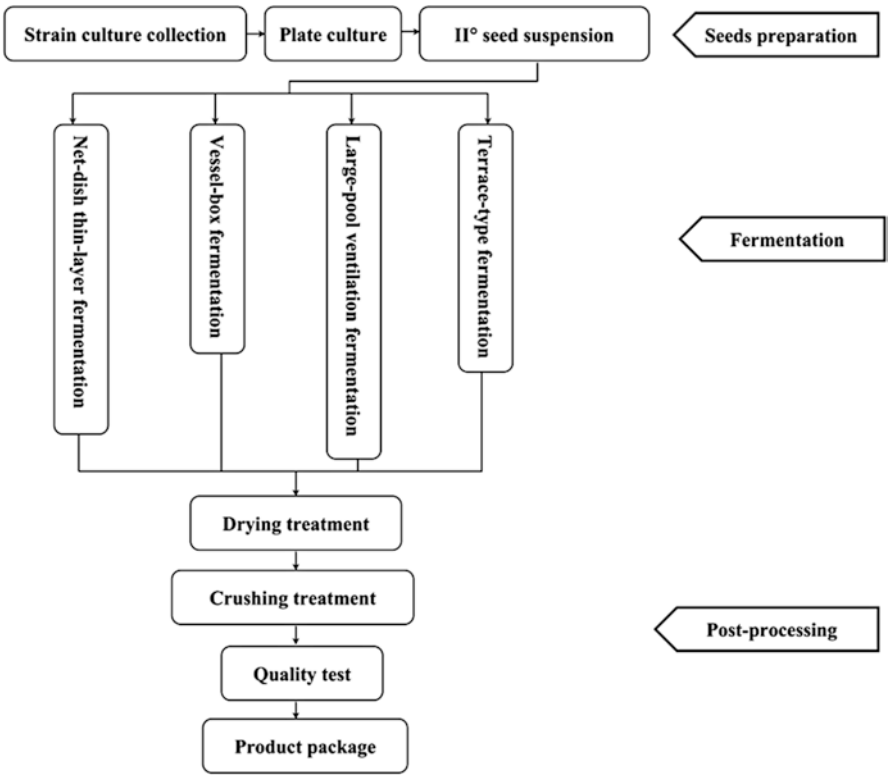

Fig. 12.3 Schematic of the solid-state fermentation of *B. thuringiensis* biopesticides

the culture medium. In addition, a certain gap between particles helps the bacteria absorb nutrients from the culture medium and is conducive to oxygen transfer, thus promoting the growth and reproduction of the bacterium of interest. High water content can lead to the agglomeration of culture medium and the decrease of porosity, thus affecting oxygen transfer. By contrast, low water content can reduce the expansion degree of culture medium and lower water activity, thus inhibiting the growth of bacteria.

Various raw materials are used for solid-state fermentation. By using industrial and agricultural by-products to replace traditional raw materials or carriers for Bt production and via optimizing the culture conditions and improving the fermentation equipment, the toxicity potency of Bt preparation by solid-state fermentation can generally be higher than that of the original culture medium. Meanwhile, the production cost can be significantly reduced. In the 1980s, most Bt solid-state fermentation factories in China used bran as the main raw material. Some investigations found that adding peanut cake into fermentation culture medium could improve the insecticidal effects of HD-1, which exerts stronger insecticidal effects than its commercial counterparts. For example, the toxicity potency of Bt preparation could

reach 7,300 IU/μl when brewer's grains are used as the main raw materials for solid-state fermentation and when the fermentation conditions are optimized via solid-state fermentation with periodical dynamic changes in air (Chen et al. 2009; Chang et al. 2010). In addition, the cost of production can be reduced by using the abandoned grass after the extraction of nicotine, solanesol, and ubiquinone 10 as the main carrier materials in the solid-state fermentation. For example, Yang et al. used bran, cottonseed cake, rice bran, and plant ash as fermentation raw materials. Using these components, an experiment was performed through Bt HD-1 solid-state fermentation experiment of 100 kg grade. The results demonstrate that the important factors that affect spore formation and toxic potency include age, fermentation temperature, initial pH value, medium water content, and plant ash content. The optimal culture conditions could make the number of fermented spore stabilize at 2.0×10^{10} CFU/g, and the fatality rate of *P. rapae* under 2,000-fold dilution is 100% (Yang et al. 1993, 1998).

Compared with submerged fermentation, solid-state fermentation has many benefits, such as lower moisture content in the culture medium, smaller wastewater and waste residue release, minimal environmental pollution, lower energy consumption, simpler equipment requirement, lower investment, and higher product concentration. Moreover, the carriers from agricultural and sideline products, such as bran, can be used as both carbon sources, and fermentation carriers can be directly dried and crushed after fermentation, which requires only simple post processing processes and is energy saving. However, solid-state fermentation also has significant disadvantages, mainly including the difficulty in controlling the fermentation temperature, humidity, pH, and oxygen supply; the low automation degree; the sensitive infectivity by other organisms; and the poor wetting performance of the products in use. An engineering solution for these technological difficulties is lacking, which restricts the early development of solid-state fermentation in China (1950–1980).

In recent years, China's environmental protection requirements on the fermentation industry have increasingly improved. Bt solid-state fermentation as an environment-friendly traditional fermentation method in Bt industrial fermentation has gradually shown its superiority to submerged fermentation and thus has considerably attracted the attention of manufacturers. Moreover, the applications of new electronic technology and computer technology, and the improvements of solid-state fermentation equipment's and fermentation control technologies have gradually increased the role and status of solid-state fermentation in the Bt fermentation industry. For example, Chen et al. first proposed the use of dynamic air pressure pulsation solid-state fermentation technology to produce Bt (Chen and Li 2002b). The use of this technology provides conditions in which the biological reaction and mass transfer rate of the cell membrane can be enhanced by the periodic stimulation of pressure pulsation. Pressure pulsation can avoid the drawbacks of mechanical agitation, improve the heat transfer and mass transfer efficiency, decrease the temperature, and lower the concentration gradients of O_2 and CO_2. This processing improves toxic efficiency. At present, the volume of reactors for pressure pulsation solid-state fermentation has been successfully increased to an industrial production scale of 70 m^3, which leads to a new vitality in the industrialization of Bt solid-state fermentation (Chen and Wang 2008; Li and Chen 2005).

2. Submerged fermentation of Bt.

Bt cells have no strict requirements for nutrients. They can grow well not only in a nutrient-enriched soluble culture medium but also in a liquid medium with high solid content. Submerged fermentation has advantages, such as high density, high control precision, and high automation degree. These advantages lead to the current Bt-industrialized fermentation production that adopts the technology of liquid-state submerged fermentation. Liquid submerged fermentation mainly involves some aspects, including the strain, culture medium, and culture conditions; the postprocessing technology; and the prevention of fermentation from infestation by phages or other microorganisms (Chang et al. 2010).

Liquid submerged fermentation has been successfully applied to Bt industrial production in the 1960s. The fermentation level and tonnage gradually increased from hundreds of liters to a few tons in the 1970s and further to 50–80 tons at present. With the application of new technologies, such as centrifugal spray and airflow smashing, the original production technologies, such as filter-press enrichment and dryer crushing, have been disused gradually. The implementation of these processes changed the formulation forms that were mainly wettable powders. The emergence of new formulation forms, such as emulsified oil, tablet, and microcapsule, has endowed the application of Bt biopesticides with more pertinence. The annual output of Bt insecticides produced by liquid submerged fermentation in China reached 30,000 tons in 1995. Three or four large manufacturers were involved, and the toxicity potency of Bt suspensions by liquid submerged fermentation was up to 3,000–5,000 IU/μl. The technological process of Bt liquid submerged fermentation is shown in Fig. 12.4.

The production level of Bt fermentation is not only determined by the performance of production strains themselves but also influenced by fermentation conditions and processes. The metabolism regulation mechanisms and possible metabolic pathways of production strains during product synthesis should be understood. Furthermore, the definite determination of the requirements of production strains for environmental conditions is the basis for mastering the metabolic variation rules of strains during fermentation. Thus, various technological conditions and parameters are effectively controlled, thereby maximizing the production capacity of strains and finally achieving the maximum economic benefits. The temperature range of Bt growth is from 20 to 42 °C. Bt does not produce endotoxins at the upper temperature limit. The optimal temperature range at which Bt produces endotoxins is 28–32 °C. In general, fermentation is performed at 30 °C. The pH range for Bt growth is 5.6–8.5. The oxygen supply during fermentation is very important because it significantly affects not only Bt growth but also endotoxin synthesis and spore germination.

The submerged fermentation of Bt liquid has been divided into batch and fed-batch fermentation. At present, large-scale Bt manufacturers mostly adopt fed-batch submerged fermentation because batch fermentation generally loads and reladles once. Moreover, the relatively high initial concentration of the culture medium allows the inhibitory effects of the substrates and metabolites on cell growth and endotoxin synthesis, and negatively influences oxygen transfer. After entering the logarithmic phase, an oxygen-deficient phenomenon is likely to occur, which significantly affects

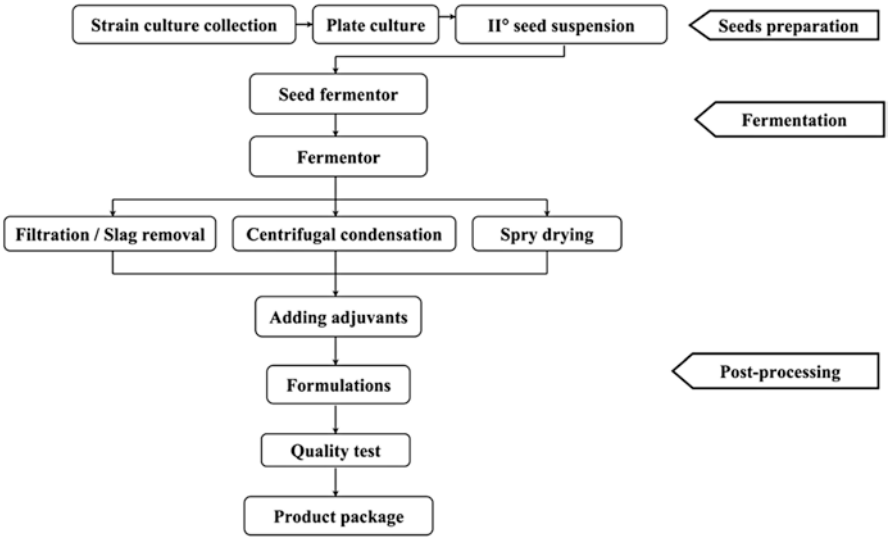

Fig. 12.4 Schematic of the submerged fermentation of *B. thuringiensis* biopesticides

the rapid reproduction of Bt cells during fermentation. Consequently, high-density Bt cells are difficult to obtain. Fed-batch fermentation technology avoids the cell flourishing, excessive oxygen consumption, and inadequate stir and ventilation equipment caused by the overloading of the culture medium in the batch fermentation. Thus, this technology has been applied in Bt fermentation. With the continuous development of theoretical research and industrial applications in recent years, several progressions from the feeding way to computer optimization control have been observed. For example, Li et al. reported that fed-batch fermentation technology significantly improves the fermentation broth dissolved oxygen (DO), thereby increasing cell density, crystal concentration, and bioassay efficacy by an average of 4.0×10^9 CFU/ml, 0.83 mg/ml, and 1,800 IU/μl, respectively, compared with batch cultures (Li et al. 2001). Yang et al. obtained maximum biomass, crystal content, bioassay virulence, and spore number values of 51.89 DCW/l, 37 mg/ml, LC_{50} of 0.29 μg/ml, and 5.7×10^{10} CFU/ml, respectively; these values are 144.3%, 137%, 169%, and 50% higher than those in batch fermentation (Chen et al. 2002; Yang and Yue 2001). Huazhong Agricultural University adopted the new Bt fed-batch fermentation technology for Bt production. The consumption of raw materials was reduced, and the fermentation period was also shortened (Zhu et al. 2003, 2004). In fact, the production cost of fermentation broth in 40 M^3 fermentation tank decreased from 350 to 175 RMB yuan/1,000 IU/μl per ton. Under the existing production equipment, the toxicity potency increased from 3,500 to 5,000 IU/μl (Chang et al. 2010; Guan et al. 1998; Huang et al. 1996; Liu et al. 2005).

The advantages and disadvantages of liquid submerged fermentation technology are as follows. During fermentation, liquid-state submerged fermentation demonstrates good liquidity, better mass transfer and heat transfer performances than

solid-state fermentation, and easy-to-control process. However, in the postprocessing process, liquid state fermentation usually needs numerous processing procedures, such as concentration, drying, and crushing, which complicate the operation. Meanwhile, effective components are prone to run off, which increases the cost of biopesticides, thereby losing the competitive advantages relative to chemical pesticides. At present, liquid-state fermentation has become widely used, and mature fermentation technology is used in industrial production. Although liquid-state fermentation technology and equipment research are both relatively sophisticated, some problems still exist, such as the high cost and low efficiency of the culture medium, thus making the production cost relatively higher.

The development of the Bt industry in China has undoubtedly gained good conditions with the emergence of China's new fermentation equipment, the increase in fermentation tonnage, the improvement of industrial fermentation and fermentation liquor processing technologies, and the diversification of formulations (Fig. 12.5). However, in terms of strain screening and fermentation technology development, the liquid-state fermentation technology in China is still relatively backward when compared with international advanced technologies. China mainly adapts batch fermentation technology, and the loss of effective components during fermentation is relatively serious. In actual industrial fermentation, Bt production should not only choose fermentation raw materials with low cost but also optimize the fermentation conditions to reduce the production cost from the two aspects of raw materials and fermentation conditions.

12.4.2 Infestation Control of Phages in Bt Production

Phage infestation has been a major threat to Bt production for a long time. Chinese scientists and enterprises have explored a series of controlled technologies in practice through close cooperation. This exploration effectively controls the reladling caused by phages. The fundamental measurement to significantly increase the toxicity potency of Bt products is adopting advanced fermentation and postprocessing technologies. For example, the risks of phages can be effectively controlled by variable temperature fermentation, thus effectively controlling the reladling rate. In previous production factories of Bt pesticides in China, the tank rate caused by phage contamination was as high as 10–30%, and some enterprises cannot keep normal production and were even forced to stop production because of the phage hazard. Through mutation breeding of heat treatment and chemical factor treatment, a HD-1 mutant strain that can still maintain normal growth and virulence level at 37 °C was obtained (Yu 1993). The sensitive period of Bt bacteria to phages can be shortened through fermentation at 33–37 °C. Combined with other comprehensive measurements, such as enhancing air purification system and improving environmental conditions, the risks of phages can be effectively controlled. In fact, data indicate that the tank rate decreased from 10% to 0.7%, which meets the international advanced level (Chen et al. 2005).

Fig. 12.5 Production of *B. thuringiensis* biopesticides by submerged fermentations in China. (**a**, **b**) Fermentors (Wuhan KeNuo Biotech, Inc.); (**c**), Seed fermentors (Wuhan KeNuo Biotech, Inc.); (**d**) Fermentation workshop (Wuhan KeNuo Biotech, Inc.); (**e**, **f**) Pesticide efficacy trials in the greenhouses (Hubei Kangxin Agro-industry Co., Ltd)

12.4.3 Post Processing Technology and Formulations of Bt Production in China

ICPs, spores, vegetative insecticidal protein, and synergistic factors, such as Zwittermicin A, are the main active ingredients of Bt. The core problem in fermented liquid postprocessing is the separation of insecticidal ingredients, such as ICPs, and their synergistic factors from the fermented liquid and the removal of more than 90% water to obtain the dry industrial raw powder. To protect the insecticidal activity of fermented liquid, the condensation process should be operated rapidly at low temperatures. The condensation of fermented liquid mainly includes

the plate–frame pressure filtration method, the vacuum condensation method, and the centrifugal separation method. The plate–frame pressure filtration method is one of the earliest used condensation methods in Bt industrial production. In this method, abundant inert fillers need to be fed before filtering to obtain a powder with efficient toxicity potency. The vacuum method by reducing pressure condenses the fermented liquid to a certain volume at low temperatures. However, it has the disadvantages of high energy consumption and low efficiency. Thus, this method is currently adopted by few enterprises. The centrifugal separation method is still the most widely used in Bt mass production. However, the separation of supernatant fluid results in the loss of synergistic ingredients and the damage of vegetative cells and spores. After removing 69% of the supernatant in the fermented liquid by centrifugal separation, the toxicity potency loss of the concentrate is 53.4%, and the crystal loss is 11.3% (Wang et al. 2011; Zhu et al. 2004). These data indicate the urgent need for Bt manufacturers to develop new postprocessing technologies and reduce the loss of insecticide activity components.

Formulation is an important factor that influences the field control effect of Bt. Bt formulations can be divided into wettable powder, suspending agent, water dispersible granules, suspension agent, microcapsule, granule, emulsified oil, and so on. The plate–frame filter technology was adapted in liquid-state fermentation from the 1950s to the 1980s in China, and it can only produce Bt suspending agents with low toxicity potency. Since the 1990s, the continuous centrifugal separation has been adopted to improve the process and increase the toxicity potency. When referring to ultrafiltration condensation and spray drying technology used in medical production, the toxicity potency of Bt products was significantly improved. Moreover, Bt wettable powder with a high content of ICPs can be produced. The requirements for production fineness and formulation processing can be met by crushing the powder using airflow smashing technology. For example, in China, disc centrifugal separators were used in the recycling of Bt pesticide production for the first time, and the recovery rate of effective components in the fermented liquid reached 75.4%. By contrast, the recovery rate of traditional plate–frame filtering recycling was only 60%. In addition, this centrifugal separation technology saved energy by 94% compared with the membrane enrichment process (Chang et al. 2010). With the new recycling process, the toxicity potency of HD-1 suspending agent products increased to 2,500 IU/μl, which further increased to more than 3,000 IU/μl in 1991–1992. Disc centrifugal separation technology combined with spray drying had been used to produce HD-1-based Bt wettable powder biopesticides and "Qingchong" powder. The content of live spores of the two products reached up to 300–400 × 10^9 CFU/g, and the toxicity potency was up to 16,000 IU/mg (Yu 1993). The quality of these two preparations was close to that of their international counterparts in the early 1990s. Since then, the technology capability of Bt powder produced by China has reached a toxicity potency of 50,000 IU/mg, and the Bt powder has begun to be exported to the United States (Yu 1993).

Currently, the formulations of Bt products produced in China are mainly Bt suspending agents and wettable powders. Suspending agents mainly have three specifications of 2,000, 4,000, and 8,000 IU/μl, and wettable powders have three specifications of 8,000, 16,000, and 32,000 IU/mg. Chinese scientists have also

developed some new formulations from 2000. For example, Liu et al. considered the exposed problems of Bt pesticides in practice, such as the susceptibility to environmental factors and the poor adhesiveness to waxy leaves, and they developed the Bt GB-111 oil agent of mineral oil type (Liu et al. 2000). This product features good adhesiveness, plating-out ability, and antirain wash capability, and overcomes the defects of existing dosage forms. As a result, the performance is improved, and the environmental pollution is significantly reduced. Nowadays, these types of products are widely used to prevent and control *H. armigera*, *P. xylostella*, *O. nubilalis*, *E. pseudoconspersa*, *C. medinalis*, *H. assulta*, *E. oblique*, and *Dendrolimus* spp., with a control efficiency reaching 85% or higher.

12.4.4 Quality Standardization of Bt Preparations

Quality standardization is a series of technical standards that are formulated after scientific research, field trial, and investigation to meet the needs of production and management. Product standardization is developed to adapt the industrial production demand. The intension of the quality standardization of Bt products includes two aspects. The first aspect is the standardization of industrial products, which refers to the products provided by the factories that must maintain the stability of quality. The second aspect is the international standardization, which refers to the products produced by different countries, production processes, and subspecies or strains of biopesticides that are comparable. For example, the quality indicators of Bt products at least include (1) the subspecies or strains of fermentation production, effective ingredients, such as parasporal crystals, thuringiensins, spores, and main adjuvant ingredients; and (2) the expression of toxicity potency, such as the number of spores and the toxicity of parasporal crystal and thuringiensin. When biological assay is performed in determining toxicity, the standard product should be used. Meanwhile, the insects that are for testing should be specified.

Chinese scientists have conducted intensive researches to determine the insecticidal activity of Bt. Investigators from Sun Yat-Sen University first used the newly hatched larvae of *Bombyx mori* to determine the virulence of Bt, whereas investigators from the Institute of Microbiology of Hubei Province assessed the virulence of parasporal crystal by using the trace drop method and then determined the virulence of purified crystal using *B. mori* and *H. armigera* larvae as indicative pests. Other investigators also detected beta-exotoxin with *M. domestica*. Meanwhile, Central China Normal University detected the activity of thuringiensin through rocket immunoelectrophoresis and enzyme-linked immunosorbent assays.

Standardization of Bt preparations is an important component in accelerating the development of Bt. In the commercialization of Bt preparations, the establishment of standardization provided the standards of quality evaluation and the corresponding detection means, which are important in standardizing the Bt market and promoting the healthy development of the Bt industry. Since the advent of Bt insecticides in 1964 in China, the content of live spores has been always viewed as the standard in testing Bt product quality. However, numerous investigations have proven that the

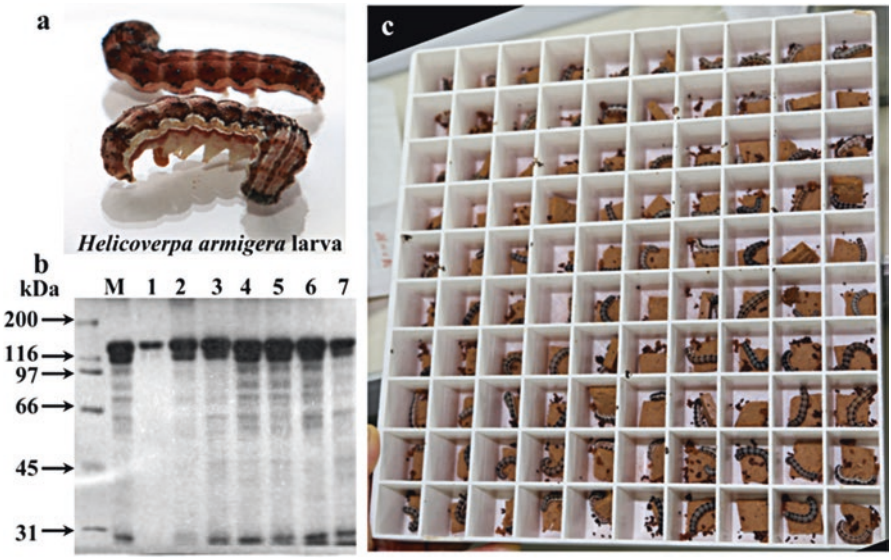

Fig. 12.6 Standard bioassays of *B. thuringiensis* biopesticides in China. (**a**), *H. armigera* larvae are used as the indicator pests; (**b**), SDS-PAGE analysis is performed to quantify the ICPs content of Bt biopesticides; (**c**), The numbers of survival pests on the 100-well culture plate are counted after feeding with Bt biopesticide-mixed feedstuff for 48 h to determine the sub-lethal toxicity of Bt pesticides

content of live spores in Bt is not always positively correlated with the toxic effect. By contrast, the toxicity potency determined by the bioassay can objectively reflect the quality of Bt insecticides. To solve the quality standardization problem of Bt insecticides in China, the Ministry of Agriculture (MOA) arranged numerous institutions to perform a large scale trial, with *H. armigera* and *P. xylostella* as the standard test insects. This work gradually perfected the bioassay technology system for Bt virulence and finally obtained the standardization of Bt quality inspection (Fig. 12.6).

12.4.5 Progress in the Preparation of Bt Standard Samples

Under the organization of the Institute for the Control of Agrochemicals, MOA, the standards of Bt preparations in China gradually achieved agreement with those of Bt preparations in Europe and America via continuous improvement and perfection. In the first step, two Bt standard samples for the bioassay, namely, CS_{3ab}-1986 (with a toxicity potency of 7,400 IU/mg) and CS_{5ab}-1987 (with a toxicity potency of 8,600 IU/mg), were prepared and validated. Second, the upgrade and update of technology standards were implemented, and another two Bt standard samples, namely, CS_{3ab}-1991 (with a toxicity potency of 15,000 IU/mg) and CS_{5ab}-1991 (with a toxicity potency of 20,000 IU/mg), were prepared. Third, on the basis of the standardization of the Bt bioassay, SDS–PAGE gel electrophoresis was introduced to detect the

ICP amount of Bt biopesticides in China, which realized the quality control of Bt fermentation and the quantitative analysis of ICPs in the products. Given its fast operation and good reproducibility, this method can be used in practical production. In 1995, the State Key Laboratory of Agricultural Microbiology in Huazhong Agricultural University prepared the third generation of Bt standard CS_{H3ab}-1995 (with a toxicity potency of 32,000 IU/mg on the test insect *P. xylostella*) and finally established a Bt quality standardization technology system that is complete, accurate, fast, and reliable. These progresses laid a fundamental guarantee for the Bt industry to be ranked in the pesticide technology market competition in China and the world. From 1988 to 1994, the bioassay standardization technology had been widely used by primary Bt manufacturers in China to perform quality testing. This phenomenon solved the standardization problem of the quality testing method in more than 20 years. The establishment and popularization of this technology significantly improved the quality management level of Bt insecticides and promoted the steady development of the production and application of Bt biopesticides in China.

12.4.6 Development of Standards of Bt Series Products in China

To regulate the industrial production and market development of the Bt industry, China enacted some industrial standards at the early phase for Bt products. These standards include HG3616-1999 Bt raw powder, HG3617-1999 Bt wettable powder, and HG3618-1999 Bt suspending agent. In 1995, the MOA departmental standard of Bt preparations (NY293-5) took effect, thus obtaining the agreement with the international standards. To further satisfy the need of Bt industrial development, China has enacted new national standards of Bt preparations, including those for Bt powder (GB/T 19567.1-2004), Bt suspending agent (GB/T 19567.2-2004), and Bt raw powder (GB/T 19567.3-2004) (the specific indicators are shown in Table 12.1).

The establishment and development of the above-mentioned Bt series standard samples and Bt product standards did not only establish China's own Bt toxin protein detection standard samples and a set of feasible methods of detecting chemical pesticide incorporation into Bt preparations but also created good conditions for improving the competitiveness of Chinese Bt biopesticides in the international market and ensuring the healthy and rapid development of China's Bt industry.

12.5 Main Restrictive Factors for the Market Development of Bt Biopesticides

The Bt industry in China has possessed a large industry scale after the development of industrialization process for more than 60 years. However, China's Bt industry still has deficiencies in terms of production, application, and market development:

Table 12.1 Chinese national standard of Bt powder (GB/T 19567.1-2004)

Testing items[a]	Indication range[b]			
	B.t.a		*B.t.k*	
	First-grade product	Qualified product	First-grade product	Qualified product
Toxin protein (130 kDa) (%) ≥	8.0	7.0	7.0	6.0
Toxicity potency (*Px* IU/mg) (*Ha* IU/mg) ≥	–	–	50,000	40,000
Toxicity potency (*Se* IU/mg) ≥	60,000	50,000	–	–
pH	5.5–7.0	5.5–7.0	5.5–7.0	5.5–7.0
Moisture (%) ≤	6.0	6.0	6.0	6.0
Fineness (75 μm) (%) ≥	98	98	98	98

[a]*Px*, *Ha*, and *Se*: *P. xylostella*, *H. armigera*, and *S. exigua*, respectively
[b]*B.t.a* denotes the Bt subsp. *aizawai*; *B.t.k* denotes Bt subsp. *kurstaki*

1. In terms of application fields, Bt products are mainly used for agriculturally important crops but seldom used in the forest protection field and the control of warehouse and health pests.
2. In terms of industrial development, numerous Bt manufacturers exist in China. However, only 3 or 4 Bt enterprises have an annual output value of more than 30 million RMB yuan. By contrast, the largest Bt manufacturer in the world, namely, Certis USA, the branch company of Mitsubishi (Japan), had a Bt sales volume of 30 million dollars in 2000. The small enterprises usually have a low production level, low preparation level, and small production scale, thus leading to a high production cost.
3. In terms of strain cultivation and production technology, the sources of strains for mass production are abundant in the countries outside China, and the efficient and broad-spectrum genetic engineering strains are widely adopted in production. In fermentation production, fed-batch fermentation is usually adopted, and the fermentation level is high. The low-temperature spray drying technology is commonly adopted, and the recovery rate of fermented products is high. The formulations are also diversiform, including powder, wettable powder, suspending agent, aqueous concentrate, oil emulsion, miscible oil, granules, tablets, emulsifiable suspension, controlled release formulations, and biological encapsulating agent. By contrast, most production strains produced by China belong to Bt subsp. *kurstaki*. Furthermore, the technology content of products produced by most enterprises is relatively low, and the formulations are also relatively few.
4. Some problems in the practical applications of Bt biopesticides are as follows. First, Bt preparations have slow efficacy and usually show pesticidal effects a week later, whereas chemical pesticides can take effect within 30 s. Second, the cost of Bt production is relatively high. The reladling rate of producing Bt preparations in China is high, and the strains are susceptible to infection by other bacteria or phages because of the absence of strict processes and quality controls, leading to the poor quality and low toxicity of fermentation products. Third, Bt has high specificity and selectivity. For example, strains with a high

virulence to *E. elutella* have a very low virulence to *L. serricorne*. Fourth, the sunlight and ultraviolet protection performances of Bt biopesticides are poor in the actual production. In the control of tobacco warehouse pests by Bt biopesticides, the effects of good control are just attributed to the low dosage or absence of sunshine and ultraviolet radiation under indoor conditions. Therefore, China should intensify the research on the control of warehouse pests by Bt. This research would provide a new pathway that accords with the Integrated Pest Management principle for the effective control of tobacco and other warehouse pests.

In addition, the development of Bt biopesticides also faces some restrictive factors in terms of marketization.

1. The price of raw materials is a restricting factor in the scale production of Bt biopesticides. The price of traditional fermentation raw materials accounts for about 35–59% of the total production cost. Therefore, the manufacturers should devote themselves in developing low-cost and locally available industrial and agricultural wastes as the raw materials. Some researchers have tried to use sludge, monosodium glutamate wastewater, and waste beer yeast decoction as the fermentation raw materials. These materials not only created favorable conditions for the promotion of Bt biopesticides but also maximized the waste resources. At present, the selection and optimization of low-cost raw materials are still in its infancy, and their enlargement and mass production are important to realize.
2. Traditional production technologies cannot satisfy the increasing application demand, and the development of efficient production process is very urgent. Although the demand for Bt biopesticides increases rapidly, the current production is far from meeting the needs of the market. This situation can be largely ascribed to the traditional fermentation method. The fermentation level can be enhanced by improving the fermentation process, fermentation equipment, and fed-batch fermentations. Furthermore, the optimization of culture medium causes the enhancement of the fermentation technology and acceleration of the industrialization process of Bt biopesticides. As confirmed by many investigations, the high-density fed-batch fermentation technology and dynamic air pressure pulsation solid-state fermentation technology, which increase Bt ICP content, would have broad application prospects.
3. The insecticidal spectrum is narrow. To further enhance the insecticidal effects of Bt, such as extending the effective persistence in the field applications and expanding the insecticidal spectrum, genetically modified Bt strains can be constructed using recombinant DNA technologies, Omics technologies, or synthetic biology technologies. In China, the R & D of novel Bt biopesticides using genetically engineered strains shows an increasing trend. For example, the genetically engineered Bt biopesticide WG-001 based on Bt and *Pseudomonas fluorescens* has passed through the safety assessment and was officially approved in 2000. Undoubtedly, an increasing number of novel Bt biopesticides that are based on engineered strains would be available in the near future.

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Chapter 13
The Role of Embrapa in the Development of Tools to Control Biological Pests: A Case of Success

Rose Gomes Monnerat, Glaucia de Figueiredo Nachtigal, Ivan Cruz, Wagner Bettiol, and Clara Beatriz Hoffman Campo

Abstract Brazil has an impressive consumption of agrochemicals, but the intensive use of synthetic agrochemicals in agriculture causes a variety of problems, such as contamination of food, soil, and water, impact animals, cause intoxication of farmers, and development of pest resistance. In addition, they are responsible for biological imbalance, changing the cycling of nutrients and organic matter by eliminating beneficial organisms, and reducing biodiversity. Over the last few decades, the Brazilian Agricultural Research Corporation (Embrapa) has generated significant basic knowledge on the biological control of agricultural and urban pests, diseases, and invasive plants, as well as their integration into existing agricultural systems. In 2013, the theme "biological control" gained even greater prominence and priority at Embrapa through the creation of a specific research portfolio for this subject. The portfolio of research, development, and innovation (R&D&I) projects for biological control has as a priority for the development of biological control technologies and their insertion in the integrated management of pests of agricultural interest that are resistant to synthetic

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chemical pesticides; those related to crops with insufficient phytosanitary support, considered as “minor crops”; to large crops and those affecting animal welfare or production facilities.

Keywords Bioinsecticides • Bioproducers • Pest control

Brazil is a world leader in the agribusiness sector, and this leadership signifies a growing dependence on imported inputs. According to the agribusiness department of the Federation of Industries of the State of São Paulo (Deagro/Fiesp), in 2012, imports of fertilizer, synthetic pesticides, machinery and implements, animal nutrition, and animal health segments exceeded 18 billion dollars. In the pesticide segment, in 2012, an increase of nearly three times higher than in 2007 was anticipated, and, currently, Brazil is also the world leader in the consumption of agrochemicals (Congressional News Agency, 05/09/2012). In 2012, the National Sanitary Surveillance Agency (Anvisa) stated that the country is responsible for one fifth of the world consumption of agrochemicals, using 19% of the pesticides produced in the world. According to the National Association of the Agricultural Defense Products Industry (Sindag), Brazilian producers are using more pesticides, whose sales between 2006 and 2012 increased from 480,100 tons to 826,700 tons thousand tons, an increase of more than 72%. Areas cultivated with grain, fibers, coffee, and sugarcane, in this interval, increased from 68.8 million to 81.7 million ha, hence less than 19%. The average consumption of agrochemicals between 2005 and 2011 increased from just over 7 to 10.1 kg/ha, representing an increase of 43.2%. The highest sales growth occurred with fungicides. Between 2006 and 2011, annual use rose from 56,000 to 174,000 tons, thus more than tripling in 5 years (Bettiol et al. 2014).

The figures on the consumption of agrochemicals in Brazil are impressive, and, admittedly, the intensive use of synthetic agrochemicals in agriculture causes a variety of problems, such as contamination of food. Studies carried out by the Program for Analysis of Agrochemical Residues in Foods (PARA), coordinated by Anvisa, found that 27% of the fruit and vegetables sold have residues of agricultural pesticides above that permitted by law. In addition pesticides contaminate the soil and water and impact animals. They cause intoxication in farmers, lead to pest resistance, and intensify the appearance of iatrogenic diseases. In addition, they are responsible for biological imbalance, changing the cycling of nutrients and organic matter by eliminating beneficial organisms and reducing biodiversity.

The indiscriminate use of pesticides concerns the various segments of society with regard to their effect and has generated at least two important consequences: (i) changes in the environmental agenda of various countries and (ii) the creation of certified food markets for the nonuse of synthetic pesticides or their proper use as organic products. Therefore, there is a growing demand for alternatives to meet environmental constraints and consumer demands. Among these alternatives, there is biological control in association with integrated pest management.

In 2009, the European Union approved a legislative package for the effective adoption of Integrated Pest Management Programs, which generated a search

for sustainable agrochemicals and offered opportunities for greater inclusion of biological control agents. In addition, the European Union approved the Biocomes project for the development of 12 biological products, in partnership with private companies, in the amount of € 12,086,533 over a period of 4 years. Actions of this nature are taking place in the Brazilian legislature, such as Senate Bill No. 679 of 2011 (Art. 21A, which created the national policy to support natural agrochemicals) and Decree No. 7794, of 08/20/2012, which instituted the National Policy on Agroecology and Organic Production.

According to the International Organization for Biological Control (IOBC), natural biological control in all world ecosystems is estimated at 85.5 million km^2, while classical biological control using exotic natural enemies for pest control has its estimated use of 3.5 million km^2 (10% of cultivated land). Augmentative biological control, which involves the release of naturally occurring natural enemies (native or exotic) for pest and disease control, is being applied to 0.16 million km^2 (0.4% of cultivated land).

In Brazil, in 2011, the market for biological control products was between US $97 million and US $194 million, equivalent to 1–2% of the sales of the synthetic agrochemicals market. It is estimated that the area treated with biological control agents in Brazil is slightly less than 8 million hectares per year. By 2016, 118 bioagent-based products were registered in Brazil, compared to the 26 existing in 2011.

Although high in absolute terms, the percentage participation is timid in crops for which biological alternatives are available (Bettiol et al. 2014). Biological control goes through a time of great repercussion and expansion in the world pesticide market, with an annual growth rate of 15% and a sales estimate of US $4 billion in 2017. The world market for agrochemicals is expected to reach $8.82 billion by 2022, according the site, http://news.agropages.com/News/NewsDetail---20170.htm.

The current profile of the biological control agent industry includes, for the most part, small and medium-sized specialized companies, few established more than 10 years ago. Despite the predominance of small and medium-sized companies, large companies, traditionally leaders in the synthetic agrochemicals market, have acquired the main biocontrol companies around the world or are reactivating divisions related to the development of biopesticides, depending on their business perspective. Given the positive scenario, biological control research represents an opportunity for innovation and competitiveness in Brazilian and world agriculture and attends to the environmental perspectives and the sustainable use of environmental services. With the market growing and estimated to reach US $25 billion by 2030, the demand to improve processes inherent in biological control will increase, generating opportunities for research and partnerships for innovation in this field. Thus, it is fundamental that public research institutions actively participate in the development of biological products.

Over the last few decades, the Brazilian Agricultural Research Corporation (Embrapa) has generated significant basic knowledge of the biological control of agricultural and urban pests, diseases, and invasive plants, as well as their integration into existing agricultural systems. One of the most successful examples of biological pest control developed by Embrapa in Brazil was the large-scale production

and the use of *Baculovirus anticarsia*. This program, implemented beginning in 1977 to control the soybean caterpillar, *Anticarsia gemmatalis*, was an example for agriculture in its simplicity and efficiency. Other viruses of the same genus, such as *B. spodoptera* and *B. helicoverpa*, for control of the corn cartridge caterpillar, *Spodoptera frugiperda* and of *Helicoverpa armigera*, respectively, were developed by Embrapa in partnership with private companies and are in the registration phase (Oliveira et al. 2006).

In the case of fungi, it is worth mentioning *Metarhizium anisopliae* for the control of grasshoppers (*Deois* spp.) and sugarcane spittlebugs (*Mahanarva fimbriolata* and *Mahanarva posticata*), used in more than 3 million hectares. Also *Beauveria bassiana*, recommended for control of the coffee berry borer (*Hypothenemus hampei*), banana root borer (*Cosmopolites sordidus*), and black coconut bunch weevil (*Homalinotus coriaceus*), had the participation of Embrapa in its development and today is the pest control bioagent that has the largest expansion in terms of area applied. Another important fungus is *Trichoderma* spp., which is used for the control of plant diseases and is being applied to more than 5 million hectares in Brazilian agriculture for the control of soil pathogens.

In terms of parasitoids, one of the most important cases is the control of the sugarcane borer (*Diatraea saccharalis*) through mass rearing in laboratory and release in the field of the wasp *Cotesia flavipes*. In the soybean crop, we highlight the parasitoid *Trissolcus basalis*, whose technology was developed and perfected by Embrapa Soybeans, and in the tomato crop the control of *Tuta absoluta* (tomato moth) by the parasitoid (*Trichogramma pretiosum*) developed by Embrapa Semi-arid.

At Embrapa Temperate Climate, the focus of studies on the biological control of insect pests is on the control of the fruit fly (*Anastrepha fraterculus* and *Drosophila suzukii*) with native parasitoids and, more recently, with microorganisms. These studies are being carried out in partnership with Embrapa Genetic Resources and Biotechnology, Embrapa Grape & Wine, Embrapa Environment, the Federal University of Pelotas, and Epagri-São Joaquim.

Parallel to the use of parasitoids, actions with the sterile male technique from the sterilization of insects via gamma radiation are being performed. At Embrapa Grape & Wine, the focus has been the production of parasitoids and sterile fruit fly males. Embrapa Temperate Climate, in partnership with the Federal University of Pelotas, Embrapa Wheat, and Embrapa Genetic Resources and Biotechnology, also studies the biocontrol of weeds, focusing on the bioprospecting of biological control agents (BCAs) for horseweed management (*Conyza* spp.) and ryegrass (*Lolium multiflorum*), which has shown multiple resistance to synthetic herbicides. These species are considered a serious problem for the soybean, corn, wheat, and rice crops in Rio Grande do Sul, justifying efforts for the rational exploitation of microbial genetic resources with deleterious potential, which cause death of the target species, without prejudice to crops of economic interest, qualifying themselves for development as potential bioherbicides. Advances in the knowledge on rhizobacteria in the control of rice root knot nematode have been achieved by Embrapa Temperate Climate, with increases in the order of 20–30% in grain yield.

Products based on *Bacillus* spp. have been extensively researched and developed in two units of Embrapa, Embrapa Corn and *Sorghum*, and Embrapa Genetic Resources and Biotechnology.

From 1992 to 1999, soil and grain dust samples were collected from different regions of Brazil in order to isolate *Bacillus thuringiensis* (Bt). More than 4,600 strains of Bt have been isolated and are stocked at Embrapa Maize and *Sorghum* Research Center, Sete Lagoas, MG. These isolates are kept in freezers and also will be lyophilized. Many strains have been characterized for many lepidopteran pests of maize, cotton, and soybean. These strains have also been characterized for insect pests of Hemiptera/Heteroptera (sucking bugs), Coleoptera (beetles), Hymenoptera (ants only) orders, and Nematodes. The main caterpillars tested are fall armyworm, *Spodoptera frugiperda*, *S. cosmioides*, *S. eridania*; sugarcane borer, *Diatraea saccharalis*; soybean looper, *Chrysodeixis* sp.; *Heliothis virescens*; and *Helicoverpa armigera.* The characterization of these Bt isolates helped the research to achieve Bt-based biological products with the main objective to decrease the use of chemical pesticides. This research includes molecular characterization, insect bioassays, bioassays with nontarget organisms, lab production of the biopesticide, and different formulations. All steps have a main goal to obtain a reliable and stable Bt-based biopesticide to be commercialized by private companies.

Embrapa Genetic Resources and Biotechnology began research into product development in 1988 when it began forming a collection of bacteria for pest control. Soil, water, and dead insect samples were collected from different regions of Brazil, and the collection currently has 2,600 strains of *Bacillus* bacteria for invertebrate control. The collection works according to international standards have been accredited according to ABNT NBR ISO/IEC 17025 and have operated since 2016 as the only Brazilian Biological Resource Center (BRC). From the assets of the collection, it initiated partnerships with private institutions for the development of fermentative processes and formulations for the synthesis of bioinsecticides (Fig. 13.1). The first partnership was established in 1992 with the company Geratec do Brasil, and as a result there was the generation of the Sphaerico Bioinsecticide, a concentrated suspension based on the bacterium *Lysinibacillus sphaericus* to control larvae of the *Culex quinquefasciatus* mosquito. The complicated bureaucratic procedures of the time to register the product caused the company to abandon the production project. In 2001, a new partnership established with the company Bthek Biotecnologia generated four products: Sphaerus SC, also based on *L. sphaericus* for control of larvae of the *Culex* spp. and *Anopheles* spp.; Bt-horus SC, based on *Bacillus thuringiensis israelensis*, for control of *Aedes aegypti* and *Simulium* spp. larvae; *fim da picada* (no more bites) SC, developed on the basis of a native strain of *Bacillus thuringiensis israelensis* for control of *Simulium* spp.; and *ponto final* (full stop), developed on the basis of a native strain of *Bacillus thuringiensis kurstaki* for control of insect larvae of the order Lepidoptera, especially *Spodoptera frugiperda*, *Anticarsia gemmatalis*, and *Plutella xylostella*. Although the four products are marketed as concentrated suspensions, their formulations have particularities defined as a function of the biology of the target and the site of application. Sphaerus and Bt-horus are suspensions containing emulsifiers to allow bacterial dispersion throughout the breeding grounds of mosquito larvae and contain substances

Fig. 13.1 Bioinsecticides developed by Embrapa Genetic Resources and Biotechnology with private institutions, based on *Bacillus thuringiensis* and *Lysinibacillus sphaericus* strains from the collection of bacteria of for pest control. Ponto final (Bthek Biotecnologia Ltda.), Inova Bti (Instituto Matogrossense do Algodão-IMAmt), Strike Bio Bti SC (STRIKE Indústria e Comércio), Bt-horus SC (Bthek Biotecnologia Ltda.), Sphaerico (Geratec do Brasil), and Sphaerus SC (Bthek Biotecnologia Ltda.)

that permit the bacteria to float, keeping them where the larvae breathe and feed. *Fim da picada*, as it is more specific for the control of larvae of black flies that live attached to substrates in river beds, has in its formulation substances that help the bacteria to float and is not deposited in the river bed after application. *Ponto final* contains a greater amount of ultraviolet protectors than the others, as well as spreaders, so that the bacteria can adhere to leaves after application and not be affected by the sun. In 2013, due to the detection of the *Helicoverpa armigera* caterpillar in Brazil and the presence of populations of *S. frugiperda* resistant to *Bacillus thuringiensis* toxins, a partnership was implemented with the Mato Grosso Cotton Institute (Instituto Matogrossense do Algodão-IMAmt), an entity formed by cotton farmers. A new screening of *B. thuringiensis* strains from the collection was carried out and from those most toxic to these two insects, the INOVA-BTK product was developed, which will be on the market in 2017. Another product, for the same purpose, was developed in partnership with company JCO Ltda. and should also be on the market in 2017. Also, in partnership with IMAmt, two products were synthesized and will be available in 2017-INOVA-Bti, developed based on the S0008 native strain of *Bacillus thuringiensis israelensis* for control of *A. aegypti*, and INOVA-Lbs, developed based on the S0126 native strain of *L. sphaericus* for control of *C. quinquefasciatus* larvae. In 2016, a partnership agreement with STRIKE Indústria e Comércio allowed the development of two products based on two other strains in the collection, the first based on the S1119 Brazilian strain of *Bacillus thuringiensis*, highly toxic to *A. aegypti* larvae, and the second based on the S0002 *L. sphaericus* strain, highly toxic to *C. quinquefasciatus* and *Anopheles* spp. The products were each developed in two formulations, concentrated suspension and tablets, and contain specific adjuvants to ensure high efficacy under Northeastern Brazilian climatic conditions. These products are called Strike Bio Bti SC, Strike Bio Bti T, Strike Bio Lbs SC, and Strike Bio Lbs T, and they are expected to be on the market in July 2017.

In 2013, the theme "biological control" gained even greater prominence and priority at Embrapa through the creation of a specific research portfolio for this subject. This portfolio aims to:

(i) Stimulate, within Embrapa, the creation of incubated companies for the development of BCAs.
(ii) Develop, together with private enterprise, products based on BCAs, available at Embrapa.
(iii) Stimulate the implementation of biological control in the field of integrated pest management.
(iv) Stimulate the use of crop and soil management techniques that favor the action of introduced or naturally occurring BCAs.
(v) Collaborate in the training of professionals for the development and use of biological control and for the implementation of the culture of the use of this technology.
(vi) Collaborate in the establishment of public policies to encourage the use of BCAs, regulation of research, and development and registration of BCA-based products.

The portfolio of research, development, and innovation (R&D&I) projects for biological control has as a priority the development of biological control technologies and their insertion in the integrated management of pests of agricultural interest that are resistant to synthetic chemical pesticides; those related to crops with insufficient phytosanitary support, considered as "minor crops"; to large crops and those affecting animal welfare or production facilities.

Five themes are addressed in the scope of the portfolio: biodiversity, incremental performance strategies of biological control agents, integration of crop protection strategies, impacts of the use of biological control agents, and incremental strategies for the adoption of biological control agents.

The "biodiversity" theme deals with the prospection and introduction of biological control agents, native or exotic, with potential knowledge, conservation, and valuation of biological control agents (activity spectrum, biogeography, secondary metabolites, genetic variability) in order to establish banks of technological assets. In this sense, Embrapa supported and institutionalized the microorganism collections by creating the Biological Resource Center for Biological Control Agents (BRC-BCAs) at Embrapa Genetic Resources and Biotechnology (Cenargen). This BRC was created more than 10 years ago, generating and establishing quality standards and, in 2016, received the accreditation of Cgcre/Inmetro by ABNT NBR ISO/IEC 17025 for five biological tests. In this structure are stored more than 7,000 strains of bacteria, fungi, and viruses toxic to invertebrates and more than 2,000 strains of bacteria and fungi antagonistic to phytopathogens or phytopathogenic to weeds of economic interest (Monnerat et al. 2007, 2012, 2014, 2015; Castro et al. 2009; Praça et al. 2009; Marques et al. 2016). In addition to serving as a reservoir of microorganisms, the BRC provides storage services for public and private institutions; characterizes and identifies microorganisms by morphological, biochemical, and molecular methods; and performs tests of efficacy, potency, persistence, and toxicity of strains and microbiological products. Information about this collection is

available "online" on the institution's website (http://alelomicro.cenargen.embrapa.br/InterMicro/index.xjs).

In addition to the BRC-BCAs, other collections are available at other research centers of the institution. At Embrapa Environment there is a collection consisting of 2,000 strains of fungi and bacteria to control plant diseases. At Embrapa Temperate Climate, 200 fungal strains for control of weeds and phytopathogens are stored. At Embrapa Corn & *Sorghum*, 5,000 strains of fungi and bacteria to control insect diseases are available. At Embrapa Soybeans 250 strains of fungi and viruses to control insects are stocked. All of these microorganisms under study at Embrapa are stored with at least two methods of preservation; a backup of each can be found in the Genetic Bank of Embrapa Genetic Resources and Biotechnology.

The second theme, incremental performance strategies for biological control agents, addresses the strategic selection of native or exotic organisms and the bioecology of BCAs, based on the ecological characteristics necessary for good persistence of activity in the field (tolerance to high or low temperature, resistance to drought, UV radiation, others). In addition, it addresses the definition of vulnerable stages in the life cycle of the target pest with a view to expanding the use of BCAs, large-scale production, and formulation methodologies and evaluating the efficiency and effectiveness of BCAs. The activities related to the second theme are usually developed in public-private partnerships, where the material pre-selected in projects of the first theme is used as the basis for the design of the product. Thus, by company standards, partners are co-owners of the technology that can be licensed to third parties.

The third theme, integration of crop protection strategies, addresses the integration of biological control agents with other management tools, such as crop practices, host resistance, natural products, synthetic pesticides, and other biological control agents. It stimulates the use of management techniques that favor the action of biological control agents, introduced or naturally occurring, and provides support systems for decision making (knowledge of the target pests and their natural enemies).

The fourth theme, impacts of the use of biological control agents, studies host specificity and persistence by monitoring population dynamics before and after release of BCAs in the field. In addition, it assesses the environmental, social, and economic impacts of the use of biological control agents.

The fifth theme, incremental strategies for adoption of biological control agents, aims to assist in the training of professionals for the development and use of biological control, defining specific requirements for handling and application and the transfer of methodologies and the BCAs appropriate for cooperatives and family farms with a focus on the production of biological control agents for their own use.

The main themes and lines of R&D&I are:

(i) Prospecting and introduction of biological control agents, native or exotic, maintaining banks of related technological assets and museums of natural enemies

(ii) Development of diagnostic kits to identify pests and their natural enemies in order to assist in the decision making on the correct management of the target pest

(iii) Evaluation of the influence of environmental factors on potential biological control agents and interaction with the target host
(iv) Risk assessment of potential biological control agents, focusing on nontarget organisms
(v) Training of professionals for the development and use of biological control and for the implementation of a culture of the use of this technology
(vi) Development of large-scale production methodologies of biological control agents
(vii) Development of formulation methodologies of biological control agents focusing on increasing competence and the persistence of their activity
(viii) Development of methodologies for evaluating the quality of products based on biological control agents
(ix) Development of processes for the integration of biological control agents with other pest management tools
(x) Agroecosystem management to promote services for the regulation of pests by naturally occurring biological control agents
(xi) Innovative strategies for the placement of biological control agents, databases, strategies, models, and geotechnologies for the characterization and monitoring of biological control agents in agricultural and natural environments
(xii) Development of methodologies for assessing the environmental, social, and economic impacts of biological control agents
(xiii) Development of techniques to monitor the presence of pests and natural enemies to determine the appropriate moment for the use of the biological control agents
(xiv) Development of methodologies to evaluate the agronomic effectiveness of potential biological control agents
(xv) Development of databases to foster regulatory institutions on biological control agents (safety, patents, environmental risks, interactions, etc.)

By working in this way, it is expected that many tools, in addition to those already developed, will be made available and that biological control products will be increasingly used in integrated pest management programs in Brazil and in the world in order to have healthier foods, a less contaminated environment, and a more sustainable planet.

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Chapter 14
Bacillus Entomopathogenic Based Biopesticides in Vector Control Programs in Brazil

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Abstract The problem of vector borne insects is a reality that attracts public health attention due to the role of such insects in transmitting diseases that affect all layers of society. At this juncture, different approaches have been developed over time in order to deal with the issue. Although infrastructure demands, such as basic sanitation, are directly linked to the population density of insect vectors, direct control measures such as insecticide application have been prioritized. Products based on microbial agents have proved to be of great value for the control of insect vectors, since they present great advantages over chemical products, such as high specificity, which results in a smaller impact on the environment. In this context, the bacteria of the genus *Bacillus* stand out as viable candidates, with real possibilities of large-scale production. Here we present a historical and current overview of the use of microbial products based on *Bacillus* spp. for the control of the main Brazilian public health targets: *Aedes* spp., *Anopheles* spp., *Culex* spp. and Simuliids.

Keywords *Bacillus* • Vector control • Microbial agents

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The microbial control of insect vectors populations has advantages over chemical substances due to their host specificity and lower impact on the environment. Among microbial agents with real potential for production in large scale are bacteria from genus *Bacillus*, particularly two species, which synthesize entomocidal toxins and stand out in the control of insect vectors: *B. thuringiensis* and *L. sphaericus*. These species are active against Diptera (Barjac and Frachon 1978), *B. thuringiensis* serovar *israelensis* (Bti) is highly toxic to mosquitoes (Culicidae) and black flies (Simuliidae), while *L. sphaericus* strains are pathogenic to some mosquitoes (Priest 1992; Regis et al. 2000a; Charles et al. 2000). Bti is lethal to *Aedes*, *Culex*, *Anopheles* and *Mansonia* species, while *L. sphaericus,* in general, is very active against *Culex*, *Anopheles* and *Psorophora* but less active against species of the genus *Aedes*. Thus, these bacteria are used in larvicidal preparations against major human vectors diseases (Priest 1992; Hougard 1998; Regis et al. 2000b).

14.1 *Aedes* spp. Control Using *Bacillus thuringiensis* Serovar *israelensis*

Dengue is one of the leading public health problems in the world. It is a tropical infectious disease caused by an arbovirus of the family Flaviviridae, genus *Flavivirus* and includes four immunological types: DEN-1, DEN-2, DEN-3 and DEN-4. The World Health Organization (WHO) estimated in 2009 that 2.5 billion people – 2/5 of the world's population – were at risk of contracting dengue fever and that around 50 million cases were reported each year. Of this total, about 550,000 needed hospitalization and at least 20,000 died as a result of the disease. In the last two decades, the incidence of dengue in the Americas has shown an upward trend, with more than 30 countries reporting cases of the disease, despite the numerous eradication or control programs that have been implemented. Epidemic peaks have been getting larger, in periods that repeat every 3–5 years, almost on a regular basis. Between 2001 and 2005 2,879,926 dengue cases were reported in this region, of which 65,235 were dengue hemorrhagic, with 789 deaths. The highest incidences in this period were reported in Brazil, Colombia, Venezuela, Costa Rica and Honduras (82% of the total) (Ministério da Saúde 2009).

In Brazil, the first epidemic documented clinically occurred in 1981–1982, in Boa Vista (RR), caused by serotypes 1 and 4. The epidemiological framework of dengue in the country in 2009 was characterized by the wide distribution of *Aedes aegypti* in all regions with a complex dynamic of virus dispersion, simultaneous circulation of three viral serotypes (DEN-1, DEN-2 and DEN-3) and vulnerability of the introduction of serotype DEN-4 (Ministério da Saúde 2009).

The largest outbreak in Brazil occurred in 2013, with around two million reported cases, and the four serotypes are still circulating in the country (Ministério da Saúde 2016a). In 2001, according to the Intensification Plan for Dengue Control Actions (PNCD) which replaced the former Plan for the Eradication of *Ae. aegypti* (PEA), an updated concept of vector control was proposed that sought to integrate various methods and strategies. It was understood from this that several rational

methods within an ecological approach should be adopted. Within the biological control section, the plan stated: "In this line, which is called biological larvicides, we now have commercial products based on *Bacillus thuringiensis* ser. *israelensis* – Bti, with good activity against *Aedes* larvae, and *Bacillus sphaericus* for larvae of *Anopheles* and *Culex*, both showing good larvicidal activity against several species of culicidae. Despite advances in this area, there are still many obstacles for using these methods in a large scale of routine operating practice, considering costs, low residual effects and intolerance to direct exposure to sunlight". According to Funasa (2002), there were plans to employ 300 tons of Bti for use in non-potable (non-drinking) water in 2002, at a cost of US$ 2.20 per kilogram, totaling US$ 600,000. For potable water, 60 tons of Bti would be used, at a cost of US$ 26.54 per kilogram, totaling US$ 1,592,400. Although other causes have influenced this plan the actions of the National Dengue Control Plan (PNCD), developed in partnership with states and municipalities, are considered to have contributed to the reduction of 73.3% of the cases in the first half of 2004, in relation to the same period of the previous year.

Data from the Health Surveillance Secretariat (SVS) of the Ministério da Saúde (Ministry of Health) show that, in the first 6 months of 2004, 84,535 people had dengue, while in 2003 the notifications reached 299,764. In 2007, the number of dengue cases has tripled, according to the SVS, up to the middle of March of that year, 85,000 notifications occurred in the states of Paraná, Mato Grosso and Mato Grosso do Sul. The largest focus, with 40,200 cases, was recorded in Mato Grosso do Sul with the DEN-3 virus spreading to other countries of the continent, such as Argentina, Uruguay and Paraguay, with 21,000 infected individuals in the same period.

In 2009, the Ministry of Health recommended the following insect control measures: the elimination of domicile and peridomicile breeding-sites, covering of water boxes, use of larvivorous fish, chemical control of larvae with temephos and of adults with malathion, as well as the use of bioinsecticides (Ministério da Saúde 2009). In the first 10 years of the twenty first century, MS advocated the use of Bti-based larvicides to control insects in locations where there were signs of resistance to the chemical insecticide temephos. Between 11th and 12th April 2012, the Secretary of Sanitary Surveillance of the Ministry of Health held the II International Seminar for Evaluation of Chemical Control Actions of *Aedes aegypti*, where chemical control methodologies and insecticide resistance management strategies were discussed. In the document prepared during the event, the use of Bti in *Aedes aegypti* control was recommended, but there were no mentions about dosage or recommendations for use (OPAS 2016a). The last data on Bti dosage for use in insect control are from 2009, where the use of Vectobac G (non-potable water) and WDG (potable water) was recommended. The dosages of Vectobac G ranged from 1.5 to 8 g, depending on the volume of the container and for WDG of 0.1–1 g (Ministério da Saúde 2009). Current data could not be obtained on the use of Bti in the control of *Aedes aegypti*, since even on the Ministry of Health webpage there is no mention of the subject. At the beginning of the 2000s, what was prescribed was as follows, and we would like to point out that some data could still be updated until 2009, through personal communications.

São Paulo state, more precisely in the Baixada Santista, started using Bti in May 2001, in the municipalities that were infected by *Ae. aegypti*, which were as follows: Santos, São Vicente, Cubatão, Guarujá and Praia Grande. According to the Regional Service-2 of the Superintendency of Control of Endemics (SUCEN) of the São Paulo State Department of Health in São Vicente, Bti was used in routine work, home to home and in the treatment of strategic points, such as buildings with commercial activity that presented a greater risk of infestation (MF Domingos, SUCEN, personal communication, 2007). Also, according to the same source, since September 2001 the use of biolarvicides was practically suspended from home to home, being replaced by mechanical control, by the use of sodium chloride and domosanitaries in the breeding-sites. The suspension of Bti was due to the difficulty of its use in drains and gutters, with the preparation floating and often causing clogging. SUCEN had no assessment of the impact of Bti in the field until October 2001, only the difficulty mentioned above, which meant a significant limitation in the use of biolarvicide. The application of Bti continued to be made, since populations of the insect still presented resistance to temephos. Two formulations of Bti: WDG were used in 1 g water boxes for each 250 L of water, and Vectobac G in the other containers, in the proportion of 1 g for each 50 L. In the coastal region of São Paulo, the annual consumption was 1000 kg of the granulated insecticide and 300 kg of the WDG (M. Silva, Control of Vectors, personal communication, 2007). The use of bioinsecticides in the region was interrupted in 2009 (D T N Conversani, regional director, personal communication, 2016).

In Natal, Rio Grande do Norte state, FUNASA found that 33.2% of the population of *Ae. aegypti* presented resistance to temephos, so the use of Bti was recommended, Natal was the first municipality to use Bti in 100% of its area. The studies carried out for the implementation of the program demonstrated that this bacterium could be an alternative for the control of the insect, since it was effective, although it was also observed that the isolated use of the biolarvicide would not solve the problem of vector control, especially in localities with poor health infrastructure (Dantas et al. 2001).

Due to a possible resistance of *Ae. aegypti* to temephos in Fortaleza, Ceará State, Bti was used to control this vector. The citizens demonstrated good acceptance identifying it as a natural product (Fernandes et al. 2001).

The city of Rio de Janeiro and others that make up the metropolitan region were using Bti-based products in vector control, but few data were available in 2001 or today.

In 11 places with high infestation rates of 5 municipalities in the State of Minas Gerais, a small scale project was carried out with the operational use of a Bti insecticide for the control of *Ae. aegypti*. The concentration used was 5 ml per m^2, presenting promising results (Hernández et al. 2001). There is no further information about what happened to this project.

14.2 *Lysinibacillus sphaericus* as a Method of Biological Control for *Anopheles* spp. in the Amazon Basin

The Amazon biome is the largest forest on the planet and is responsible for regulating various environmental components at the global level, and is governed by a natural system of water, with ebbs and flows that annually modify the environment and determine the living conditions in this region (Tadei et al. 1998, 2002, 2010, 2016).

The two great rivers of the Amazon (Solimões and Negro) have heterogeneous ecosystems that provide different breeding sites for Culicidae. The geologically older Negro river has acidic black water, is rich in dissolved fulvic and humic compounds, and has low productivity and electrical conductivity (Walker 1995). This environment is conducive to the breeding of anophelines in areas close to human habitation (Tadei et al. 1998, 2002, 2010).

Malaria in humans has worldwide occurrence and affects about 214 million people, with approximately 438,000 deaths per year (WHO 2016). In 2015, over 3.2 billion people in 95 countries and territories were at risk of contracting the disease and 1.2 billion are at high risk (Who 2016). The Americas had approximately 128 million of people in areas at risk of malaria transmission (Opas 2016b). In Brazil 138,697 cases of the disease were recorded in 2015. However, 98% of the cases of malaria occur in the Amazon region (Ministério da Saúde 2016b).

The region of the Amazon basin is associated with anthropic and environmental factors, human activities such as the occupation of urban and peri-urban spaces in an uncontrolled manner, construction of hydroelectric power plants, irrigation projects, fish ponds, exploitation of fossil fuels, minerals and natural gas, forest fires, deforestation and construction of roads, favor malaria transmission (Tadei et al. 1988, 1998, 2016; Confaloniere et al. 2014; Hahn et al. 2014).

The Western Amazon region has natural characteristics favorable for the development of fish farming. The government encourages this enterprise since it generates income and jobs for the population (Tadei et al. 2005, 2007, 2016; Rodrigues et al. 2008, 2013; Silva et al. 2013).

However fish ponds become breeding sites for *A. darlingi* in the vicinity of Manaus, the state of Amazon promoting conditions for oviposition of *Anopheles* females and larval development. Studies on biological control in fish ponds have noted an abundance of *A. darlingi,* corresponding to 54% of larvae collected. Other species present in the ponds were *Anopheles braziliensis* Chagas, 1907, *A. triannulatus*, *A. nuneztovari* and *A. albitarsis*, the latter with low abundance (Rodrigues et al. 2008).

Unlike the natural conditions in the Amazon, fish ponds are a permanent breeding site because water supply tanks are not influenced by the pulse of flood and ebb, keeping the vector breeding conditions at high levels throughout the year (Tadei et al. 2005, 2007, 2016; Rodrigues et al. 2008, 2013; Silva et al. 2013).

Anopheles darlingi, the most important species involved in malaria transmission in the Amazon Basin, presents high behavioral variability, genetic plasticity and resilience, adapting very well to environmental changes, particularly those caused by anthropic activities (Tadei et al. 1988, 2016; Scarpassa and Conn 2007; Silva et al. 2010).

The population density of *A. darlingi* and consequently the occurrence of the disease respond to the hydrological cycle. This annual phenomenon influences the seasonality of species, which leads to an increase in breeding sites in flooded forests of black waters (Igapós), such as the Negro river and intensifies human/vector contact and hence, malaria cases (Tadei et al. 1998, 2002, 2007, 2010, 2016; Wolfarth et al. 2013).

Anopheline control actions, directed to the adult stage, are carried out by means of chemical insecticides sprayed on the inner walls of residences and aerospace application (thermonebulization), aimed at reducing adult density and contact between man and vector. However, the constant use of insecticides promoted the development of resistance of *Anopheles* (Tadei et al. 2002, 2010; Ministério da Saúde 2015).

Because of the territorial dimensions of the Amazon and the characteristics of sites breeding of anophelines, which occur in large collections, makes the application of larvicides impossible. Thus the procedure is restricted to the vicinity of inhabited areas and fish ponds (Tadei et al. 2002, 2007, 2016).

Biological control is a measure directed to the control of immature forms of the vectors through biological and pathogenic agents. In this context the use of bacterial entomopathogens, such as *Lysinibacillus sphaericus* Neide, 1904 (Ls) and *Bacillus thuringiensis* ser. *israelensis* Berliner, 1915 (Bti), is an alternative to conventional control measures against *Anopheles* larvae that consider the preservation of the environment (Barjac 1990).

The effectiveness of formulated *L. sphaericus* products against larvae of different species of *Anopheles* has been evidenced in laboratory and field conditions such as fish ponds, gold miner-excavation pools and pottery lakes in the Amazon region (Oliveira 2005; Rodrigues et al. 1999, 2008, 2013; Galardo et al. 2013; Ferreira et al. 2015).

The mortality rate of larvae of three species of the Amazonian anophelines, *Anopheles nuneztovari*, *A. darlingi* and *Anopheles braziliensis* exposed to the Ls strain 2362, the WHO standard, was evaluated under laboratory conditions. The authors reported that *A. nuneztovari* was the least susceptible to this insect pathogen, while *A. braziliensis* was the most susceptible, followed by *A. darlingi* (Rodrigues et al. 1999).

Larvicidal activity of 20 strains of Ls isolated from soil samples collected in different regions of the Amazon, against larvae of *A. darlingi*, was evaluated by Litaiff et al. (2008) under laboratory conditions, in order to determine the toxicity through LC_{50} and to compare the power of the strains isolated in relation to the standard strain (Ls 2362). The authors reported high effectiveness for five strains and IB15 was about 50% more powerful than the standard strain.

Field tests conducted in the central Amazon using Bti and Ls showed greater effectiveness in black water than white water (Rodrigues et al. 2013). The reduction in larval percentage indicated a rapid effect of Ls within 24 h after its application (Tadei et al. 2007; Rodrigues et al. 2008).

Bacterial biological control was also evaluated for possible effects on macroinvertebrates, which coexist with mosquitoes. An estimate of these insects was made in four fish ponds around Manaus, and a total of 12,495 specimens of aquatic insects

were found, including some important indicators of water quality. After 15 days of Ls application, approximately 100% of aquatic insects recovered their pre-application abundance levels (Sampaio et al. 2005).

Another study carried out by Ferreira et al. (2015) in fish ponds showed that bioinsecticides were found to be efficient in the immature control after 24 h for the application. The results also indicated that the diversity and richness were not affected by the application of Ls. Thus, the results showed no effect of the larvicide on the general insects present in fish ponds.

The efficiency and persistence of different formulations of Ls 2362 (VectoLex® CG, and Spherimos® Griselesf® FC) were evaluated in fish ponds and lagoons formed as a result of the extraction of materials for the manufacture of bricks and ceramics (Rodrigues et al. 2008). The formulated VectoLex® CG and Spherimos® FC proved effective in the fish farming on the second day after application, remaining active until the 35th and 21st day, respectively. Despite the VectoLex® CG having reduced the larvae indices on the second day after application, in the lagoons of pottery, the three formulated products presented low activity in these breeding sites, due to the high level of clay and other suspension components giving high turbidity (Rodrigues et al. 2008).

Galardo et al. (2013) also measured the larvicidal activity of Ls, VectoLex® CG, against the larvae of *A. darlingi* in ponds, formed as a result of mining activity in the State of Amapá/Brazil, over 52 weeks. The results showed impacts on larval density 48 h after the application of Ls. The authors also reported 78% reduction of the last larval stage (L_4) and 93% of pupae.

Another perspective of the use of Ls, in the control of anopheline larvae, is the spread of the bacterium by the adult vector. Schlein and Muller (2015) conducted a study where *Anopheles sergentii* was fed with sugar baits containing Ls, in order to evaluate the power of dispersion of this bacterium by an adult and, consequently, evaluate the preservation of infection in breeding sites-larval habitats. The authors observed a reduction of approximately six times the number of larvae and about 60% in adult density, in the treated area after 10 days. The results showed that is possible to have the transfer of Ls, via infected adults, allowing the introduction of pathogens into the breeding sites of immature forms. This is an innovative procedure, which allows the spread of Ls, in areas of difficult access.

The characteristics of the Amazon ecosystem and the breeding sites of *A. darlingi*, denote the need for specific control measures and lower environmental impact. Studies conducted in laboratory and field conditions demonstrated the effectiveness of the application of Ls, in control of larvae of *Anopheles* sp.

Another positive aspect of this application is that Ls does not change the composition of associated fauna, in fish ponds, which is relevant marker of environmental quality. These tanks, located in the peri-urban area of Manaus/AM, Brazil, represent an important economic activity for the local population, contributing to a better socio-economic level and the quality of life.

However, fish farming tanks have all conditions for the reproduction of anophelines, since the daily feed offered to the fish accumulate in the banks. These margins of the tanks need to be continually managed to reduce the lateral vegetation, thus undoing the conditions for the development of the vector.

Based on the above considerations, we can conclude that *L. sphaericus* is important as a method of biological control in Amazon, being a safe alternative in breeding sites where the use of chemical insecticides is not feasible, as in fish ponds.

14.3 *Bacillus* Entomopathogenic Based Biopesticides in *Culex* spp. Control Programs in Brazil

The mosquito *Culex quinquefasciatus* is present in the vast majority of the 5570 Brazilian cities and in thousands of villages. This mosquito, whose presence is a real or potential risk for transmission of pathogens to humans, besides being a nuisance in its own right, is present and reproductively active all year round throughout the tropical zone of the country.

A notable biological characteristic of this species is the aggregation of their offspring in aquatic bodies where organic materials are abundant. The high densities commonly observed in *C. quinquefasciatus* populations is a result of an evolutionary strategy developed by this species, which involves the aggregation of their eggs rafts that can bring together more than 200 eggs, and carrying an intraspecific pheromone to attract other ovipositing females. As a result, where environmental conditions are favorable, it is frequent the occurrence of very high population densities. The consequences are high nuisance to people, and high vectoring capacity. On the other hand, a high concentration of aquatic forms in breeding sites, well-typed and easy to locate, make the control of this species through the use of larvicides viable, which is less evident in the case of mosquitoes species that spread their eggs more and in a diversity of unstable aquatic containers, as for instance *Aedes aegypti*.

C. quinquefasciatus has transmitted the worm *Wuchereria bancrofti*, an etiologic agent of lymphatic filariasis (LF), in some Brazilian big cities for five or more decades, despite the fact that the transmission process of this worm is highly inefficient, so that the production of an infection case requires intense exposure to mosquito bites for long periods. It is known that the establishment and maintenance of LF transmission is only possible where environmental conditions allow for the maintenance of very high mosquito population density. Since the beginning of this century Brazilian health authorities have been concerned about the extensive and intensive presence of this mosquito and the consequent risk of introduction and establishment of West Nile virus (WNV) transmission. This led the Ministry of Health to prepare a preventive monitoring and alert system for emergency actions (Brasil 2011). Moreover, this concern is now renewed in view of the possibility of involvement of this species in the transmission of Zika virus, currently circulating in the country (Ayres 2016).

Despite this scenario regarding disease transmission and major nuisance generated by established populations of this mosquito everywhere, only two large control programs are currently in operation in Brazil, both using entomopathogenic bacteria as larvicides.

The largest, and longest, program for *C. quinquefasciatus* control based on bacterial larvicides was established in 2002 in Recife city, the capital of Pernambuco state, which together with two cities (Olinda and Jaboatão dos Guararapes) located in its metropolitan area are currently the last LF endemic focus in Brazil. The mosquitogenic conditions in Recife, highly favorable to the maintenance of mosquito high densities, explains why the city remained for many decades as the main Brazilian focus of *Wuchereria bancrofti* transmission and is now the main site of ZKV transmission.

The Recife Health Department decided to include actions for vector control integrated to the main intervention of the Filariasis Control Program (FCP), that is, mass treatment of residents with a microfilaricide as recommended by the WHO Global Program for Elimination of Limphatic Filariasis (WHO 1997, 2007). The reduction of man-vector contact was thus the second line of the defined strategy for eliminating LF from Recife (Albuquerque et al. 2013). Studies on the vector carried out in districts of the city played a role in the program planning. Focusing on environment conditions, *Culex* population dynamics and integrated control measures and including a large scale trial of a *L. sphaericus* larvicide against *C. quinquefasciatus*, those studies contributed for planning operational methods and the option for biological control (Regis et al. 1995, 2000b; Silva-Filha et al. 2001).

Vector control operations were installed progressively, initially covering 13 districts, reaching 55 districts 3 years later and since 2006 including all 94 districts of Recife. The control actions include environmental management and education, besides the biological control with microbial larvicide. The initial mapping of mosquito breeding sites indicated that cesspits, tanks, channels and drains were the main breeding places, the cesspits representing 80% of them and also the most productive sites. A product based on *L. sphaericus* strain 2362 (VECTOLEX L®, Valent Biosciences) has been applied every 2 months at a rate of 3–5 g/m^2, covering all positives breeding sites found. A toggle scheme with Bti is scheduled to happen every 5 years to avoid the development of resistance in the mosquito population to *L. sphaericus* (Ls). Furthermore, a susceptibility surveillance schedule of the target population to Ls has been held every 6 months since 2003, the susceptibility status of field collected larvae is measured by the Reference Service on Culicidae Vector Control of the Research Center Aggeu Magalhães – FIOCRUZ-PE. This continuous surveillance allowed detecting, on an occasion, just a low level resistance to Ls (Silva-Filha et al. 2008). It is important to note that the Dengue Control Program in Recife is based on the use of Bti against *Ae. aegypti*, applied every 2 months throughout the city.

Data from Recife Health Department show success of the FCP with regard to the decline of endemic filariasis in the city. In 2003, 907 infection cases were recorded, in 2011 four infection cases, a 99% reduction of incidence (Recife 2013) and in 2014 only one case was detected (Recife 2015). It is, however, impossible to measure what was the contribution of a likely reduction in the amount of vectors for these good results.

In São Paulo city, a program to reduce the strong discomfort caused by *Culex* in an area of the city has been implemented for decades by the Municipality through its Health Surveillance Coordination. Since 2003, the program Integrated Management

of *C. quinquefasciatus* in the Pinheiros River, SP, adopted the use of microbial larvicides, associated with environmental care as the removal of aquatic vegetation. It covers the Pinheiros River region, 26.4 km long and 70 m wide, crossing densely populated neighborhoods of the city. Transformed into a channel with stagnant water and rich organic matter content, it is a vast breeding place of *Culex* spp., predominantly (>95%) *C. quinquefasciatus*. An Ls-based larvicide is applied over 9 months per year, in rotation with a *B. thuringiensis* ser. *israelensis* (Bti)-based larvicide used over the other 3 months. The frequency of treatments is about 1 per month, depending on the intensity of infestation by *Culex* larvae, which is weekly monitored. Over 13 years, a mean of ten treatments per year were carried out. The larvicides are applied using an aeroboat at a rate of 5 g/m^2 through a device that dispenses the granules on the water surface at a rate of 4 kg/min, covering a track of 10 m wide, on each side of the channel with a length of 26.4 km, a total area of 54 ha treated. The susceptibility of *Culex* larvae to *L. sphaericus* has been periodically monitored in collaboration with the Department of Entomology, Fiocruz-PE (Silva-Filha et al. 2008) and the Department of Zoology, Unicamp (Andrade et al. 2007).

The impact of the program actions on the local mosquito population is evaluated through periodical measurement of adult mosquitoes and larvae-pupae density, in 54 sampling stations. The program records positive results, as for instance, a significant reduction in the number of adults collected in traps: from 56,593 in 2003 dropped to 1888 in 2013 (Dini et al. 2008).

The control of *C. quinquefasciatus* populations in Recife and São Paulo, with continued use of microbial larvicides for 13 years without resistance development, and no exposure of operators, residents and non-target species to toxic products, demonstrates once again that: (i) the biological control of these mosquitoes using entomopathogenic bacteria as larvicides is feasible and sustainable; (ii) it is essential to monitor the susceptibility of the target population to the biological control agent; (iii) the alternate use of larvicides based on Ls and Bti in rotation is able to avoid the selection of resistant mosquito population, as has been recommended and demonstrated since the record of occurrence of resistance to Ls binary toxin (Regis and Nielsen-LeRoux 2000; Regis et al. 2001; Ferreira and Silva-Filha 2013; Silva-Filha et al. 2014).

14.4 Simuliid Control Programs Using *Bacillus thuringiensis* Serovar *israelensis*

In Brazil black flies are recognized mainly for the inconvenience caused by the reactions and minor injuries associated with their bites and the transmission of *Onchocerca volvulus* restricted to the far north of the state of Roraima in the Brazilian Amazon (Moraes et al. 1974).

Thus, the importance of black flies in Brazil is closely related to the nuisance caused by excessive bites in high-density regions. This stands out as an economic problem in agricultural regions in the south of the country and in tourist resorts along the slopes of the massif of the Serra do Mar in the southeast.

Black flies are holometabolous insects whose breeding-sites are lotic environments. Immature forms, according Cummins's (1973) general classification for trophic categories of aquatic insects, are gatherer filtering larvae that feed on suspended particles in water. This feeding behavior, associated with the larvicides' mode of action, makes larvae the most susceptible stage of the insect's life cycle and consequently the main target of control strategies.

Before the 1970s black fly control activities consisted of organochlorine applications. This practice has been replaced by use of temephos, an organophosphate, with less environmental impact.

Since 1984, with the report of development of resistance to temephos in black fly populations in Rio Grande do Sul (Ruas Neto 1984a) and São Paulo (Andrade et al. 1987), researchers started to look for alternatives to chemical control.

Studies conducted by Ruas Neto (1984b) in Rio Grande do Sul and Araújo-Coutinho and by Lacey (1990) in São Paulo presented *Bacillus thuringiensis* ser. *israelensis* (Bti) efficiency against *Simulium pertinax,* the most prevalent species of Simullidae in Serra do Mar, in São Paulo state (Araújo-Coutinho et al. 1988) and Rio Grande do Sul (Souza et al. 1994).

Currently there are two major programs of black flies biological control using Bti: one in Rio Grande do Sul state and another on the north coast of São Paulo state.

São Paulo State Program started in 8the 1980s, in four municipalities in Serra do Mar slope, comprising an area of 893 km^2 and covering 1456 breeding sites, which are treated in a maximum length of 5 km from areas of human concentration (Araújo-Coutinho 1995a).

Applications are made every 2 weeks through the use of a watering can calibrated to deliver 15 g of the commercial product per liter of water per minute (15 ppm) (Araújo-Coutinho 1995b). The breeding-sites are high drainage rivers, crystal clear and with a high number of falls, of variable sizes and flow rates, most are small and medium, which determines the points of application. Because of this, the effective observed carrying of the Bti larvicides is approximately 500 m.

The program's goal is to establish a maximum density in the areas of human concentration of ten blackflies per person per hour. The program efficiency is assessed through catches with entomological nets during 15 min in the main urban areas of the region. The results of these catches are matched with complaints of the population to the health unit of the municipality responsible for control activities.

This program began in 1986 and was fully implemented in 1989. At that time, monthly consumption was about 500 L of commercial product, representing 25% of the monthly cost of the program (Araújo-Coutinho 1995a), and after 30 years of beginning the program has efficiently and satisfactorily achieved their goal, and currently the program is conducted in 1805 breeding-sites and 7316 breeding application points in the municipalities of Caraguatatuba, Ihabela and Ubatuba, since the municipality of San Sebastian interrupted aplications. Nowadays, the monthly consumption of Bti is around 700 L.

In Rio Grande do Sul, the program is conducted in 68 municipalities, with a total area of 37,850 km^2 (Souza et al. 1994). The methodology has significant differences from the other program, especially in relation to the concentration used. Thus a certain concentration is not used based on diagnostic dose, but established by a sliding

scale, correlating flow, concentration and carrying, so the concentration and quantity of the product changes, according to the flow rate, aiming to obtained the expected carrying. This sliding scale determines concentrations ranging from 12 ppm to 16 m^3 per minute to 50 ppm to less than 0.6 m^3 per minute. The results showed a larval mortality rate of 90% and reducing the attack rate in localities up to 4 km away from the treated breeding (Souza et al. 1994). There are no data available on the number of breeding-sites treated and the amount of larvicide consumed in the program.

Over the past three decades, several small programs were implemented in municipalities in the states of São Paulo, Paraná, Espírito Santo, Santa Catarina and Rio de Janeiro, however, it was not possible to obtain data on their results and even if these programs were interrupted or not.

The high cost of Bti-based products in Brazil is certainly a limiting factor to the expansion of its use, since they are employed in areas where the black flies do not pose health problems and are funded mainly by small municipalities.

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Chapter 15
Resistance of Mosquitoes to Entomopathogenic Bacterial-Based Larvicides: Current Status and Strategies for Management

Maria Helena Neves Lobo Silva-Filha

Abstract The entomopathogenic bacteria *Bacillus thuringiensis* serovar. *israelensis* (Bti) and *Lysinibacillus sphaericus* have successfully been used to control insects of public health relevance, including those from the genera *Aedes*, *Anopheles*, *Culex*, and *Simulium*. These bacteria display a specific mode of action that relies on unique interactions which makes them the most selective agents currently available to control Diptera larvae. They produce crystalline insecticidal proteins that act on the larval midgut through their interaction with specific receptors. *L. sphaericus* presents a single major larvicidal factor, the binary (Bin) protoxin, whose action relies on the binding to one class of receptors, while Bti crystals contain four main protoxins (Cry4Aa, Cry4Ba, Cry11Aa, Cyt1Aa) which display interactions with a group of distinct midgut receptor molecules. The mode of action of *L. sphaericus* displays a greater potential for resistance selection, compared to Bti which has no record of insect resistance to date. These major mosquitocidal toxins and their interaction with midgut target sites, as well as resistance issues related to their utilization, are summarized in this chapter.

Keywords Vector control • Bti • Cry toxins • *Lysinibacillus sphaericus* • Bin toxin • Receptors

Among the microbial control agents available, *Bacillus thuringiensis* serovar. *israelensis* (Bti) and *Lysinibacillus sphaericus* have been employed for the production of biolarvicides aimed at the control of dipterans of medical importance (Lacey 2007). Some strains of these bacteria produce crystalline inclusions that contain protoxins with high and selective larvicidal action against some species of Diptera. These

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protoxins act by ingestion and are processed into toxins in the midgut in order to target the epithelium through specific receptors. Bti was the first *Bacillus thuringiensis* (Bt) serovariety characterized as active against Diptera (de Barjac 1978), among several described (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt). Soon after its discovery and characterization, Bti was introduced in a large-scale program to control *Simulium* in the Onchocerciasis Control Program carried out in West Africa (Guillet et al. 1990), and its effectiveness led to Bti adoption in other programs worldwide (Regis et al. 2001). The major insecticidal factor in Bti-based biolarvicides is the crystal that contains both three-domain-type Cry toxins and cytolytic or Cyt toxins. These crystals have high potency and a selective spectrum for Culicidae and also target some species of Simuliidae and Chironomidae (Lacey 2007). The greatest limitation of Bti activity under field conditions is its degradation due to solar radiation and other environmental factors, and suitable formulations and application strategies are needed to achieve optimal field performance. *L. sphaericus*' mosquitocidal properties were first described in 1965, in the K strain isolated in moribund *Culiseta incidens* larvae, by Kellen, followed by the discovery of the SSII-1 strain by Singer in 1973. However, both strains displayed low to moderate toxicity to larvae (Lacey 2007) and only in the 1980s were highly toxic strains (e.g., 1593, 2362, 2297) identified, leading to the production of commercial biolarvicides (Charles et al. 1996). The powerful action of these strains is mainly associated with the production of crystals, during bacterial sporulation, that contain the binary (Bin) protoxin which remains the major insecticidal protein produced by *L. sphaericus* (Berry 2012). The spectrum of *L. sphaericus* action is more limited than Bti, and it targets only culicids. This chapter aims to summarize current knowledge of the interaction of these insecticidal toxins with the midgut receptors of mosquito larvae and the implications for the selection of resistance and management strategies.

15.1 Mode of Action of Bacterial Toxins Employed for Mosquito Control

The larvicidal toxins produced by *L. sphaericus* and Bti can be defined as "bacterial disruptors of insect midgut membranes," and they are classified as mode of action group 11 (Moa11), according to the Insect Resistance Action Committee (www.irac-online.org). As described, these proteins take the form of protoxins enclosed in crystals, and, after ingestion and midgut processing by serine proteases, they are converted into toxins. These interact with specific receptors located on the midgut epithelium, leading to cytopathological alterations and larval mortality (Charles et al. 1996). *L. sphaericus* strains can produce mosquito-active toxins including the binary (Bin), the group of so-called mosquitocidal toxins (Mtx1, Mtx2, Mtx3, and Mtx4), a second binary Cry48Aa-49Aa toxin and the S-layer envelope protein (Berry 2012). This chapter will focus on the mode of action of the Bin protoxin crystal, since this is the active ingredient of all *L. sphaericus*-based biolarvicides currently available for mosquito control. The Bin spectrum of action is limited to

mosquito larvae and includes species from the genera *Culex*, *Anopheles*, *Aedes/Ochlerotatus*, *Psorophora*, and *Mansonia.* The most susceptible are *Culex* spp., in particular those from the *Culex pipiens* complex, followed by *Anopheles* species (Arredondo-Jimenez et al. 1990; Davidson 1989; Rodrigues et al. 1999). In the *Aedes* genus the response varies, with some species susceptible (e.g., *Ochlerotatus atropalpus*, *Aedes vexans*) and others refractory to Bin toxin, such as *Aedes aegypti* (Berry et al. 1993). As previously described, Bti has a broader spectrum since it is active against Culicidae, Simuliidae, and Chironomidae species (Goldberg and Margalit 1978; Lacey 2007; Rodcharoen et al. 1991). Mosquito larvae susceptibility to *L. sphaericus* has been reviewed by Lacey (2007) and Silva-Filha et al. (2014).

15.1.1 Bti Toxins

The protoxins found in Bti crystal are encoded by genes located on the pBtoxis megaplasmid (Berry et al. 2002), and the most common found are members of the Cry family, such as Cry4Aa (125 kDa), Cry4Ba (135 kDa), Cry11Aa (68 kDa), and a cytolytic toxin Cyt1Aa (28 kDa). Cry10Aa and Cyt2Ba toxins also exhibit activity against Diptera and can be detected in crystals produced by some strains. Cry and Cyt are pore-forming toxins, a family of bacterial toxins that are able to insert into the cell membrane of their hosts (de Maagd et al. 2003). Bti crystals have important larvicidal features, such as a diversity of Cry and Cyt protoxins, optimal ratio of toxins in crystals, and synergistic action of Cyt toxin, which can act as a surrogate receptor for the Cry toxins. The two toxin families display different features: Cry toxins interact with receptors to attain the pre-pore oligomeric form in order to insert themselves in cell membranes to form pores, while Cyt toxin has a cytolytic action and interacts directly with cell membranes (Soberón et al. 2007). Crystals containing both Cry and Cyt protoxins are characteristic of dipteran-active *B. thuringiensis* (Bt) strains.

The structure of Cry toxins shows three domains that have been characterized by crystallography and functional studies (Boonserm et al. 2005; de Maagd et al. 2003). Functionally, loops from domains II and III are responsible for interaction with specific receptors, and domain I is involved in membrane insertion, oligomerization, and pore formation (de Maagd et al. 2003). Cyt toxin has a single α-β domain, and, as described, it has cytolytic activity, acting directly on cell membrane to form pores (Bravo et al. 2007). Toxins from the Bti crystal act in synergy, and the activity of the whole crystal is far more effective than that of any individual toxins, or their combination (Crickmore et al. 1995). The Bti mode of action involves ingestion and solubilization of crystals under alkaline midgut conditions, activation of protoxins into toxins, binding to receptors, and pore formation in the cell membrane resulting in a colloid-osmotic lysis (Bravo et al. 2007; Knowles and Ellar 1987). After proteolytic cleavage at the N- and C-termini of protoxins, active Cry toxins have the ability to interact specifically with midgut microvilli (Beltrão and Silva-Filha 2007; Hofte and Whiteley 1989). Cyt toxin is able to insert itself into the cell membrane and synergizes the binding of Cry toxins, as described below.

The general Cry toxin mode of action has been explained including the hypothesis that action is based on the toxin binding to receptors followed by pore formation and a second hypothesis in which the toxins are able to activate intracellular signaling pathways that lead to cell death (Pigott and Ellar 2007; Vachon et al. 2012). A detailed outline of the Bt mode of action was presented in a previous chapter. Briefly the Bravo model, based on the action of Cry1A toxin in larvae of the lepidopteran *Manduca sexta*, showed that activated Cry toxins bind initially to GPI-anchored receptors such as alkaline phosphatases (ALPs) and N-aminopeptidases (APNs) with relatively low affinity, but toxins then bind with higher affinity to transmembrane cadherins (CADRs). Binding to CADRs promotes toxin oligomerization, which, under this conformational change, binds then to a second receptor, either APN or ALP again, but now with greater affinity (Bravo et al. 2004). After this binding step, Cry toxin can insert itself in the membrane and provoke pore formation. The Zhang model argues that Cry1A monomer toxin binding to the CADRs triggers a signaling mechanism that activates a cell death pathway (Zhang et al. 2006). It has been suggested that both mechanisms may occur simultaneously. CADRs, ALPs, APNs, and an α-amylase have been identified in *Ae. aegypti* and *Anopheles* larvae as Cry11Aa and Cry4Ba receptors (Bayyareddy et al. 2009; Likitvivatanavong et al. 2011). Cyt1Aa is a strategic component of Bti crystal because it can also act as a Cry toxin receptor. Cry11Aa and Cry4B can bind specifically to Cyt1Aa, subsequently enhancing Cry toxin binding to midgut microvilli receptors and inducing the formation of the pre-pore structure, which is able to insert itself in membranes and form pores in cells (Bravo et al. 2007; Cantón et al. 2011; Pérez et al. 2005, 2007). The two-receptor model proposed for Cry toxins active to Lepidoptera (Bravo et al. 2004) can also be applied to Bti Cry toxins. In this case, the Cyt toxin may play a role equivalent to that of a cadherin receptor, which is able to promote oligomer formation and lead to the subsequent binding step with high affinity to the GPI-anchored midgut receptors. Besides this set of midgut proteins that act as receptors (Likitvivatanavong et al. 2011), other molecules may also be involved in the mode of action of Bt toxins such as the immune defense involving the MAPK p38 pathway (Cancino-Rodezno et al. 2010, Torres-Martinez et al. 2016), ABC transporter proteins (Gahan et al. 2010), and other Cry-binding molecules that have been identified by proteomic approaches (Bayyareddy et al. 2009; Cancino-Rodezno et al. 2012; Stalinski et al. 2016). The Bti mode of action has been characterized by a complex set of events that do not favor the selection of resistance, as will be discussed in the next section.

15.1.2 Lysinibacillus Sphaericus Binary Toxin

The binary (Bin) protoxin is a heterodimer composed of two subunits BinA (42 kDa) and BinB (51 kDa) proteins which is produced during sporulation and deposited as a parasporal crystalline inclusion within the exosporium (Kalfon et al. 1984). The subunits are produced in equimolar amounts and form a co-crystal in

sporulating *L. sphaericus*. The first selective step of *L. sphaericus* is the need for ingestion of crystals by larvae, followed by their solubilization in the alkaline environment of the gut, and activation of the protoxin forms into toxins by proteolytic cleavage, mediated by midgut proteinases (Charles et al. 1996). The subunits BinA and BinB are converted into active polypeptides of 39 and 43 kDa, respectively, due to cleavage of residues from the N- and C-termini (Broadwell et al. 1990). The processing and the presence of equimolar amounts of both subunits are essential factors in achieving optimal activity of this toxin (Nicolas et al. 1993). For *C. pipiens* larvae, the BinB component of the toxin is responsible for binding to the receptor, while the BinAt subsequently binds to BinB or the BinB-receptor complex (Charles et al. 1997). The functional domains of these subunits have been investigated through mutagenesis to identify regions and specific amino acids involved in binding to the Cqm1 receptor, binding between the two subunits and in vivo toxicity to larvae. N- and C-termini of BinA may be involved in the interaction of the BinB subunit (Kale et al. 2013; Oei et al. 1992). The N-terminal region of BinB (residues 33–158) is needed for receptor binding, and some residues identified are critical for this interaction (Romão et al. 2011; Singkhamanan et al. 2013).

In highly susceptible species of the *C. pipiens* complex, Bin toxin displays a marked and regionalized binding to the gastric caeca and posterior midgut. In *Anopheles gambiae* larvae the binding pattern is less clearly defined, and for *Ae. aegypti*, which is refractory to Bin toxin, this interaction is rather nonspecific compared to the two previous species (Davidson 1989). Quantitative binding assays between the Bin toxin and midgut microvilli of *C. pipiens* larvae have demonstrated high affinity (K_d 5–20 nM), while a lower affinity (K_d 30–110 nM) has been found for *An. gambiae* and *An. stephensi*, which are, overall, five- to tenfold less susceptible (in vivo) than *C. pipiens* (Nielsen-Leroux and Charles 1992; Nielsen-Leroux et al. 1995, 2002; Silva-Filha et al. 1997). For the refractory *Ae. aegypti* larvae, only a very low level of specific Bin toxin binding to the midgut is detected (Nielsen-Leroux and Charles 1992). Toxin binding to the midgut receptors of susceptible species leads to cytopathological alterations that have been described in Bin-treated *C. pipiens* larvae. These include disruption of microvilli, cytoplasmic vacuolization, mitochondria swelling, and breakdown of the endoplasmatic reticulum (Charles 1987; de Melo et al. 2008; Silva Filha and Peixoto 2003; Singh and Gill 1988; Tangsongcharoen et al. 2015). Other sites can also be affected as neural tissues and muscles (Singh and Gill 1988). The mode of action of the Bin toxin, following receptor binding, remains unclear, but there is evidence that the Bin toxin can form pores in the cell membranes, like the pore-forming toxins of *B. thuringiensis* and other bacteria (Pauchet et al. 2005; Schwartz et al. 2001). The vacuolization of target cells accompanied by the uptake of toxins into vesicles is also a marked effect of Bin intoxication (Davidson 1988). Bin toxin induces cell autophagy and displays a mechanism that prevents toxin degradation (Opota et al. 2011). The crystal structure of the BinB subunit has revealed features that support its action through pore formation, as proposed by previous studies (Srisucharitpanit et al. 2014). A recent study that revealed the BinAB structure suggests that BinA has the capacity to interact with the complex of BinB bound to the receptor, for co-internalization (Colletier et al. 2016).

The availability of midgut molecules that act as receptors for the Bin toxin is crucial for determining the susceptibility status of mosquito species for this toxin. Furthermore, Bin toxin resistance mechanisms found to date are associated with the failure of Bin toxin binding to those midgut receptors, as presented in Sect. 15.2. The receptors of the Bin toxin, which have been characterized in three susceptible species, are α-glucosidases bound to the midgut epithelium and named Cpm1 (*C. pipiens* maltase 1) (Darboux et al. 2001, Silva-Filha et al. 1999), Cqm1 (*C. quinquefasciatus* maltase 1) (Romão et al. 2006), and Agm3 (*An. gambiae* maltase 3) (Opota et al. 2008). *Ae. aegypti* displays the Aam1 protein (*Aedes aegypti* maltase 1), which is an ortholog with 74% identity to the Cqm1 receptor; however, Aam1 is not able to bind to the Bin toxin (Ferreira et al. 2010, 2014). These α-glucosidases (EC 3.2.1.20), belonging to the α-amylase family that plays a role in digestion, have the ability to hydrolyze α-1-4 links between glucose residues of carbohydrates (Krasikov et al. 2001). The Cpm1 α-glucosidase was the first receptor characterized for the Bin toxin in *C. pipiens* larvae (Darboux et al. 2001), and it shares 97% and 66% identity with the Cqm1 and Agm3 orthologs, respectively. These genes are organized in three exons and two introns, and their open reading frames encode the Cpm1/Cqm1 and Agm3 proteins with 580 and 588 residues, respectively. They display four conserved α-glucosidase domains, predicted consensus N-glycosylation sites, and a conserved sequence for a glycosylphosphatidylinositol (GPI) anchor (Darboux et al. 2001; Opota et al. 2008; Romão et al. 2006). The expression of the receptors as membrane-bound proteins is essential for the activity of Bin toxin, and mutations in their genes, which prevents the expression of these molecules as GPI-bound proteins to the midgut, are the most important resistance mechanism found in *C. pipiens* larvae. *Ae. aegypti* refractoriness seems to be based on the lack of ability of Aam1 to bind Bin toxin (Ferreira et al. 2010), although this protein is correctly located in the midgut through a GPI anchor. Minor differences in the amino acids of the Cqm1 and Aam1 protein sequence seem to be responsible for their capacity to interact or not with the Bin toxin. The N-terminal segment of Cqm1 (S129–A312) is responsible for binding to the Bin toxin, and a group of six amino acids within this region is critical for the ability of Cqm1 to bind the Bin toxin. These amino acids are not conserved in Aam1 and may be responsible for the refractoriness of this species (Ferreira et al. 2010, 2014).

15.2 Resistance Reports, Mechanisms, and Diagnosis

15.2.1 Investigation of Bti Resistance

Bacillus thuringiensis (Bt)-based biolarvicides have been used for pest control since the 1960s (Bravo et al. 2011), and field resistance to Bt toxins has already been reported for some species (Bravo et al. 2011). On the other hand, resistance to Bti biolarvicides has not been recorded to date. In the 1980s, very soon after its discovery, Bti was introduced for simulid and culicid control in a number of countries

(Margalit and Dean 1985). In some areas of Germany, Switzerland, and France, Bti has been employed, mainly for *Aedes* spp. control, for more than 30 years without reports of resistance as reviewed by Ferreira and Silva-Filha (2013). The screening of the susceptibility of mosquito populations to Bti, before the introduction of this biolarvicide, has also provided baseline data for the natural variations occurring in several areas. *Culex pipiens* populations, without previous Bti exposure, have shown susceptibility variations ranging from less than 3- to 12.5-fold (Vasquez et al. 2009; Wirth et al. 2001). *Aedes aegypti*, *Aedes albopictus*, and *Aedes rusticus* populations of different origins and never exposed to Bti showed a slight variation between 1.5- and 3.9-fold (Araujo et al. 2013; Kamgang et al. 2011; Liu et al. 2004; Loke et al. 2010; Marcombe et al. 2011, 2014; Pocquet et al. 2014). The susceptibility of Bti-treated populations, compared to laboratory colonies or untreated field samples used as references, was also similar to those observed in non-treated samples, confirming the lack of Bti resistance in those populations after exposure (Ferreira and Silva-Filha 2013). The only exception to these findings is the report of two *C. pipiens* populations in New York State (USA), which had a history of Bti spraying and displayed resistance ratios (RR) at LC_{95} of 14- and 41-fold (Paul et al. 2005). In this study, data from the pretreatment period was not available, and it was not possible to conclude whether the decreased susceptibility found was a consequence of Bti treatments.

Selection studies using whole Bti crystals performed under laboratory conditions also failed to show significant susceptibility alterations. Several attempts showed a maximum increase of around threefold in the lethal concentration of Bti for the selected colonies, which was the same as the natural variation found among untreated populations, as previously described (Ferreira and Silva-Filha 2013; Wirth 2010). In conclusion, resistance to Bti crystals, which are the active ingredient of commercially available biolarvicides, has not been reported to date. Although resistance to the whole Bti crystal has not been detected, larvae resistance to individual toxins from the crystal has been demonstrated by artificial selection assays using single Cry toxins (Cadavid-Restrepo et al. 2012, Georghiou and Wirth 1997, Paris et al. 2011). It was also demonstrated that an *Ae. aegypti* colony selected with Bti, but without decreased susceptibility to Bti, nevertheless displayed resistance ratios (RR) of 68-, 9-, and 9-fold for Cry4Aa, Cry4Ba, and Cry11Aa, respectively (Tetreau et al. 2012). This result shows that the action of single toxins can be affected, although the synergy provided by the set of toxins is able to prevent the selection of resistance to the whole Bti crystal. In some Bti-selected colonies, molecules that act as receptors for Cry toxins, such as ALPs and APNs, have been shown to be under-expressed (Stalinski et al. 2016). Alteration of these molecules may be responsible for the reduction of susceptibility to individual toxins found in this laboratory colony. However, as described, the synergy promoted by toxins, in particular the role of Cyt1Aa as a receptor for Cry toxins, is a key factor in overcoming failures related to alterations of Cry binding to midgut receptors. Data show that a decrease in susceptibility to individual Cry toxins does not evolve to Bti resistance but these can be used as markers to access the level of selection pressure imposed on a certain population (Tetreau et al. 2013b). The synergism of Bti toxins confers a great advantage

provided by their interaction with midgut cells, but other mechanisms of resistance unrelated to this step in the mode of action could potentially occur, although these have not yet been specifically recorded for Bti. These may include failures in proteolytic processing and innate immune response, which are currently under study (Cancino-Rodezno et al. 2010; Tetreau et al. 2013a). To date, Bti is still the biolarvicide available for mosquito control that has the most selective spectrum of action and lack of recorded field resistance, after decades of use. These major advantages are the result of the multiple set of toxins found in Bti crystals, the synergy of toxins, and the strategic role of Cyt toxin in overcoming failures occurring at the level of larvae midgut receptors, which has been the most important mechanism behind refractoriness to bacterial insecticidal toxins.

15.2.2 Lysinibacillus Sphaericus Resistance

The insecticidal activity of *L. sphaericus*, unlike Bti, is based on the action of one toxin that targets a single class of receptors (Nielsen-Leroux and Charles 1992), and this is a critical factor for the selection of resistance. *L. sphaericus* displays a high larvicidal activity in combination with effective performance under field conditions, although the potential for selection of resistance to the Bin toxin remains its major disadvantage. This section will summarize the major resistance reports available in the literature and advances in its management. Bin toxin resistance has been reported in field populations of *C. pipiens*/*C. quinquefasciatus* exposed to this agent and also in colonies selected with *L. sphaericus*, under laboratory conditions. The first report was of a *C. pipiens* population from France exposed to *L. sphaericus* for about 5 years that displayed high resistance levels (RR>20,000) (Sinègre et al. 1994). Subsequently, resistance cases of *C. quinquefasciatus* or *C. pipiens* populations were recorded in India (Rao et al. 1995), China (Yuan et al. 2000), Tunisia (Nielsen-Leroux et al. 2002), and Thailand (Mulla et al. 2003), along with a second resistant population (BP) in France (Chevillon et al. 2001; Nielsen-Leroux et al. 2002). There are also examples of *L. sphaericus* utilization for *C. quinquefasciatus* control programs in two urban areas in Recife and São Paulo city, in Brazil, which did not lead to resistance (Silva-Filha et al. 2008). It is likely that factors such as the interruption of treatment and/or rotation with Bti recorded in these areas may have disrupted the selection pressure. Selection performed under laboratory conditions using *L. sphaericus* has also confirmed that larvae may achieve high levels of resistance to the Bin toxin (RR ≈ 100,000) (Amorim et al. 2007; Pei et al. 2002; Rodcharoen and Mulla 1994; Wirth et al. 2000). The resistance reports available indicated that prolonged and intensive utilization of *L. sphaericus*, as the sole agent for control, may result in selection of high resistance in the treated populations.

The mechanism of resistance identified in some laboratory-selected and field-derived *C. pipiens* colonies is caused by target site alteration. In such cases, previous studies have shown that protoxin from crystals can be correctly processed, but the activated Bin toxin fails to bind to the midgut epithelium, due to lack of functional

receptors (Darboux et al. 2002; Guo et al. 2013; Nielsen-LeRoux et al., 1995, 2002; Oliveira et al. 2004). There are only two cases reported to date, resistant SPHAE (France) and TUNIS (Tunisia) field-derived colonies, in which there are functional binding receptors on the midgut and the resistance mechanisms remain unknown (Nielsen-Leroux et al. 1997, 2002). To date, the lack of receptors in the larval midgut is the major resistance mechanism for the Bin toxin (Silva Filha et al. 2014), and this occurs due to mutations in the *cpm1*/*cqm1* genes that prevent the expression of these midgut-bound α-glucosidases. Resistance to *L. sphaericus* was found to be monofactorial and recessively inherited in all the cases studied to date (Amorim et al. 2007; Nielsen-Leroux et al. 1995, 2002; Oliveira et al. 2004).

The identification of the genes coding for the Cpm1/Cqm1 receptors in *C. pipiens*/*C. quinquefasciatus* (Darboux et al. 2001) opened the way for investigations of the molecular basis of resistance. Eight alleles from *cpm1*/*cqm1* genes associated with Bin resistance have been characterized in populations from the USA, Brazil, France, and China (Chalegre et al. 2012, 2015; Darboux et al. 2002, 2007; Guo et al. 2013; Menezes et al. 2016; Romão et al. 2006). Seven of these alleles (*cpm1GEO* from the USA; $cqm1_{REC}$, $cqm1_{REC\text{-}2}$, $cqm1_{REC\text{-}D16}$, and $cqm1_{REC\text{-}D25}$ from Brazil; $cpm1_{BP}$ from France; *cqm1R* from China) were characterized by mutations, as transitions or deletions that generate a premature stop codon in their open reading frames. As a consequence, their transcripts code for truncated proteins, without the GPI anchor which is located at the C-terminus of protein. The loss of the GPI anchor prevents the protein localizing to the midgut surface, and the Bin toxin can no longer bind to the epithelium in order to produce its toxic effect and larvae mortality. Only one allele was found in a resistant population from France; $cpm1_{BP}$-*del* has a mutation that produces a different effect. In this case the mutant protein retained the predicted GPI anchor, but a 198 bp internal deletion, provoked by the insertion of a retrotransposon, generates an alternative splicing event, and the resulting transcript codes for a protein with internal deletion of 66 amino acids. This protein is unable to bind to the Bin toxin, despite being correctly located on the epithelium (Darboux et al. 2007). This mechanism prevents Bin interaction with the midgut epithelium, and it is responsible for the high level of resistance exhibited by larvae that are homozygous for the allele. Similarly, *Ae. aegypti* larvae are naturally refractory due to the lack of functional receptors in the midgut (Nielsen-Leroux and Charles 1992). Larvae express the Aam1 α-glucosidase, which is a Cqm1 ortholog that, although located in the midgut, does not have the ability to bind to the Bin toxin and thus prevents the toxic action of the Bin toxin on *Ae. aegypti* (Ferreira et al. 2010). The characterization of these mutations indicates that *cpm1*/*cqm1* is a highly polymorphic gene and six mutations, of the eight described, are located in the same region. These mutations can have a high impact because, unlike those observed in resistance genes of other insecticidal compounds (e.g., pyrethroids) which often cause only a reduction in their capacity to bind to the active ingredient (Du et al. 2013; Rinkevich et al. 2013), they generate full refractoriness, as seen in the case of Bin receptors, which become absent from the midgut.

Resistance to *L. sphaericus* needs be monitored, since the selection of homozygous individuals can lead to serious operational failures. *L. sphaericus* resistance is

also likely to be associated with discrete biological costs rather than marked impact on the fitness of resistant individuals, as has often been reported in the literature (Anilkumar et al. 2008). Some *L. sphaericus*-resistant colonies, for instance, have been maintained for more than 200 hundreds generations, under laboratory conditions (Chalegre et al. 2015). One direct consequence of *L. sphaericus* resistance in these insects is the potential lack of the Cpm1/Cqm1 α-glucosidase. However, *C. quinquefasciatus* larvae display a set of other α-glucosidases (Gabrisko 2013; Romão et al. 2006), and hypothetically, the lack of Cqm1 may be compensated by other α-glucosidases expressed in the larvae midgut. The role played by Cqm1 and the other α-glucosidases in larvae physiology has not yet been elucidated, but the long-term maintenance of these resistant colonies suggests that these insects could be successfully established which increases concerns about *L. sphaericus* resistance.

Monitoring the susceptibility of populations exposed to *L. sphaericus* is thus crucial for the effectiveness of this biolarvicide. Bioassays to determine the lethal concentrations of Bin toxin to larvae are the main tool used to evaluate susceptibility. However, *L. sphaericus* resistance is recessively inherited, and heterozygous individuals carrying *r* alleles are susceptible and can thus barely be detected by this tool. On the other hand, the identification of mutations of the *cqm1* gene that confer resistance has enabled the development of PCR screens which have enhanced the capacity to directly monitor these recessive genes in population samples. Screening of these genes in *C. quinquefasciatus* populations in the city of Recife (Brazil) has revealed four of these alleles: $cqm1_{REC}$, $cqm1_{REC\text{-}2}$, $cqm1_{REC\text{-}D16}$ and $cqm1_{REC\text{-}D25}$ (Chalegre et al. 2009, 2012, 2015). $cqm1_{REC}$, which was primarily identified in a laboratory-selected colony, was found to occur in Recife city areas at a frequency in the order of 10^{-3} in samples of untreated populations, while a significantly higher frequency (≈0.05) was recorded in larvae samples from a *L. sphaericus*-treated area. Furthermore, although the four alleles were found in Recife city, $cqm1_{REC}$ was detected in all populations at a higher frequency, compared to the other alleles (Menezes et al. 2016). The dataset reported the frequency of these alleles in Recife populations and indicated that $cqm1_{REC}$ may be a marker for the surveillance of resistance in *C. quinquefasciatus* populations from those areas. The frequency of other *L. sphaericus* resistance alleles in the geographical areas in which they were originally detected, or abroad, has not been studied.

15.3 Management Strategies to Prevent *L. sphaericus* Resistance

The *L. sphaericus* resistance recorded in exposed populations from different countries highlights the need to design strategies to manage resistance to this agent. One of the most important approaches is the use of multiple strategies to reduce the density of mosquitoes. This is crucial for reducing insecticide use and hence the corresponding selection pressure that is caused by its use (Becker et al. 2003). It is highly recommended that environmental strategies be introduced to reduce the

number of active breeding sites and to keep larvicide application at the minimum level possible, in order to prevent the onset of resistance. However, if resistance is detected in an exposed population, the interruption of *L. sphaericus* treatment is the primary measure to be taken. Mosquitoes are *r*-strategists, and populations can recover rapidly after interruption of the control interventions. The interruption of treatments, per se, allows the immigration of susceptible individuals from surrounding areas and leads to the dilution of resistance alleles. Reversal of *L. sphaericus* resistance is facilitated by the recessive inheritance of this phenotype (Amorim et al. 2007, 2010; Chevillon et al. 2001, Nielsen-Leroux et al. 1995, 1997, 2002; Oliveira et al. 2004). In a Chinese field population, a high resistance level (22,000-fold) was recorded, and, 6 months after stopping treatment, the resistance ratio decreased to sixfold (Yuan et al. 2000). The second strategy to be implemented is the replacement of *L. sphaericus* by other insecticides with different modes of action. Among the commercially available agents to be used in association with *L. sphaericus*, Bti-based biolarvicides are considered the most promising option because their toxins and mode of action are unrelated to the Bin toxin, as described previously in this chapter. Other dipteran-active *Bacillus thuringiensis* (Bt) strains also produce toxins that do not display cross-resistance to Bin toxin, such as those from *Bacillus thuringiensis* serovar. *medellin* (Btmed) and *Bacillus thuringiensis* serovar. *jegathesan* (Btjeg), although commercial products are not available to date. There are also other mosquitocidal toxins produced by *L. sphaericus*, that are effective on Bin-resistant mosquito strains, such as the Mtx and Cry48–49 toxins (Berry 2012). However, the expression of these toxins in native strains has limitations in terms of optimal amounts and stability. Further biotechnological development is needed for the production of biolarvicides based on these toxins. Recombinant *L. sphaericus* strains containing Bti toxins have been developed, and these have been shown to be active against larvae from Bin-resistant colonies. However, these modified strains showed low expression and/or instability of Bti proteins (Federici et al. 2010; Gammon et al. 2006). The integration of the Bin toxin into Bti strains has also been performed, and the recombinant constructs successfully produced Bti and Bin toxins with improved toxicity (Park et al. 2005). Products based on such recombinant bacteria have not been developed for field utilization but are a promising prospect (Federici et al. 2010).

Nowadays, it is strongly recommended that Bti be used in combination with *L. sphaericus*, since Bti commercial products are already available, are effective in overcoming Bin-resistance, and have a long history of successful field utilization. Bti can be used in rotation or mixed with *L. sphaericus*, and this strategy can be introduced for prevention or reversal of *L. sphaericus* resistance. Both rotation and mixtures may be effective, but mixtures may be more efficient in delaying the onset of resistance (Zahiri and Mulla 2003). Based on this successful association of the complementary features of *L. sphaericus* and Bti, commercial products containing a mixture of crystals produced by each agent in a single product have been developed (Anderson et al. 2011). These aim to target a wider range of mosquito species in a variety of settings. Successful trials have been carried out to control *Culex* and *Aedes* species that colonize typical breeding sites in urban areas and to control other

mosquito species that occur in wetlands in environmentally sensitive areas (Anderson et al. 2011; Cetin et al. 2015; Dritz et al. 2011). These multi-toxin products have shown promising results and can be used in mosquito control programs as a safe tool with a low potential for resistance selection. In a broader view, other agents may also be considered for use in management of *L. sphaericus* resistance, and these may include biological control agents such as predators (fish, aquatic insects), entomopathogenic fungi, and nematodes (Hurst et al. 2006; Keiser et al. 2005; Lacey 2007; Lingenfelser et al. 2010). Spinosins are another group of larvicides that have been recently introduced for mosquito control, and field trials showed successful results (Hertlein et al. 2010). Synthetic insecticides, such as insect growth regulators, are another category to be considered, since these have a mode of action distinct from *L. sphaericus* and a relatively safe spectrum of action (Giraldo-Calderon et al. 2008, Guidi et al. 2013). In conclusion, resistance can be counteracted, and *L. sphaericus* is an effective component to be employed in association with other control measures in integrated programs in order to reduce mosquito populations.

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Chapter 16
The Importance of *Bacillus thuringiensis* in the Context of Genetically Modified Plants in Brazil

Deise Maria Fontana Capalbo and Marise Tanaka Suzuki

Abstract Sustainable agriculture requires alternative interventions for pest control and management. In this context the use of microorganisms pathogenic to pests has become even more studied and widespread, especially in the successful case of bioinsecticides based on *Bacillus thuringiensis*. With advances in recombinant DNA biotechnology, *B. thuringiensis* has continued to show its potential with the insertion of its insecticide-encoding genes into plants, which thus become resistant to a varied range of pest insects. These Bt plants, often containing multiple Bt genes, are commercially available. And today, after assessing biosafety in several countries around the world, they are adequate to control pests without significant harm to humans or to the environment. If the required safety conditions are maintained, a greater use of these plants is anticipated, guaranteeing an effective tool for an environmentally friendly agriculture.

Keywords Biosafety • Bt plants • Agriculture

Insect pest control in the field has been done with chemical insecticides since the early 1940s. These insecticides controlled many pests but were associated with environmental problems, toxicity to humans and animals, and were not very specific, affecting "nontarget" organisms. Twenty-first-century sustainable agriculture increasingly requires alternative interventions for pest control and management, ones that are safe and, if possible, reduce human contact with pesticides. As an option, the use of microorganisms pathogenic to pests (insects or invasive plants) has become more studied and disseminated, proving to be safer and increasing the activity of other natural enemies. Such was the successful case using bioinsecticides based on *Bacillus thuringiensis* (Bt).

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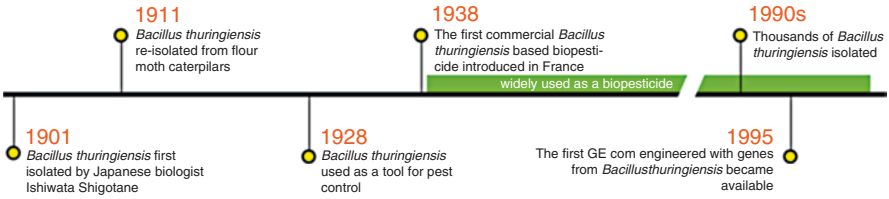

Fig. 16.1 Advances in the use of *Bacillus thuringiensis* for control of agricultural pests in the twentieth century as presented by Niederhuber (2015)

To achieve successful biological control, it was necessary, over time, to search for, among many aspects, a greater number of insect pathogenic strains, a wider range of action respect to target organisms, a higher yield in toxin production, and better characteristics for fermentation process. In addition, several Bt strains have been improved using recombinant DNA technologies.

Regarding the use of biotechnology, it has been systematically applied in the improvement of agriculture since the beginning of the twentieth century, with the development of cell and plant tissue culture, followed by the rescue of immature embryos in vitro (1930s), mutagenesis and selection (1940s), anther culture (1950s), somaclonal variation (1960s), recombinant DNA technology or genetic engineering (1970s), selection assisted by molecular markers (1980s), and genomics, proteomics, metabolomics, and bioinformatics (1990s).

Figure 16.1 summarizes the advances in the use of Bt and the development of GM plants throughout the twentieth century (Niederhuber 2015).

In the twenty-first century, innovations in biotechnology have continued to leverage advances in agriculture, including RNA interference (RNAi) and transgenic technology and, much more recently, genome editing. Several of these utilize new methodologies for genetic improvement, and a summary of them can be found in Whelan and Lema (2015).

With so many new techniques since the advent of recombinant DNA technology, it has become possible to alter parts of the genome of any organism in order to favor desired traits (Arantes 2003). The said modified organism is called a genetically modified organism – GMO – according to Art. 3, section V, of the Brazilian Federal Law No. 11,105/2005 – "available in < http://www.ctnbio.gov.br/index.php/content/view/11992.html". It is important to mention that "transgenesis" is a particular case of genetic modification in which a DNA sequence, total or partial, of an (exogenous) organism is transferred to another organism of a species other than its own, thus sexually incompatible. "Cysgenesis" uses the same transgenic techniques, but DNA transfer occurs in the same species or between species that cross in the wild. Although not synonyms, the terms have been grouped and referred to in this chapter under the designation of GMOs. Other designations may also be cited, such as LMOs (for living modified organisms, adopted by the Cartagena Protocol), GMF for GM food, GMA for GM animal, GMV for GM vegetable, and GMM for GM microorganism").

This has led to a significant change in the technological standards of various sectors, including agriculture, and, as a consequence, the entire production chain related

to it. Thus, the main contribution of modern biotechnology to agriculture was the possibility of creating new varieties from the transfer of genes, including between two distinct species, aiming to aggregate an attribute of economic interest, such as:

(a) Plants resistant to biotic (pathogens, insect pests, and weeds) and abiotic stresses (salinity, dryness, cold, flood, heat)
(b) Improving the quality of agricultural products such as extended shelf or storage life; differential or biofortified nutritional content (proteins, fibers, oils, carbohydrates, vitamins/minerals, and phytochemicals); removal of antinutrients, allergens, and/or toxins; and better quality of wood fiber
(c) Plants used as bioreactors to obtain drugs and/or industrial products
(d) Faster-growing plants
(e) Plants with greater efficiency in the conversion of biomass, in the use of water and/or nitrogen fixation of the soil
(f) Plants with greater potential for biofuel production and phytoremediation

According to James (2015), the global cultivated area between 1996 and 2015 for GM plants was approximately 2 billion hectares. Of this total, 1 billion hectares were planted with soybean, 0.6 billion hectares with maize, 0.3 billion hectares with cotton, and 0.1 billion hectares with canola. The United States continues to lead adoption with 70.9 million hectares (39% of the global). Brazil is the second largest producer, with 44.2 million hectares planted (representing 25% of the global). Another interesting fact is that about 20 million farmers are involved in this production, wherein ~90% of them are small farmers distributed in 28 countries, of which 20 are developing countries and only 8 are industrialized countries.

16.1 Bt Plants

Since the late 1990s it has been possible to insert *cry* ("the cry gene is the gene that codes for the formation of the toxic protein Cry responsible for the insecticidal activity of Bt") genes into plants, forming the so-called Bt plants. These plants produce their own Cry proteins, protecting them from the attack of insects that are susceptible to such protein. The production of this plant occurs initially in the laboratory and passes through monitored processes of release in greenhouse and field and later by processes of commercial liberation denominated biosafety processes (Capalbo et al. 2014).

Prior to being introduced into a plant, the *cry* genes must be altered in their DNA sequence by site-directed mutagenesis. This is necessary so that the differences in the mechanisms of expression between prokaryotic and eukaryotic organisms do not block or decrease gene expression (Capalbo et al. 2005).

The advances are continuous, and the perspectives expand with each new technique or added knowledge. Thus many Bt plants have emerged safer and focused not only on insect control but also on the conservation of established ecological conditions in the growing areas. Researchers have developed plants in which proteins can be expressed only where and when necessary through the use of specific

tissue promoters, specific time, or inducible promoter genes. These and other precautions are taken to minimize the development of insect resistance to Cry proteins and the gene flow to wild varieties, since in the Bt plant the toxin is available in its active form rather than in the form of protein crystal.

When the first Bt plants were commercialized, the use of a refuge area was suggested or required for greater resistance control. For the use of Bt maize in Brazil, the producer must comply with two rules: the coexistence, required by law, and the Insect Resistance Management (IRM) rule recommended by the National Technical Biosafety Commission (CTNBio). This means that a 100 m border should be used to isolate transgenic corn crops from other crops. Some alternatives are offered with respect to border size if rows of non-transgenic maize of equal size and in the same cycle of transgenic maize are sown. Obviously, monitoring of plant infestation is also important because, depending on the non-GM hybrid and infestation intensity, the producer may need to adopt complementary control measures. For fall armyworm the refuge area should not be more than 800 m away from transgenic plants. This is the maximum verified distance for the dispersal of adults in the field. Also, according to the CTNBio recommendation, in the area of refuge the use of other control methods is allowed, so long as Bt-based bioinsecticides are not used. Further details on minimum distances between GM and non-GM maize commercial crops, aiming at coexistence between production systems, can be obtained in Mendes et al. (2009) and CTNBio (2007).

The non-Bt refuge area generally means a loss in production since it will be intensely available to insect attack. Everything becomes even more difficult when crop rotation is considered throughout the year in planted areas, and additional care must be taken to ensure that the same area is not cultivated with the same Bt gene inserted in different crops, which would intensify exposure to the toxin and the likelihood of developing resistance, endangering this important ally.

In the search for alternative solutions to resistance development, new technologies were developed expressing more than one Bt protein. These technologies are called pyramiding, stacking, or second generation (ISAAA 2013), referring to the process of combining two or more genes of interest in the same plant. An example of such plants is a soybean or maize that expresses two or more Bt genes whose proteins have different modes of action. But they may also be plants expressing one gene for insect resistance and another for herbicide tolerance. A list of stacked products that are available on the world market can be found in the Center for Environmental Risk Assessment (CERA) database (Center for Environmental Risk Assessment).

From the point of view of science and plant breeding, it is desirable to be able to insert several characteristics simultaneously at the same *locus*; this action is known as "molecular stacking." In these new events, the inserted characteristics will behave essentially as a single gene, making the introgression of the new characteristic (or set of them) much simpler (Que 2010).

From the standpoint of the rural producer, the "staked" plant, when compared to the single-trait variety, offers a wider opportunity to overcome various problems in the field, such as pest insects, invasive plants, plant diseases, and environmental stresses, which favors the increase of productivity at the field level (ISAAA 2013).

16.2 Security of Bt Plants and Their Adoption

Brazil is a country with great potential for the development of agricultural biotechnology, it has great biological diversity, and it is a large potential source of natural molecules for different purposes. Among the developing countries, Brazil stands out because it has a strong national agricultural research system, consolidated after many years of scientific research aimed at making better use of its natural diversity: tropical and subtropical climates, ecosystems with agricultural aptitude, and germplasm selected and adapted for great variability. The diffusion of GMOs into Brazilian agriculture is only inferior to the United States, due to the robust and workable regulatory process in place since 2005 (in Brazil, authorization for experimental and commercial planting is obtained from the National Biosafety Commission – CTNBio), which ensures the safe use and practice of GMOs.

In order to make biotechnology products safely available to society, a biosafety analysis of the products generated must be rigorously structured and executed. This analysis measures potential risks and their probability of occurrence. As the potential risks include both environmental aspects and effects on human and animal health, the biosafety assessment of transgenic plants considers the two aspects together. The term "biosafety" in its broader application refers to the actions of prevention, minimization, or elimination of risks inherent to the activities of research, production, teaching, technological development, and provision of services that may compromise the health of humans, animals, and plants or the quality of the work carried out (Teixeira and Valle 1996).

In its narrower sense, linked to the legal framework that supports the use of recombinant DNA technology in Brazil, the term biosafety has been applied to the care of the protection of humans and the environment when GMOs and their derivatives are involved. The Brazilian biosafety legislation in force – Law No. 11,105 of March 24, 2005, which replaced Law No. 8974, of January 5, 1995 – establishes safety standards and mechanisms to supervise activities involving GMOs and their derivatives, in addition to regulating items II, IV, and V of paragraph 10 of article 225 of the Federal Constitution, which deals with the protection of the environment.

It is not the intention in this chapter to detail these regulations nor the way in which risk analysis of GMOs is conducted for biosafety purposes, but it is very important to point out that there are specific regulations for such an analysis and there are also bodies responsible for standards and their monitoring, both with regard to human and animal health and with regard to environmental issues (Capalbo et al. 2005, 2009, 2015; Hagler et al. 2010).

CTNBio is a multidisciplinary collegiate body, created through the same Law No. 11,105, whose purpose is to provide technical advisory support and advice to the Federal Government in the formulation, updating, and implementation of the National Biosafety Policy on GMOs, as well as in establishing technical safety standards and technical advice concerning the protection of human health, living organisms, and the environment for activities involving the construction, testing, cultivation, handling, transportation, commercialization, consumption, storage, release, and disposal of GMOs and derivatives.

16.3 Bt Plants in Brazil

The Bt plants approved by CTNBio for commercialization in Brazil are indicated in Table 16.1, along with pyramid (or stacked) Bt plants. More information on other events that are not listed in the Table can be found in the CTNBio database (http://ctnbio.mcti.gov.br/liberacao-comercial?p_p_id=110_INSTANCE_SqhWdohU4BvU&p_p_lifecycle=0&p_p_state=normal&p_p_mode=view&p_p_col_id=column-2&p_p_col_count=3&_110_INSTANCE_SqhWdohU4BvU_struts_action=%2Fdocument_library_display%2Fview_file_entry&_110_INSTANCE_SqhWdohU4BvU_redirect=http%3A%2F%2Fctnbio.mcti.gov.br%2Fliberacao-comercial%2F-%2Fdocument_library_display%2FSqhWdohU4BvU%2Fview%2F1684467%3F_110_INSTANCE_SqhWdohU4BvU_redirect%3Dhttp%253A%252F%252Fctnbio.mcti.gov.br%252Fliberacao-comercial%252F-%252Fdocument_library_display%252FSqhWdohU4BvU%252Fview%252F614405%253F_110_INSTANCE_SqhWdohU4BvU_redirect%253Dhttp%25253A%25252F%25252Fctnbio.mcti.gov.br%25252Fliberacao-comercial%25253Fp_p_id%25253D110_INSTANCE_SqhWdohU4BvU%252526p_p_lifecycle%25253D0%252526p_p_state%25253Dnormal%252526p_p_mode%25253Dview%252526p_p_col_id%25253Dcolumn-2%252526p_p_col_count%25253D3&_110_INSTANCE_SqhWdohU4BvU_fileEntryId=1712293#/liberacao-comercial/consultar-processo).

16.4 Bt Plants in the Rest of the World

Table 16.2 lists the countries that commercially produced Bt plants between 1996 and 2015, according to the ISAAA GM Approval Database.

16.5 Final Considerations

Bacillus thuringiensis presents more than 100 years of action in the control of agricultural pests. Even with the relatively recent development of genetically engineered modifications, Bt remains one of the most effective and safe control agents. Bt is a highly effective control agent as a biopesticide and meets the demands for use in biotechnological applications (as in the generation of Bt plants). The action of Bt toxins expressed in Bt plants is specifically directed toward the target insect, which has shown less environmental impact than methods that do not use Bt.

Bt plants possess advantages when used correctly in compliance with regulations and technical guidelines. In these cases, they have been very advantageous by virtue of:

- Facilitating pest management by allowing the farmer a longer protection during plant growth and reducing the handling and application of toxic insecticides.

Table 16.1 Summary table of genetically modified plants containing Bt gene, authorized by CTNBio for commercialization in Brazil, until August 15, 2016

Product	Commercial name	Donor organism	Characteristic[a]	Protein inserted	Applicant	Approval year
Soybean	**Intacta RR2 PRO**	Agrobacterium tumefaciens/Bacillus thuringiensis	**TH and RI**	**CP4-EPSPS Cry1Ac**	**Monsanto**	**2010**
	b	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**Cry1Ac** **Cry1F** **PAT**	**Dow AgroSciences**	**2016**
Maize	YieldGard	*Bacillus thuringiensis*	RI	Cry1Ab	Monsanto	2007
	TL	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**Cry1Ab** **PAT**	**Syngenta**	**2007**
	Herculex	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**Cry1F** **PAT**	**DuPont and Dow AgroSciences**	**2008**
	YR YieldGard/RR2	Agrobacterium tumefaciens/Bacillus thuringiensis	**TH and RI**	**CP4-EPSPS Cry1Ab**	**Monsanto**	**2009**
	TL/TG	Bacillus thuringiensis/Streptomyces viridochromogenes/Zea mays	**TH and RI**	**Cry1Ab** **PAT** **mEPSPS**	**Syngenta**	**2009**
	Viptera-MIR162	*Bacillus thuringiensis*	RI	VIP3Aa20	Syngenta	2009
	HR Herculex/RR2	Bacillus thuringiensis/Streptomyces viridrochromeogenes/Agrobacterium tumefaciens	**TH and RI**	**Cry1F** **PAT** **CP4-EPSPS**	**DuPont**	**2009**
	Pro	*Bt*	RI	Cry1A.105 Cry2Ab2	Monsanto	2009
	TL TG Viptera	Bacillus thuringiensis/Streptomyces viridochromogenes/Zea mays	**TH and RI**	**Cry1Ab VIP3Aa20 mEPSPS**	**Syngenta**	**2010**

(continued)

Table 16.1 (continued)

Product	Commercial name	Donor organism	Characteristic[a]	Protein inserted	Applicant	Approval year
Maize	**Pro2**	Bacillus thuringiensis/Agrobacterium tumefaciens	**TH and RI**	**Cry1A.105 Cry2Ab2**	**Monsanto**	**2010**
				CP4-EPSPS		
	YieldGard VT	Agrobacterium tumefaciens/Bacillus thuringiensis	**TH and RI**	**CP4-EPSPS Cry3Bb1**	**Monsanto**	**2010**
	Power Core PW/Dow	Bacillus thuringiensis/Streptomyces viridochromogenes/Agrobacterium tumefaciens	**TH and RI**	**Cry1A.105 Cry2Ab2**	**Monsanto and Dow AgroSciences**	**2010**
				Cry1F		
				PAT		
				CP4-EPSPS		
	HX YG RR2	Bacillus thuringiensis/Streptomyces viridochromogenes/Agrobacterium tumefaciens	**TH and RI**	**Cry1Ab**	**DuPont**	**2011**
				Cry1F		
				PAT		
				CP4EPSPS		
	TC1507xMON810	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**Cry1F**	**DuPont**	**2011**
				Cry1Ab		
				PAT		
	MON89034 × MON88017	Bacillus thuringiensis/Agrobacterium tumefaciens	**TH and RI**	**Cry1A.105 Cry2Ab2 Cry3Bb1 CP4-EPSPS**	**Monsanto**	**2011**
	Herculex XTRA™ maize	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**Cry1F PAT Cry34Ab1 Cry35Ab1**	**DuPont and Dow AgroSciences**	**2013**
	Viptera4	Bacillus thuringiensis/Streptomyces viridochromogenes/Zea mays	**TH and RI**	**Cry1Ab PAT VIP3Aa20 mCry3A**	**Syngenta**	**2014**
				mEPSPS		

Maize	MIR 604	*Bt*	RI	mCry3A	Syngenta	2014
	b	Bacillus thuringiensis/Streptomyces viridochromogenes/Agrobacterium tumefaciens	**TH and RI**	**Cry1F**	**DuPont (RN 15)**	**2015**
				Cry1Ab		
				PAT		
				VIP3Aa20		
				CP4-EPSPS		
	b	Bacillus thuringiensis/Streptomyces viridochromogenes/Agrobacterium tumefaciens	**TH and RI**	**Cry1F**	**DuPont (RN 15)**	**2015**
				PAT		
				VIP3Aa20		
				CP4-EPSPS		
	b	Bacillus thuringiensis/Streptomyces viridochromogenes/Bacillus thuringiensis	**TH and RI**	**Cry1F**	**DuPont (RN 15)**	**2015**
				PAT		
				VIP3Aa20		
	b	Bacillus thuringiensis/Agrobacterium tumefaciens	**TH and RI**	**VIP3Aa20**	**DuPont (RN 15)**	**2015**
				CP4-EPSPS		
	[b]	*Bt*	RI	Cry1Ab VIP3Aa20	DuPont (RN15)	2015
	b	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**Cry1F**	**DuPont**	**2015**
				PAT		
				VIP3Aa20		
				Cry1Ab		
	b	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**eCry3.1Ab Cry1Ab**	**Syngenta**	**2015**
				Vip3Aa20		
				Cry3A		
				Cry1F		
				PAT		
				mEPSPS		

(continued)

Table 16.1 (continued)

Product	Commercial name	Donor organism	Characteristic[a]	Protein inserted	Applicant	Approval year
Maize	**b**	Bacillus thuringiensis/Streptomyces viridochromogenes/Agrobacterium tumefaciens/Sphingobium herbicidovorans	**TH and RI**	**Cry1A.105 Cry2Ab2**	**Dow AgroSciences**	**2016**
				Cry1F		
				PAT		
				CP4-EPSPS		
				AAD-1		
		Bacillus thuringiensis/Streptomyces viridochromogenes/Agrobacterium tumefaciens	**TH and RI**	**Cry2Ab2 Cry1A.105**	**Dow AgroSciences**	**2016**
				Cry3Bb1/CP4 EPSPS		
				Cry1F		
				PAT		
				Cry34Ab1 Cry35Ab1		
Cotton	Bolgard I	*Bacillus thuringiensis*	RI	Cry1Ac	Monsanto	2005
	Bolgard I Roundup Ready	Bacillus thuringiensis/Agrobacterium tumefaciens	**TH and RI**	**Cry1Ac**	**Monsanto**	**2009**
				CP4-EPSPS		
	WideStrike	Bacillus thuringiensis/Streptomyces viridochromogenes	**TH and RI**	**Cry1Ac**	**Dow AgroSciences**	**2009**
				Cry1F		
				PAT		
	Bolgard II	*Bt*	RI	Cry2Ab2	Monsanto	2009
				Cry1Ac		

Cotton	**TwinLink**	Bacillus thuringiensis/Streptomyces hygroscopicus	**TH and RI**	**Cry1Ab**	**Bayer**	**2011**
				Cry2Ae		
				PAT		
	Glytol x TwinLink	Zea mays/Bacillus thuringiensis/ Streptomyces hygroscopicus	**TH and RI**	**Cry1Ab**	**Bayer**	**2012**
				Cry2Ae		
				2mEPSPS		
	Bolgard II Roundup Ready Flex	Bacillus thuringiensis/Agrobacterium tumefaciens	**TH and RI**	**Cry1Ac Cry2Ab2**	**Monsanto**	**2012**
				CP4-EPSPS		

Adapted from table provided by CTNBio (http://ctnbio.mcti.gov.br/liberacao-comercial?p_p_id=110_INSTANCE_SqhWdohU4BvU&p_p_lifecycle=0&p_p_state=normal&p_p_mode=view&p_p_col_id=column-2&p_p_col_count=3&_110_INSTANCE_SqhWdohU4BvU_struts_action=%2Fdocument_library_display%2Fview_file_entry&_110_INSTANCE_SqhWdohU4BvU_redirect=http%3A%2F%2Fctnbio.mcti.gov.br%2Fliberacao-comercial%2F-%2Fdocument_library_display%2FSqhWdohU4BvU%2Fview%2F1684467%3F_110_INSTANCE_SqhWdohU4BvU_redirect%3Dhttp%253A%252F%252Fctnbio.mcti.gov.br%252Fliberacao-comercial%252F-%252Fdocument_library_display%252FSqhWdohU4BvU%252Fview%252F614405%253F_110_INSTANCE_SqhWdohU4BvU_redirect%253Dhttp%25253A%25252F%25252Fctnbio.mcti.gov.br%25252Fliberacao-comercial%25253Fp_p_id%25253D110_INSTANCE_SqhWdohU4BvU%252526p_p_lifecycle%25253D0%252526p_p_state%25253Dnormal%252526p_p_mode%25253Dview%252526p_p_col_id%25253Dcolumn-2%252526p_p_col_count%25253D3&_110_INSTANCE_SqhWdohU4BvU_fileEntryId=1712293#/liberacao-comercial/consultar-processo) (accessed in Dec. 14 2016).
The pyramid (or stacked) Bt plants are in bold
[a]*TH* tolerant to herbicide, *RI* resistant to insect
[b]Awaits denomination

Table 16.2 Bt plants and the countries where they were commercially produced between 1996 and 2015 (As presented by ISAAA GM Approval Database)

Bt crop	Country
Cotton	Argentina, Australia, Brazil, Burkina Faso, Canada, China, Colombia, Costa Rica, the European Union (EU), India, Japan, Mexico, Myanmar, New Zealand, Pakistan, Paraguay, the Philippines, Singapore, South Africa, South Korea, Sudan, Taiwan, the United States (USA)
Eggplant	Bangladesh
Maize	Argentina, Australia, Brazil, Canada, Chile, China, Colombia, Egypt, the EU, Honduras, Indonesia, Japan, Malaysia, Mexico, New Zealand, Panama, Paraguay, the Philippines, the Russian Federation, Singapore, South Africa, South Korea, Switzerland, Taiwan, Thailand, Turkey, the United States, Uruguay, Vietnam
Poplar	China
Potato	Australia, Canada, Japan, Mexico, New Zealand, the Philippines, the Russian Federation, South Korea, the United States
Rice	China, Iran
Soybean	Argentina, Australia, Brazil, Canada, China, Colombia, the EU, Japan, Mexico, New Zealand, Paraguay, the Philippines, the Russian Federation, South Africa, South Korea, Taiwan, Thailand, Turkey, the United States, Uruguay, Vietnam
Tomato	Canada, the United States

- Since Bt plants do not require the application of broad spectrum pesticides, the beneficial organisms (nontarget) are not affected and can thus proliferate, which indirectly allows the control of secondary pests.
- Another advantage already detected was the lower incidence of mycotoxins in maize, since there are no physical damages caused by pests that allow the entry of opportunistic pathogenic microorganisms. The fungi that produce these mycotoxins can be lethal to humans and animals.
- James (2015) points out that in the last 19 years of commercialization, profits obtained with Bt plants reached US$ 86.9 billion and that for the year 2014 profits alone was US$ 9.8 billion.
- In addition, Bt plants are another tool in the arsenal required for the control of pests that are more difficult to control with traditional pesticides.

With the increase in the world's population and the decrease in available arable land, it is more and more necessary to explore options that allow greater productivity in the same area. When used side by side with other agricultural practices, insect resistance technology (Bt plants) can bring many positive gains in productivity, benefiting the farmer, the productive chain, and, especially, the final consumer.

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Chapter 17
Resistance of *Spodoptera frugiperda* to *Bacillus thuringiensis* Proteins in the Western Hemisphere

Samuel Martinelli, Renato Assis de Carvalho, Patrick Marques Dourado, and Graham Phillip Head

Abstract Resistance to *Bacillus thuringiensis* (*Bt*) Cry1 insecticidal proteins expressed in genetically modified plants (*Bt* maize and other *Bt* crops) has been documented in the fall armyworm (FAW; *Spodoptera frugiperda* [J.E. Smith]) in South America. The factors that led to the onset of resistance include less-than-optimal product characteristics (dose) and poor compliance with the requirement for structured refuges of non-*Bt* maize. In this article, we review the documented cases of resistance in FAW and explore the path forward to the implementation of effective insect resistance management (IRM) programs to support the sustainable deployment of this technology, particularly in tropical regions. Effective IRM plans require effective product design and management of *Bt* maize technology. Due to the challenges presented in tropical regions, the development of effective *Bt* maize pyramids combining highly effective and novel modes of action is fundamental to a successful IRM strategy. The integration of IRM and business imperatives through the development of a multilayer, multi-stakeholder strategy to ensure the proper use of the technology, and particularly to adequate compliance with refuge requirements, is another critical element of an IRM strategy for *Bt* crops.

Keywords *Bt* maize • Fall armyworm • Insect resistance management • Refuge • *Spodoptera frugiperda*

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Genetically modified plants (GM) containing genes from the soil bacterium *Bacillus thuringiensis* (*Bt*), which produces a number of insecticidal proteins, have been extensively adopted globally. The area planted with crops containing *Bt* traits was approximately 83.7 Mha worldwide in 2015 (James 2015). The benefits realized from adopting *Bt* crops are substantial and include effective management of target insect pests (Wu et al. 2008; Hutchison et al. 2010; Waquil et al. 2013), decreased use of synthetic insecticides (Lu et al. 2012; Tabashnik et al. 2013; Brookes and Barfoot 2016), conservation of beneficial nontarget organisms (natural enemies) (Wolfenbarger et al. 2008; Naranjo 2009; Tian et al. 2012, 2013), and increased crop yield and farm income (Brookes and Barfoot 2016). For these reasons, the use of *Bt* plants in integrated pest management (IPM) programs has been extensively adopted by growers globally (James 2015; Brookes and Barfoot 2016).

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is an important pest of maize and other economically important crops in the Western Hemisphere, particularly in warm-winter areas such as Central and South America (Cruz et al. 2012; Nagoshi et al. 2014, 2015). FAW is a migratory and highly polyphagous pest species that does not have the ability to enter diapause, an inactive state that allows insects to survive prolonged periods of non-conducive conditions such as extreme cold or drought (Luginbill 1928; Sparks 1979). FAW comprises two genetically and behaviorally separate strains that occur sympatrically throughout North and South America (Pashley 1986). The "corn" strain (CS) is associated with maize and sorghum plants, whereas the "rice" strain (RS) is preferentially found in rice and turfgrass (Nagoshi et al. 2007). These "host strains" of FAW are morphologically indistinguishable; however, polymorphisms in the mitochondrial cytochrome oxidase subunit I (COI) gene proved to be capable of consistently differentiating and identifying CS and RS haplotypes (Levy et al. 2002). The COI marker also defined the further subdivision of CS into four haplotype groups (Nagoshi et al. 2007). In the United States, due to the absence of a diapause trait in FAW, this pest species of tropical origin must migrate northward to reinfest cropping areas in temperate areas (Westbrook et al. 2016). FAW is frequently reported as far north as Canada (Mitchell et al. 1991). The primary overwintering regions in the southern portions of Florida and Texas, extending into Mexico, are assumed to be the source of practically all FAW infestations in the United States and Canada (Nagoshi et al. 2014). This knowledge about the biology and ecology of FAW was used to simulate migratory flight of FAW moths from distinct winter-breeding source areas in the United States (Westbrook et al. 2016).

In tropical and subtropical regions of Central and South America, where cropping systems are especially favorable for the buildup of insect pest populations, the use of *Bt* maize technologies has provided an efficient and environmentally safe tool to manage FAW populations and damage (see Table 17.1) (Okumura et al. 2013; Waquil et al. 2013; Trumper 2014). The main threat to the sustained use of *Bt* crops to manage insect populations is the evolution of resistance by target pests (Carrière et al. 2015; Gould 1998; Tabashnik et al. 2013). The risk of resistance evolving to *Bt* crops is frequently linked to the expression of insecticidal proteins in all plant parts throughout the season (constitutive expression), thereby exposing all individuals

Table 17.1 Summary of *Bt* maize events (and stacks) to manage FAW, and respective *Bacillus thuringiensis* insecticidal proteins expressed, approved for cultivation in Brazil and Argentina

	Class of *Bt* proteins		
Event/stack	Cry1	Cry2	Vip3A
MON 810; *Bt*11	Cry1Ab		
TC1507	Cry1F		
MON 89034	Cry1A.105	Cry2Ab2	
MON 89034 × TC1507	Cry1A.105; Cry1F	Cry2Ab2	
MIR162			Vip3Aa20
*Bt*11 × MIR162	Cry1Ab		Vip3Aa20
MON 810 × TC1507 × MIR162	Cry1Ab; Cry1F		Vip3Aa20

Data from ISAAA – International Service for the Acquisition of Agri-Biotech Applications (http://www.isaaa.org/gmapprovaldatabase/)

in local insect pest populations to selection (Caprio et al. 2000). In order to proactively delay the onset of resistance to *Bt* crops, insect resistance management (IRM) programs have been designed and implemented worldwide. The characteristics of tropical regions considerably increase the overall risk for the development of resistance to all tactics used in insect management, including *Bt* proteins expressed by GM plants. For example, the warmer seasonal temperature patterns that allow growers to manage their crops within longer growing seasons (Hollingsworth 2011) also allow for multiple generations of the pest to be exposed to selection every crop season (Martinelli and Omoto 2005). In Brazil, for example, maize is cultivated in two consecutive growing seasons (first crop and second crop). FAW populations are able to build up considerably under such conditions, which favor multiple and overlapping generations of this pest (Omoto et al. 2016). These bioecological factors favor high levels of selection pressure against technologies used to control FAW, including insecticides (Carvalho et al. 2013) and *Bt* proteins expressed in GM plants (Farias et al. 2014a; Bernardi et al. 2015a; Omoto et al. 2016).

In general, the sustained use of *Bt* technologies requires the implementation of refuges coupled with the deployment of *Bt* plants with high killing power over the individuals within an insect pest population (e.g., high-dose plants and/or *Bt* pyramids) (see Sect. 17.2). Despite efforts to effectively delay the evolution of resistance to *Bt* crops, field-evolved resistance has been documented in *Busseola fusca* (Füller) resistant to Cry1Ab maize in South Africa (Van Rensburg 2007), in FAW resistant to Cry1F maize in Puerto Rico and in Brazil (Storer et al. 2010; Farias et al. 2014a), in *Pectinophora gossypiella* (Saunders) resistant to Cry1Ac cotton in India (Dhurua and Gujar 2011), and in *Diabrotica virgifera virgifera* LeConte resistant to Cry3Bb1 maize in the United States (Gassmann et al. 2011). Among the cases of field-evolved insect resistance to *Bt* crops/proteins documented thus far, three common factors are prominent and apparently are the main causes for resistance: (1) deployment of non-high-dose single-mode-of-action *Bt* plants, (2) poor implementation of refuges, and (3) high insect pressure in tropical/subtropical environments. In this chapter, we will discuss the theory behind the management of insect resistance to *Bt* crops and lay out cases of field-evolved resistance to *Bt* crops in FAW documented in the

Western Hemisphere. We will also provide a critical analysis for the likely causes of resistance and indicate the main imperatives regarding product design and usage of *Bt* crops to achieve effective IRM.

17.1 Resistance Management

The definition of insect resistance to *Bt* proteins and crops has been a subject of ongoing debate. The National Research Council (1986) defined insecticide resistance as "genetically heritable changes in a population causing a reduction in susceptibility to a specific insecticide" without addressing on the impact of resistance on product performance at the field level. Tabashnik and colleagues (2008, 2009) and Tabashnik (1994) adapted the definition from NRC to resistance to *Bt* proteins and *Bt* crops and defined field-evolved (or field-selected) resistance as a genetically based decrease in susceptibility of a population to a toxin (e.g., *Bt* protein) caused by exposure of the population to the toxin in the field. However, this definition of field-evolved resistance does not imply loss of economic efficacy of a *Bt* protein and/or *Bt* crop in the field (Tabashnik et al. 2009) because it does not take into consideration factors such as the impact of the magnitude of resistance (temporally or spatially) or the extent of changes in product efficacy. Rather, it is frequently contingent on the reading of laboratory results, without taking into consideration pest ecology and how the interaction between the pest and the *Bt* product influences selection pressure; thus, field-evolved resistance may or may not result in changes in susceptibility on *Bt* crops (Sumerford et al. 2013; Tabashnik et al. 2003). Moreover, according to Sumerford et al. (2013), in order to confirm insect resistance to a *Bt* protein or a *Bt* crop in the field, it is essential to prove (1) the heritable basis for the shift in susceptibility and (2) the ability of individuals possessing the alleles causing the shift in susceptibility to complete their development on plants expressing the *Bt* protein(s), enabling them to inter-mate and transmit resistance alleles to their offspring.

17.2 Management of Insect Resistance to *Bt* Crops

IRM programs for *Bt* crops are designed and implemented to slow the rate at which insect resistance evolves and thus to maximize the effective life of a *Bt* crop, but not to prevent resistance (Head and Greenplate 2012). The rate at which insect resistance may evolve to *Bt* crops is affected by several factors, including the use of a product that results in the mortality of all or nearly all of the heterozygous insects (causing resistance to be functionally recessive), the frequency of the resistance alleles in the insect pest population, and the availability of refuge areas formed by non-*Bt* plants, which provide susceptible insects to contribute to dilution of the

resistance alleles through random mating with fully resistant (homozygous) insects (Gould 1998; Roush 1994; Tabashnik et al. 2013). Furthermore, the rate of resistance evolution is significantly influenced by the number of *Bt* proteins (and independent modes of action) expressed in a *Bt* crop and by the level and consistency of expression of each *Bt* protein (Head and Greenplate 2012). For instance, based on mathematical modeling, resistance should evolve more slowly to *Bt* crops with multiple *Bt* proteins than to those with a single *Bt* protein (Roush 1998). Additionally, the ideal expression pattern of each *Bt* protein in a *Bt* crop is season long, at a level high enough to control target insects that are heterozygous for any resistance genes (EPA 1998).

The IRM programs implemented for the first generation of *Bt* crops in the United States (single-mode-of-action products) were based on the "high-dose + refuge" approach. The expectation was that a *Bt* plant that met the "high-dose" standard would be able to control 99.99% of the target insect pest population. The US EPA recommended five different methods, each with its individual practical difficulties, for determining if a *Bt* plant meets the high-dose criterion (Caprio et al. 2000; EPA 1998). Several authors have demonstrated that the value of refuges to resistance management is greater when the high-dose criterion is achieved (Tabashnik et al. 2013). However, several factors can diminish the expected value of the refuge approach for IRM. For example, not all *Bt* plants are capable of fully controlling all of the target species, given the naturally inherited variation in susceptibility to *Bt* proteins found in certain insect species. In that case, resistance is less likely to be functionally recessive. Three other elements that may cause a deviation from the ideal high-dose scenario are the presence of resistance alleles at higher-than-expected frequency, refuges that are not as productive as expected regarding the number of susceptible adults emerging off the non-*Bt* plants, and poor overall refuge compliance or incorrect management of refuge areas (Storer et al. 2012b).

The design of *Bt* plants that expressed two or more distinct *Bt* proteins that control (kill) the same insect pest species (*Bt* pyramids) was proposed to mitigate some of the risk related to deploying single-mode-of-action *Bt* plants (Carrière et al. 2015; Head and Greenplate 2012; Roush 1994; Storer et al. 2012b). In addition, *Bt* pyramids can expand the spectrum of insect control relative to the use of single-toxin *Bt* crops and can therefore be more efficient at controlling target insect species and reducing crop damage. For these reasons, *Bt* pyramids have become increasingly important for IPM and IRM (Carrière et al. 2015). There are three key conditions for *Bt* pyramids to be an effective IRM tactic: (1) high mortality of homozygous susceptible insects is achieved by each component of the pyramid (stack) alone, such that there is "redundant killing"; (2) the probability that cross-resistance between the components is low or absent; and (3) the pattern and expression level of each *Bt* protein in the pyramid (stack) is similar (Storer et al. 2012b). Roush (1994) found that if two insecticidal traits (e.g., *Bt* proteins) each provide >95% control of susceptible insects and there is, for instance, a 10% refuge, the number of generations until resistance occurs can increase 25- to >100-fold relative to a single-trait plant. According to Roush (1998), with the use of *Bt* pyramids, most resistance alleles are

eliminated from an insect population when *Bt* proteins control (kill) individuals that are homozygous for susceptibility to one *Bt* protein, even when resistance to the other toxin is not recessive. Thus, the ability of each *Bt* protein in a pyramid to control susceptible insects is more central to IRM than is recessive inheritance of resistance (Roush 1998). As with single-mode-of-action plants, however, deviations from ideal conditions may reduce the IRM value of a *Bt* pyramid. For example, survival of susceptible insects in a *Bt* pyramid that is greater than expected, cross-resistance between the *Bt* proteins in pyramids, and poor compliance with refuge recommendations all have the potential to compromise the effectiveness of a *Bt* pyramid at delaying the onset of insect resistance (Carrière et al. 2015, 2016).

17.3 Documented Cases of Resistance to *Bt* Proteins in FAW

17.3.1 Puerto Rico

The first case of documented field-evolved resistance to *Bt* proteins in FAW came from Puerto Rico (Storer et al. 2010, 2012b). The Cry1F resistance in the FAW population from Puerto Rico was shown to be autosomal and highly recessive, with limited cross-resistance to Cry1Ab and Cry1Ac (Storer et al. 2010). The combination of several factors played a very important role leading to the evolution of resistance to Cry1F protein, expressed in TC1507 maize, in Puerto Rico (Storer et al. 2010, 2012a). The fact that Puerto Rico is an island limits insect migration from external environments and, as a consequence, likely constrains the genetic variability. It also has a tropical climate conducive to year-round cultivation of maize, increasing the number of FAW generations potentially exposed to the selection pressure of TC1507. Drought conditions were experienced in 2006/2007, which reduced the availability of alternative hosts for FAW, limiting the contribution of those plants to the pool of susceptible insects available in the landscape (Storer et al. 2010). Upon an initial confirmation of field resistance in 2006 and as a part of the IRM program for TC1507, the company registrant of that technology stopped the commercial sale of the product (Herculex – TC1507) in Puerto Rico (Storer et al. 2012b). Resistance to Cry1F in FAW in Puerto Rico was found to be stable (Storer et al. 2012b) and was still present even after 4 years without commercial planting of Cry1F maize, with no significant fitness costs associated with resistance (Jakka et al. 2014; Velez et al. 2013). Notwithstanding a prior report of no detectable levels of Cry1F resistance in FAW impacting the US mainland (Storer et al. 2012b), field resistance of FAW to Cry1F maize was documented in the southeastern United States (Huang et al. 2014; Niu et al. 2013, 2014). Although the geographical range and distribution of Cry1F resistance in FAW in the mainland United States remains unclear, the cause for resistance in this case is likely to be migration of Cry1F-resistant FAW from Puerto Rico (Huang et al. 2014).

17.3.2 Brazil

Unexpected damage caused by FAW in TC1507 maize was initially observed in the state of Bahia, Brazil, 3 years after the product was deployed in that country (Farias et al. 2014a). A significant decrease in susceptibility to Cry1F was detected in FAW across Brazil over growing seasons from 2010 to 2013, especially in areas with intensive maize production, high adoption of *Bt* technologies, and poor compliance with refuge recommendations (Farias et al. 2014b). Farias et al. (2015) found that the Cry1F resistance allele in FAW from Bahia was not completely recessive on TC1507 maize when compared with a susceptible laboratory strain. Through leaf tissue dilution bioassays, Farias et al. (2015) concluded that TC1507 was not a high-dose product for FAW. Their results showed high survivorship of susceptible FAW larvae at a 25-fold dilution (i.e., 76.8%). Separately Cry1F-resistant heterozygous larvae exhibited 8.3% survival in a Cry1F leaf bioassay (for the same maize hybrid and growth stage). Similar results were found by Santos-Amaya et al. (2016). These results taken as whole indicate the high likelihood of a significant survivorship of FAW larvae carrying at least a single copy of the Cry1 resistance allele after exposure to TC1507, consequently fueling the evolutionary process that led to field-evolved resistance to Cry1F. No relevant fitness costs were observed in a near-isogenic Cry1F-resistant strain of FAW, indicating stability of resistance to Cry1F protein in the absence of selection pressure (Horikoshi et al. 2015).

Farias et al. (2016) reported that the Cry1F resistance allele was common in several states across Brazil early in 2012 and that the detected frequency of the allele in association with the geographically widespread of resistance suggested a higher-than-anticipated initial frequency of the resistance allele at the time that TC1507 maize was launched there. The estimated resistance allele frequencies varied from 0.009 to 0.277, with the collections from western Bahia (northeast Brazil) showing the highest Cry1F resistance frequencies. Additionally, the frequency of the Cry1F resistance allele increased more than threefold (3×) in the 3 months from the first to the second maize growing season in a particular county in the State of Paraná, Brazil. According to Farias et al. (2016), this result indicated a potential increase in the selection pressure on *Bt* maize associated with the consecutive planting of technologies containing insecticidal proteins with the same or similar modes of action.

17.3.3 Argentina

In Argentina, anecdotal observations of unexpected damage caused by FAW on Cry1F maize (TC1507) were reported in 2012 and 2013 (Trumper 2014). Moreover, Flores and Balbi (2014) documented unexpected survivorship of FAW larvae in laboratory and field trials in Argentina. As in Brazil, low levels of compliance with the structured refuge recommendation were the likely explanation for the shift in the Cry1F susceptibility of FAW populations in Argentina (Trumper 2014).

17.4 Genetics of Cry1F Resistance in FAW

Leite et al. (2016) subjected a field-collected FAW population to four generations (rounds) of laboratory selection, yielding a strain highly resistant to Cry1F. This resistance was demonstrated by the survival of insects reared on leaves of TC1507 maize plants and by the more than 300-fold resistance level measured in bioassays with purified Cry1F protein. Reciprocal crosses between control and Cry1F-selected strains revealed that the resistance was autosomal and incompletely recessive and the response obtained in a backcross of the F1 generation with the resistant strain was consistent with simple monogenic inheritance. Additionally, there were no apparent fitness costs associated with resistance either for survival or larval growth on non-*Bt* maize leaves. These findings provide experimental evidence for rapid evolution of Cry1F resistance in FAW in the laboratory and further reinforce the potential of this species to evolve field resistance to the TC1507 maize and to subsequently impact the efficacy of other Cry1 proteins to due to cross-resistance (see Sect. 17.6).

Santos-Amaya et al. (2016) continuously exposed FAW to the TC1507 event for 11 generations, which resulted in more than 183-fold resistance to Cry1F in the two strains studied. This high resistance level enabled the insects to complete larval development on the *Bt* maize plants. Genetic analyses using concentration-response bioassays with progenies from reciprocal crosses between resistant and susceptible insects indicated that the inheritance of the resistance was autosomal, partially recessive, and without maternal effects (i.e., sex linkage). Backcrosses of the F1 progeny with the resistant parental strains revealed that the resistance in the two selected strains was conferred by a single locus or set of tightly linked loci. These results support some of the assumptions of the strategy in use for management of FAW resistance to *Bt* Cry1F maize, but survival rates of heterozygotes on the *Bt* plants were higher than 5%, supporting the assertion that Cry1F maize (TC1507) does not produce a high dose of the insecticidal protein (i.e., resistance is partially recessive) for FAW.

17.5 Resistance to Cry2Ab2 Protein in FAW

Niu et al. (2016b) detected a major Cry2Ab2 resistance allele using a leaf tissue F_2 screen to screen field-collected population of FAW and estimate the allele frequency. FAW field populations were collected in the United States from Texas, Louisiana, Georgia, and Florida. A conservative estimate of the frequency of major Cry2Ab2 resistance alleles in FAW from the four states was 0.0023 with a 95% CI of 0.0003–0.0064. In addition, six FAW families were considered likely to possess minor resistance alleles at a frequency of 0.0082 with a 95% CI of 0.0033–0.0152. The F_2 screen identified at least 1 (GA-15) out of the 215 two-parent families of FAW from populations collected in the southern United States to possess a major resistance allele to the Cry2Ab2 protein. Larvae from the GA-15 family survived well on whole maize plants expressing Cry2Ab2 protein and demonstrated a significant level (>15-fold) of resistance when fed with the same protein incorporated in an

artificial diet. Prior to the study by Niu et al. (2016b), there had been no information available about the frequency of Cry2Ab2 resistance alleles in FAW. The detection of the major resistance allele coupled with the relatively more common "minor" resistance alleles in the field populations of FAW may have important implications for resistance management.

17.6 Studies of Cross-Resistance Among Cry Proteins

Despite early indications that cross-resistance among Cry1 proteins was limited, Hernández-Rodríguez et al. (2013) studied whether the chimeric Cry1A.105 protein had shared binding sites in FAW with Cry1A proteins, with Cry1Fa, or both. The authors concluded that Cry1A.105, Cry1Ab, Cry1Ac, and Cry1Fa competed with high affinity for the same binding sites in FAW, which might explain the cross-resistance among Cry1 proteins. Therefore, cross-resistance among Cry1 proteins may occur through the alteration of shared binding sites. On the other hand, Hernández-Rodríguez et al. (2013) and several other reports indicated that Cry2Ab2 had a different mode of action from that of Cry1F and Cry1A proteins. For that reason, cross-resistance between Cry2Ab2 and Cry1F or Cry1A proteins is not likely (Hernández-Rodríguez et al. 2013). Not surprisingly, low levels of cross-resistance between Cry1F and Cry1A.105 proteins were detected in a Cry1F-resistant FAW strain isolated through an F_2 screen from a field population sampled in south Florida (Huang et al. 2014).

Bernardi et al. (2015a) detected moderate levels of cross-resistance among Cry1 proteins in FAW. The authors tested a Cry1F-resistant FAW strain in bioassays using solubilized proteins (Cry1A.105 and Cry2Ab2) and in leaf tissue trials. The results confirmed moderate levels of cross-resistance among Cry1F, Cry1A.105, and Cry1Ab in FAW. Consistent with other reports, no significant levels of cross-resistance were found between Cry1F and Cry2Ab2, and MON 89034 maize (which expresses Cry1A.105 and Cry2Ab2) in combination with appropriate management practices continues to provide effective control of FAW in Brazil (Bernardi et al. 2015a).

MON 810 maize, which expresses the Cry1Ab protein, significantly contributed to IPM programs for FAW in Brazil (Waquil et al. 2013), but it is not considered a high-dose product for FAW primarily due to the moderate activity of Cry1Ab against this species. The deployment of MON 810 in an environment with widespread resistance to Cry1F documented cross-resistance among Cry1 proteins, and low compliance with structured refuge recommendations led to the documentation of field-evolved resistance to Cry1Ab in FAW in Brazil (Omoto et al. 2016). Results from laboratory (in vitro) and field (in planta) monitoring programs consistently indicated a shift in the susceptibility of FAW to the Cry1Ab protein. According to Omoto et al. (2016), the contribution of the selection pressure of the Cry1Ab protein expressed in MON 810 to the onset of resistance could not be distinguished from impacts from cross-resistance between Cry1Ab and Cry1F proteins, given that resistance to the latter was well documented in Brazil (Farias et al. 2014a, b, 2015, 2016) as was cross-resistance to Cry1Ab (Bernardi et al. 2015a).

Huang et al. (2016) estimated the frequency of Cry1A.105 resistance alleles in field populations of FAW, collected from three locations in the US states of Louisiana and Florida in 2011. A total of 18 FAW families, 4 from Louisiana, and 14 from Florida were identified to be potentially positive families carrying resistance alleles to Cry1A.105. Whole-plant tests confirmed that the four potentially positive families that were evaluated in this test possessed major resistance alleles to Cry1A.105 maize plants. Based on the similar performance of the 18 families in the leaf tissue F_2 screen, the 14 other potentially positive families that were not retested in the whole-plant assay, due to the unsuccessful establishment of progeny populations, most likely possessed major resistance alleles to the Cry1A.105 maize plants. Therefore, all 18 potentially positive families identified in the leaf tissue F_2 screen were also considered as possessing major resistance alleles when calculating resistance allele frequency. The corresponding frequency of alleles for resistance to Cry1A.105 maize was estimated to be 0.0158 with a 95% credibility interval (CI) of 0.0052–0.0323 for the Louisiana populations and 0.0559 with a 95% CI of 0.0319–0.0868 for the Florida populations. These resistance allele frequencies are greater than those estimated for other insect pests and *Bt* proteins. The authors were able to establish two resistant strains in the laboratory (FL32 and FL67), which survived on whole Cry1A.105 maize plants and demonstrated a significant level (>116-fold) of resistance to the Cry1A.105 protein in a diet-incorporated bioassay. These findings suggest that resistance in FAW to single-gene Cry1A.105 maize in the southeast United States is not rare, most likely due to the selection of Cry1F resistance and its cross-resistance to Cry1A.105.

Niu et al. (2016a) evaluated the survival and plant injury of the two Cry1A.105-resistant strains (FL32 and FL67) established by Huang et al. (2016), along with a susceptible population and two F1 heterozygous genotypes, on commercial and experimental *Bt* maize hybrids/lines containing single or pyramided *Bt* genes. These *Bt* maize hybrids/lines consisted of five single-gene *Bt* maize products containing Cry1A.105, Cry2Ab2, Cry1F, or Cry1Ab protein and three pyramided *Bt* maize products expressing Cry1A.105/Cry2Ab2, Cry1A.105/Cry2Ab2/Cry1F, or Cry1Ab/Vip3A. Resistance in FL32 and FL67 on leaf tissues of Cry1A.105 maize was recessive to incompletely recessive, while on whole Cry1A.105 plants, it was moderate to incompletely dominant. This variation in dominance level observed on different test plant materials suggests that careful experimental designs are needed for evaluating the "high-dose" qualification of *Bt* maize against FAW. Their results showed that both Cry1A.105-resistant populations of FAW were highly cross-resistant to Cry1F maize. The cross-resistance was incompletely recessive for FL32 but dominant for FL67 in the leaf tissue bioassay, while it was incompletely dominant in the whole-plant tests for both populations. The non-recessive resistance could be one of the factors that led to the rapid development of resistance to Cry1F maize in some field populations of FAW. The Cry1A.105-resistant strains of FAW were not cross-resistant to Cry2Ab2 or Vip3A.

Yang et al. (2016) investigated whether a Cry1F-resistant FAW strain selected on *Bt* maize was also resistant to *Bt* cotton containing similar or different *Bt* genes. The Cry1F gene in the cotton plants (event DAS-24236-5) is a synthetic, plant-optimized,

full length version of the Cry1F gene, whereas in maize (TC1507), it is a plant-optimized version of a truncated Cry1F gene. The authors evaluated the survival, growth, development, and plant injury of the Cry1F maize-susceptible (SS), heterozygous (RS), and resistant (RR) genotypes of FAW on one non-*Bt*, three single *Bt*, and five pyramided *Bt* cotton products. The results showed that Cry1F-maize-resistant FAW was also highly resistant to *Bt* cotton expressing the Cry1Ac and Cry1F proteins. This outcome suggested that the dissimilar forms of the Cry1F gene inserted in the maize and cotton plants apparently did not change the mode of action/binding of the Cry1F protein. These results also demonstrated that the highly resistant FAW selected with *Bt* maize are susceptible to pyramided *Bt* cotton expressing Cry2A and/or Vip3A. Horikoshi et al. (2016) observed similar results, demonstrating that resistance in FAW to Cry1 proteins expressed in *Bt* maize plants can compromise *Bt* cotton technologies expressing similar *Bt* proteins.

17.7 Implications of Cross-Resistance for FAW Resistance Management

The use of *Bt* maize plants with less-than-ideal IRM fit (e.g., less-than-high-dose technologies, components of *Bt* pyramids with cross-resistance to other *Bt* proteins in landscape) combined with low compliance with the structured refuge recommendation seems to be a common theme across the resistance cases and issues documented in FAW across southern South America. A consequence of these findings is a direct reduction in the number of effective modes of action to manage FAW infestations and resistance to *Bt* maize insecticidal proteins. For example, the cross-resistance between Cry1F and Cry1A.105 could significantly reduce the activity of the Cry1A.105 protein in MON 89034 maize, leaving the Cry2Ab2 protein in MON 89034 only partially protected against Cry1F-resistant FAW and stressing the importance of effective implementation of refuges (see Sect. 17.6).

The deployment of MIR162 (Vip3Aa20) maize represents an effective new and unique mode of action added to the maize cropping system. MIR162 achieved the high-dose requirements for FAW (Bernardi et al. 2015b), and the frequency of Vip3Aa20 resistance in Brazilian FAW populations sampled across the country was low (Bernardi et al. 2015c). Pyramided *Bt* maize and *Bt* cotton containing Cry2A and/or Vip3A genes should still provide a means for managing the Cry1F resistance in FAW (Horikoshi et al. 2016). However, there is a high risk of resistance to the current pyramided *Bt* crops evolving in areas where resistance to Cry1F maize is widespread (Horikoshi et al. 2016; Santos-Amaya et al. 2015). Recent studies characterized field-relevant Vip3Aa20 resistance in strains isolated through F_2 screens and showed that the inheritance of Vip3Aa20 resistance was autosomal, recessive, and monogenic (Bernardi et al. 2016). Despite the IRM value of adding Vip3Aa20 to *Bt* pyramids, due to the evolution of resistance to Cry1 proteins and limited activity of Cry2Ab2 against FAW, it is reasonable to assume that the IRM value of *Bt* pyramids combining current available insecticidal proteins is diminished in Brazil and in other areas across South America.

17.8 Conclusions

The current situation highlights the importance of enhancing compliance with the non-*Bt* maize refuge recommendations along with the adoption of best management practices (BMPs) to ensure longer durability of these technologies in tropical and subtropical regions of South America. The low compliance with the refuge recommendations for *Bt* maize technologies in Brazil is connected to an array of factors that highlight the need to effectively engage key stakeholders across the seed supply chain. For instance, the majority of growers in Brazil gain access to *Bt* and non-*Bt* maize seeds and to technical information on the use of these technologies through outside resources such as seed distributors and coops. Therefore, achieving reasonable refuge compliance at the field level requires the proper engagement of key stakeholders through the active implementation of multilayer, multi-stakeholder IRM strategies. Overall, IRM plans should be designed to ensure (1) reasonable refuge seed supply in the marketplace, (2) the existence of marketing programs to incentivize refuge seed sales, and (3) a plan for surveying refuge compliance at the field level. Developing and implementing multilayer, multi-stakeholder programs to ensure reasonable refuge compliance is critical to supporting the use of current *Bt* maize technologies. It is also imperative for the sustainable use of the next generation of GM technologies carrying insect control traits. The design of the next generation of GM technologies for insect control should combine multiple novel insecticidal traits with no cross-resistance to the current Cry1 proteins and with high activity against the same target pests. GM maize products with superior IRM fit in tropical regions, in combination with effective management of these technologies at the field level, will be the foundation for sustainable use of these technologies.

In the light of the global challenges to the implementation of IRM plans, particularly in South America, Excellence Through Stewardship (ETS; http://excellencethroughstewardship.org/), a global industry-coordinated organization that promotes the adoption of stewardship programs for agricultural biotechnology, developed a Guide for Resistance Management for Biotechnology-Derived Plant Products. The goal of the ETS/IRM Guide is to provide guidance to company members for developing and implementing processes around resistance management of *Bt* crops. In addition, the Brazilian Seed Association (ABRASEM) has developed an online system to help growers select appropriate non-*Bt* maize hybrids to be planted as refuge in different regions of Brazil (http://www.abrasem.com.br/sistema-de-selecao-de-cultivares-para-refugio/). The sustained use of *Bt* crops depends upon initiatives like these to ensure all key stakeholders understand their roles in IRM and are committed to the success of IRM strategies.

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