Chapter 13
Bacillus thuringiensis

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13.1 Introduction

Bacillus thuringiensis (*Bt*) is an aerobic Gram-positive bacterium from the Bacillaceae family that produces protein crystalline inclusions named 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, spiderwebs, and stored grain (Federici 1999; Glare and O'Callaghan 2000; Valicente and Barreto 2003). Crystal proteins are composed of one or more proteins, Cry or Cyt (cytolytic protein), and they are named delta (δ) endotoxins. These are the factors that determine Bt pathogenicity (Schnepf et al. 1998) and may show different forms as shown in Fig. 13.1 (Valicente and Souza 2004). 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 not forming any crystals, which were identified by Estruch et al. 1996. Some other important proteins are also produced, such as Cyt, β-exotoxins, and Sip proteins. However, the most studied are the *cry* genes/Cry proteins. The identification of a *Bt* strain to subspecies is done using the flagellar antigen H, e.g., *Bacillus thuringiensis* sv *kurstaki*. However, 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*, *cry2A*, and *cry2B*, and strain HD-73 *(Bt sv kurstaki)* harbors only *cry1Ac* gene. These toxic proteins from Bt are used in pest control as a biological pesticide or as transgenic plant expressing these proteins. This is a very useful and powerful tool in integrated pest management (IPM). In this work, we describe the genetic variability

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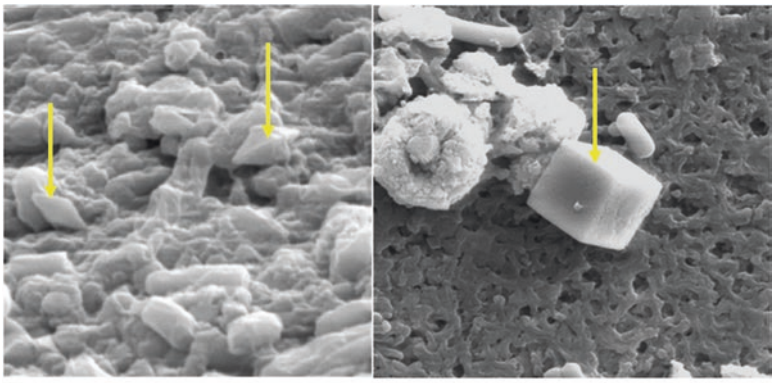

Fig. 13.1 Crystal of *Bacillus thuringiensis* showing a bipyramidal shape (20.000 ×) (**a**) and cuboid shape (30.000 ×) (**b**). (Valicente and Souza 2004)

and molecular characterization of *Bacillus thuringiensis*, and the importance of finding the appropriate strain for a specific insect pest. We also provide the description of the Cry proteins and the nomenclature of Cry proteins, as well as their importance for insect specificity, their mode of action, and how *Bacillus thuringiensis* is used as biological pesticides.

13.2 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 different isolates with different purposes. The most common techniques used are polymerase chain reaction (PCR) (Cerón et al. 1994, 1995; Bravo et al. 1998; Shangkuan et al. 2001; Lima et al. 2002; Valicente et al. 2010), repetitive element polymorphism (REP-PCR) (Higgins et al. 1982; Stern et al. 1984; Sharples and Lloyd 1990; Versalovic et al. 1991, 1994; Rademaker and de Bruijin 1997; Louws et al. 1999; Shuhaimi et al. 2001; da Silva and Valicente 2013), amplified fragment length polymorphism (AFLP) (Vos et al. 1995; Arnold et al. 1999; Ridout and Donini 1999; Mueller and Wolfenbarger 1999; Grady et al. 2001; Ticknor et al. 2001; Burke et al. 2004; Hill et al. 2004; Abreu et al. 2007; Valicente and da Silva 2014), and plasmid pattern characterization (Gilson et al. 1984; Birge 1994; Berry et al. 2002; Gitahy et al. 2005; Loeza-Lara et al. 2005; Roh et al. 2007; Ramírez and Ibarra 2008; Fagundes et al. 2011). *Bt* proteins can be characterized according to their size in SDS PAGE.

13.3 Cry Proteins

Cry proteins are the most studied toxins and they are toxic to insects from the orders Lepidoptera, Coleoptera, Hemiptera, Neuroptera, Orthoptera, Siphonaptera, Thysanoptera, and Blattodea (Isoptera) (Glare and O'Callaghan 2000) and also to nematodes. *Bacillus thuringiens*is may be used as biological pesticides in transgenic plants (*Bt* plants). In 1998, Crickmore et al. proposed a new nomenclature to 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/. Most of *cry* genes are present in the plasmids and not in the chromosomes. *Bt* strains may be used to produce biological pesticides in fermentation systems, and *Bt* genes can be used in plant transformation against insect pests.

13.4 Nomenclature of Cry Proteins and Their Importance Towards Insect Specificity

Crystal proteins receive a classification based on four hierarchical categorizations consisting of numbers, uppercase letters, and lowercase letters (e.g., Cry1Ab1). The classification is given depending on the sequence homology that is shared with other Cry proteins. A different number (first category) is given to a protein if it has less than 45% identity or homology with all other Cry proteins (e.g., Cry1, Cry2, Cry3 (...) Cry65, Cry74) (Crickmore et al. 2016). The second category is the capital letter, which is given if the protein has less than 78% but more than 45% homology with a specific group of Cry proteins (e.g., Cry1A, Cry1B, Cry1C). The third category, which is a lowercase letter, is given to distinguish proteins sharing more than 78% and less than 95% identity with the other Cry proteins (e.g., Cry1Aa, Cry1Ab, Cry1Ac). The number added at the end is to distinguish proteins that share more than 95% identity but are not identical and should be considered variants of the same protein (Cry1Ab1, Cry1Ab2, Cry1Ab3, etc.) (Crickmore et al. 1998, 2016). This classification is also used for Vip proteins.

Cry proteins show specific toxicity to certain insect orders. Cry1 proteins are active against Lepidoptera, Cry2 against Lepidoptera and Diptera, Cry3 against Coleoptera, etc. With the evolution of molecular techniques, PCR (polymerase chain reaction) can differentiate the *Bt* genes that are present in a certain strain or *Bt* isolate. Cry2Aa protein is toxic to lepidopterans and dipterans, and the Cry2Ab2 and Cry2Ac3 proteins are toxic only against lepidopterans. Another example is the Cry5Ac1 protein that acts against Hymenoptera (ants) and the Cry5Ba3 and Cry5Ca1 proteins have a toxic action against nematodes. There is no *Bt* protein that has insecticidal action against bees, although bees are insects of the order Hymenoptera (Dr. Neil Crickmore – personal information, 2016). This factor is important in determining the toxicity of a particular pest, insect, or group of insects.

Cry proteins that have three domains (domains I, II, and III) are called 3d-Cry. Cry toxins belonging to the 3-domain Cry toxin family show clear differences in their amino acid sequences, but all have in common a conserved 3-domain structure (de Maagd et al. 2001, 2003; Bravo et al. 2007; Pardo-López et al. 2013). Toxins are considered viable alternatives for the control of insect pests in agriculture and vectors of importance in public health (Crickmore et al. 2016). These proteins are highly specific to target insects, killing a limited number of species. The toxins are innocuous to humans, vertebrates, and plants and are completely biodegradable (Pardo-López et al. 2013). Bt 3d-Cry toxins show toxic activity against insects of orders Lepidoptera, Diptera, Coleoptera, and Hemiptera (low to moderate toxicity in aphids) and nematodes (Van Frankenhuyzen 2009, 2013). In general, domain I is the domain of the perforation and is subject to proteolytic cleavage, domain II or central domain is involved in toxin-receptor interactions, and domain III is involved in the junction of galactose, receptor junction, and pore formation (Bravo et al. 2007; de Maagd et al. 2001, 2003; Pardo-López et al. 2013).

13.5 Mode of Action of Cry Proteins

The mode of action of *Bt* toxins described in the literature refers to Cry1 proteins, which have a toxic action against Lepidoptera. The mode of action of *Bt* toxins has been well studied by some research groups; this way one can better understand the steps for the death of the insect as well as the resistance of this insect to a certain protein. The mode of action of 3d-Cry toxins against lepidopterans can be understood at the molecular level as follows: the interaction of Cry1 toxins with different proteins present in the midgut of Lepidoptera in a complex process involving several proteins such as cadherin, aminopeptidase N, and alkaline phosphatase (Pigott and Ellar 2007; Soberón et al. 2009). According to Pardo-López et al. 2013, the main steps of this mode of action are as follows:

1. Caterpillars ingest 3d-Cry protoxins, which are solubilized in the midgut due to the high pH and reducing conditions and, activated by proteases of the intestine, generating the toxic fragment.
2. The 3d-Cry monomeric toxin binds to alkaline phosphatase and aminopeptidase receptors, and with a low interaction and affinity, the toxin is then located near the membrane.
3. The 3d-Cry monomeric toxins bind to the cadherin receptor in a high affinity binding, and this interaction induces a proteolytic cleavage at the N-terminus at the end of the toxin, including the α-helix domain.
4. The cleaved 3d-Cry protein is then capable of oligomerizing in a pre-pore oligomer toxin.
5. The oligomeric 3d-Cry structure binds to the alkaline phosphatase and aminopeptidase receptors with high affinity.
6. The pre-pore inserts into the membrane causing the pore to form.

The mode of action of the Cry proteins mentioned above is always related to Cry1 proteins – which have activity against Lepidoptera. These steps should not be used as a mode of action for insects from other insect orders. There are orders of insects that do not have intestinal receptors and not all insects have a medium intestine with alkaline pH.

These *Bt* genes may be used in plant transformation, generating transgenic crops protected against specific insect pests.

13.6 *Bacillus thuringiensis* Used as Biological Pesticides

Bacillus thuringiensis needs sources of nitrogen, carbon, mineral salts, and oxygen to grow. Various agricultural and industrial by-products, such as maize glucose, soybean flour soy extract, peanuts, sugarcane molasses, and liquid swine manure, are carbon- and nitrogen-rich and may be used as sterilized raw materials in biopesticide production. Tirado-Montiel et al. (2001) first tested the use of wastewater sludge for biopesticide production, although the entomotoxicity level reported was low. Many authors reported the use of by-products and raw materials to develop *Bt*-based biopesticides (Salama et al. 1983; Obeta and Okafor 1984; Morris et al. 1997). Moreover, the low cost of by-products as nutrient source in fermentation media for *Bt* biopesticide production has received little attention. However, a higher level of entomotoxicity is desired to reduce the production costs of biopesticides. Valicente and Mourão (2008) also reported that a carbon/nitrogen proportion and mineral salts (all expressed in 0.1–0.002 gl^{-1} of $FeSO_4$, 0.02 gl^{-1} of $ZnSO_4$, 0.02 gl^{-1} of $MnSO_4$, 0.3 gl^{-1} of $MgSO_4$) are essential to promote adequate *Bt* growth.

Bacillus thuringiensis production in Cuba is done in 185 "Centros de Reproducción de Entomófagos y Entomopatógenos (Cree)" distributed all over the country (Pérez and Vásquez 2001), and the *Bt* produced is stored for 3 months in temperatures up to 25 °C. According to Aranda et al. (2000), *Bt* is the most studied entomopathogen in Cinvestav (Centro de Investigaciones y de Estudios Avanzados de IPN), in México, and UNAM (Universidad Nacional Autónoma de México). Also according to Aranda et al. (2000), Peru produces *Bt* with activity against *Anopheles albimanus* (Wiedemann) (Culicidae).

Bravo and Ceron (2004) reported the production of *Bt*-based biopesticide in different formulations (wettable powder, granules, etc.) to control mosquitos.

Arcas et al. (1984, 1987) studied the first aspects of *Bt* fermentation in Argentina at the University of La Plata. In the 1990s, Dr. Graciela Benintende and Jorge Cozzi developed technology to produce a *Bt*-based biopesticide against lepidopteran pest.

In Brazil, one *Bt*-based biopesticide was registered for fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Noctuidae). The biopesticide "Crystal®" (Fig. 13.2) produced by Farroupilha/Lallemand is the result of an agreement with Embrapa Maize and Sorghum Research Center.

Fig. 13.2 *Bacillus thuringiensis*-based biopesticide "Crystal®" produced by Farroupilha/Lallemand in Agreement with Embrapa Maize and Sorghum. (Photo: Fernando H. Valicente)

13.7 Final Considerations

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium that produces protein crystalline inclusions named Cry proteins during the stationary phase encoded by different *cry* genes. *Bacillus thuringiensis* can be found in different substrates such as soil, water, plant surfaces, dead insects, grain dust, spiderwebs, and stored grain. Crystal proteins are composed of one or more proteins, Cry or Cyt (cytolytic protein), and they are named delta (δ) endotoxins. These are the factors determining Bt pathogenicity to insects and nematodes. Many *Bt* strains also produce other types of insecticidal proteins, such as the Vip proteins (vegetative insecticidal proteins), and do not form any crystals. Some other important proteins are also produced, such as β-exotoxins and Sip proteins. These toxic proteins from Bt are used in pest control as a biological pesticide or as transgenic plant expressing these proteins. This is a very useful tool in integrated pest management (IPM).

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