Journal of Industrial Microbiology & Biotechnology (1997) 19, 202–219

Bacillus thuringiensis: from biodiversity to biotechnology

DL Prieto-Samsónov, RI Vázquez-Padrón, C Ayra-Pardo, J González-Cabrera and GA de la Riva

Laboratory of Environmental Biotechnology, Plant Division, Centre for Genetic Engineering and Biotechnology (CIGB), PO Box 6162, 10600 Havana, Cuba

Bacillus thuringiensis **is a Gram-positive bacterium, widely used in agriculture as a biological pesticide. The biocidal activity mainly resides in a parasporal protein inclusion body, or crystal. The inclusion is composed of one or more types of δ-endotoxins (Cry and Cyt proteins). Cry proteins are selectively toxic to different species from several invertebrate phyla: arthropods (mainly insects), nematodes, flatworms and protozoa. The mode of action of the insecticidal proteins is still a matter of investigation; generally, the active toxin is supposed to bind specific membrane receptors on the insect midgut brush-border epithelium, leading to intestinal cell lysis and subsequent insect death by starvation or septicemia. The toxin-encoding *cry* genes have been extensively studied and expressed in a large number of prokaryotic and eukaryotic organisms. The expression of such genes in transgenic plants has provided a powerful alternative for crop protection.**

Keywords: delta-endotoxin; insecticidal crystal protein; ion-channel; heterologous expression; transgenic plants; pesticide

Introduction

The spore-forming soil bacterium *Bacillus thuringiensis* (*Bt*.) is widely used in agriculture as a biological pesticide; this microorganism is characterized by the production of crystal-shaped parasporal inclusion bodies (Figure 1), composed of one or more types of insecticidal crystal proteins (ICP), or δ-endotoxins: 'crystal' (Cry) and 'cytolytic' (Cyt) proteins [90]. The biocidal activity mainly resides in these proteins; they are selective for several groups of invertebrates (lepidopteran, dipteran and coleopteran insect species; arachnids; nematodes; flatworms; protozoa) [62] and their activity seems to be related to specific receptor-binding by the toxin (at least in the insect larvae, in the case of Cry proteins). Each crystal protein has its own toxicity spectrum. Since Cry proteins combine high potency for target organisms with safety for other animals, they are a valuable tool for pest management [25]. The most studied *Bt*. strains are toxic to insects [62]. Formulations of *Bt*. have been used for more than three decades as biological agents for controlling agricultural pests and insect vectors of human and animal diseases.

Cloning and heterologous expression of *cry* genes in *Escherichia coli* [156] and *Bacillus subtilis* [161] have permitted the study of individual δ-endotoxins. The transfer of *cry* genes to plants has provided a powerful tool for crop protection against insect pests, by generating resistant plant varieties. Different plant species such as tomato, tobacco, potato, cotton, maize and sugarcane have been genetically engineered for the production of insect-resistant cultivars [11,52,107,139,140,178].

In this review we present updated information on the study of *Bacillus thuringiensis*, emphasizing the bacterial biodiversity, structure and action mechanism of ICPs, and heterologous expression of their coding genes in genetically-modified organisms including transgenic plants.

Correspondence: Dr GA de la Riva, Laboratory of Environmental Biotechnology, Plant Division, Centre for Genetic Engineering and Biotechnology (CIGB), PO Box 6162, 10600 Havana, Cuba. E-mail: Gustavo.Riva@cigb.edu.cu
Received 25 February 1997; accepted 15 August 1997

Classification of *Bacillus thuringiensis*

Bacillus thuringiensis (Bt.) is a Gram-positive bacterium belonging to the *Bacillaceae* family; it is closely related to *Bacillus cereus*. The only notable phenotypic difference between them is the production of an insecticidal crystal. However, there are many crystal-producing strains without known insecticidal activities. Additionally, many acrystalliferous strains have been described; they can easily be obtained by chemical mutagenesis or by plasmid-curing, resulting in cells phenotypically indistinguishable from *B. cereus*. In 1980, O'Donnell *et al* proposed a method based on pyrolysis gas-liquid chromatography to discriminate between these two bacterial species [129]. However, more recent studies of a series of molecular markers in a large set of strains have indicated that *B. thuringiensis* and *B. cereus* should be considered as only one species [33].

The *B. thuringiensis* strains are usually classified into serotypes according to their H flagellar antigenic determinants [45,46]; to date, up to 55 different serotypes have been defined and ranked as subspecies (M Lecadet, Institut Pasteur, France, personal communication). Serotype is not directly correlated with specific toxicity towards a given insect. In addition to the H-antigen classification system, Ohba *et al* [133] reported a serotyping methodology based on extracellular heat-stable somatic antigens (HSSA), which seem to be more genetically stable.

Bacillus thuringiensis crystal protein(s). Diversity

Most δ-endotoxins are encoded by *cry* genes. More than 50 *cry* genes have been cloned, sequenced and extensively studied. The toxins were originally classified by Höfte and Whiteley [89] into four classes, according to their amino acid sequence homology and insecticidal specificity: Cry I

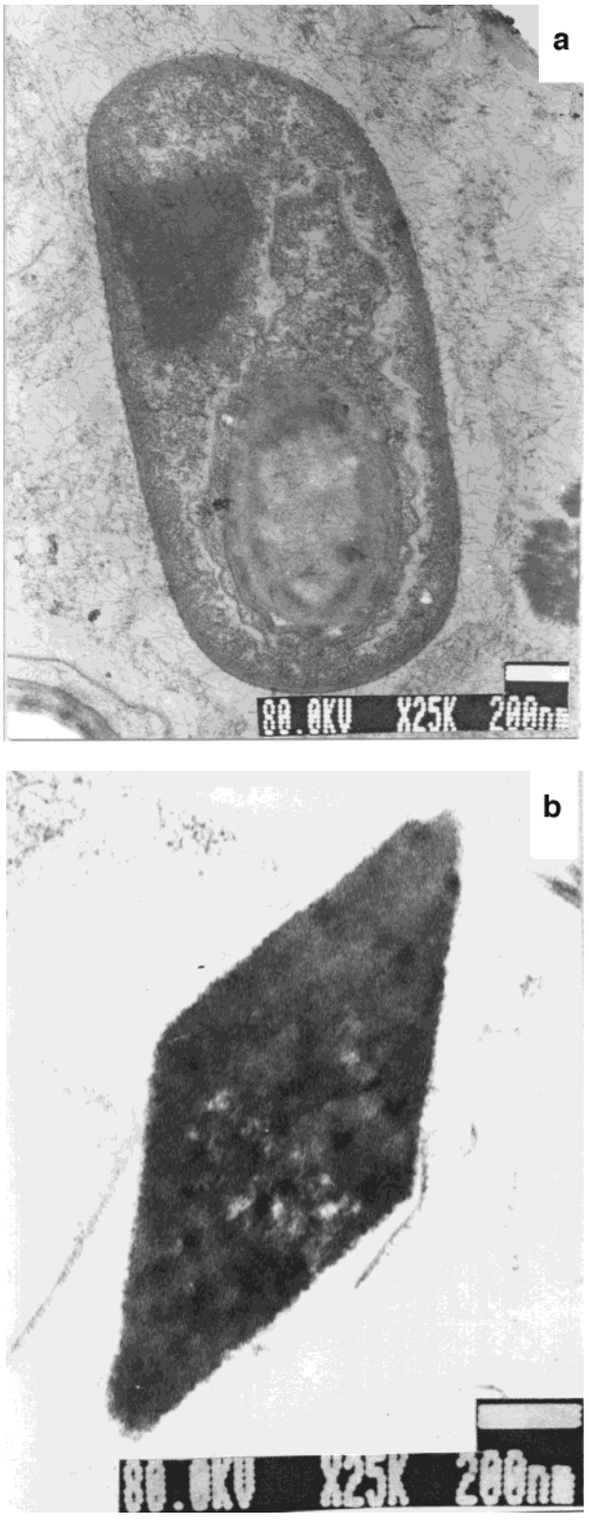

Figure 1 Transmission electron microscope view of sporulating *Bacillus thuringiensis* var *kurstaki* HD-1 (a) and bipyramidal crystal parasporal body characteristic for this strain (b).

(Lepidopteran-specific), Cry II (Lepidopteran and Dipteran-specific), Cry III (Coleopteran-specific) and Cry IV (Dipteran-specific). Two additional classes—Cry V and Cry VI—were added by Feitelson *et al* [62] for the nematode-active toxins. Recently, Crickmore *et al* established a new homology-based classification system; Cry proteins are now distributed into 19 different classes [42] (see the *Bt*. WWW page at http: //www.susx.ac.uk and users/bafn6/Bt/index.html). The authors of this new classification system argue that several ICPs are toxic to more than one order of insects; thus, the similitude in amino acid sequences of all known Cry proteins was taken as the only criterion for distributing them into classes and subclasses. Some classes, such as Cry6 and Cry15, do not show any significant homology with the rest of the Cry proteins [42] (Figure 2).

The cytolytic and hemolytic non-specific toxic proteins (Cyt proteins) produced by several *Bt*. strains (mainly isolates of the serovar *Bt*. subsp *israelensis*) have been classified traditionally as δ-endotoxins. However, their amino acid sequences are not related to those of the Cry proteins; thus they comprise an independent family. The studies on the Cyt protein structure and mode of action have shown substantial differences among the *cry* δ-endotoxins. For these reasons, we will analyze them separately. Interestingly, *cry*-like genes were found recently in the anaerobic bacterium *Clostridium bifermentans*. The gene product showed a remarkable mosquitocidal activity, and the names Cry16A and Cry17A were proposed for these first 'non-*Bt*.' Cry proteins [16]. This is also the first reported case of a secreted or excreted mosquitocidal toxin derived from an anaerobic bacterium.

B. thuringiensis crystal proteins. Structure and mechanism of action

Cry toxins resemble, structurally and functionally, the colicin-like and diphtheria bacterial toxins, and thus can be considered as members of an ion-channel protein family [136]. All of these proteins have high specificity for the target organisms and complex interdomain relationships. To date, these properties make the Cry family one of the most studied toxin groups with both industrial and academic interest. ICPs have been characterized to a current total number of 59 primary sequences and two deduced three-dimensional structures at the atomic level.

Cry proteins are produced as protoxins forming parasporal bodies; to become active, they must be activated by drastic proteolytic processing in the larval midgut. The active fragment is located in the N-terminal region of the ICP polypeptide [89]. Alignments of the sequences of active toxins with different specificities revealed five conserved stretches limited by hypervariable sequences, which could be present in the Cry proteins belonging to a given group, depending on the subclasses. For example, the ICPs Cry 1,3,4,7,8 and 9 have five intra-subclass conserved blocks while Cry 5,12,13,14 proteins contain only four of those blocks. The other subclasses contain only one or two conserved blocks [76,89,112].

A major advance in understanding the δ-endotoxin function was achieved in 1991 by Li and Ellar [112]. From

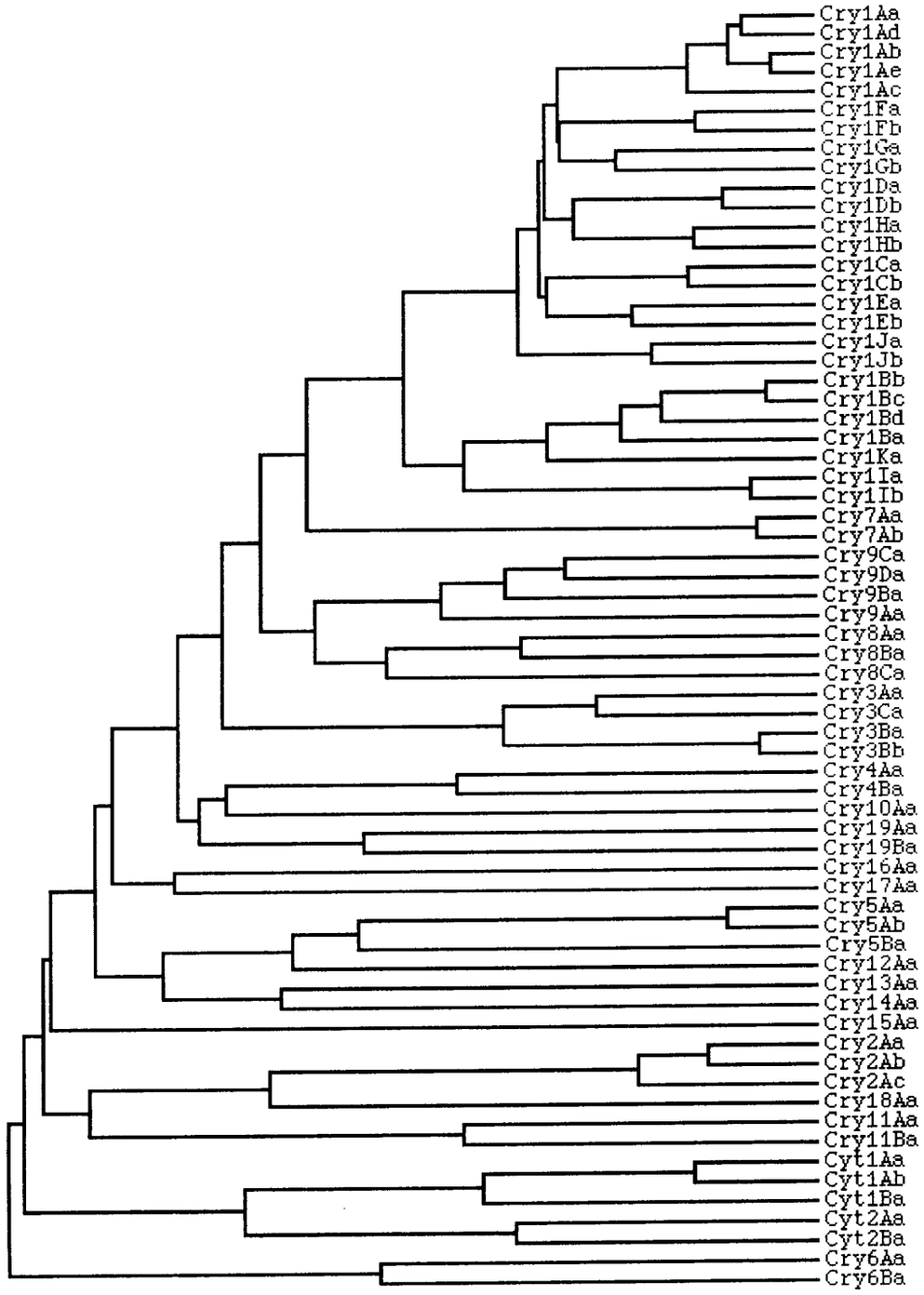

Figure 2 Amino acid homology between *Bacillus thuringiensis* δ-endotoxins. The dendogram was used to compare the amino acid sequences of 54 active toxin regions. (The original picture was taken from http:www.susx.ac.uk/ and users/bafnG/bt/index.html).

X-ray diffraction data they deduced the three-dimensional structure of the Cry3A activated protein (Figure 3). Recently, those results were complemented with a three-dimensional solution of the Cry1Aa protein structure from *Bt*. subsp *kurstaki* HD-1. In both cases, the molecules showed a globular wedge form composed of three closely packed domains which correspond to consecutive segments of the linear amino acid sequence [76].

In the deduced three-dimensional structure of Cry3A three domains are clearly distinguished. Domain I is composed of a seven-helix bundle with a central amphipathic helix (α5), thought to be the pore-forming region. Five of the helices are more than 30 Å long and thus could be capable of spanning the hydrophobic core of the membrane bilayer [76]. Von Tersch *et al* [187] showed that domain I of the coleopteran-active Cry3Bb toxin is sufficient for

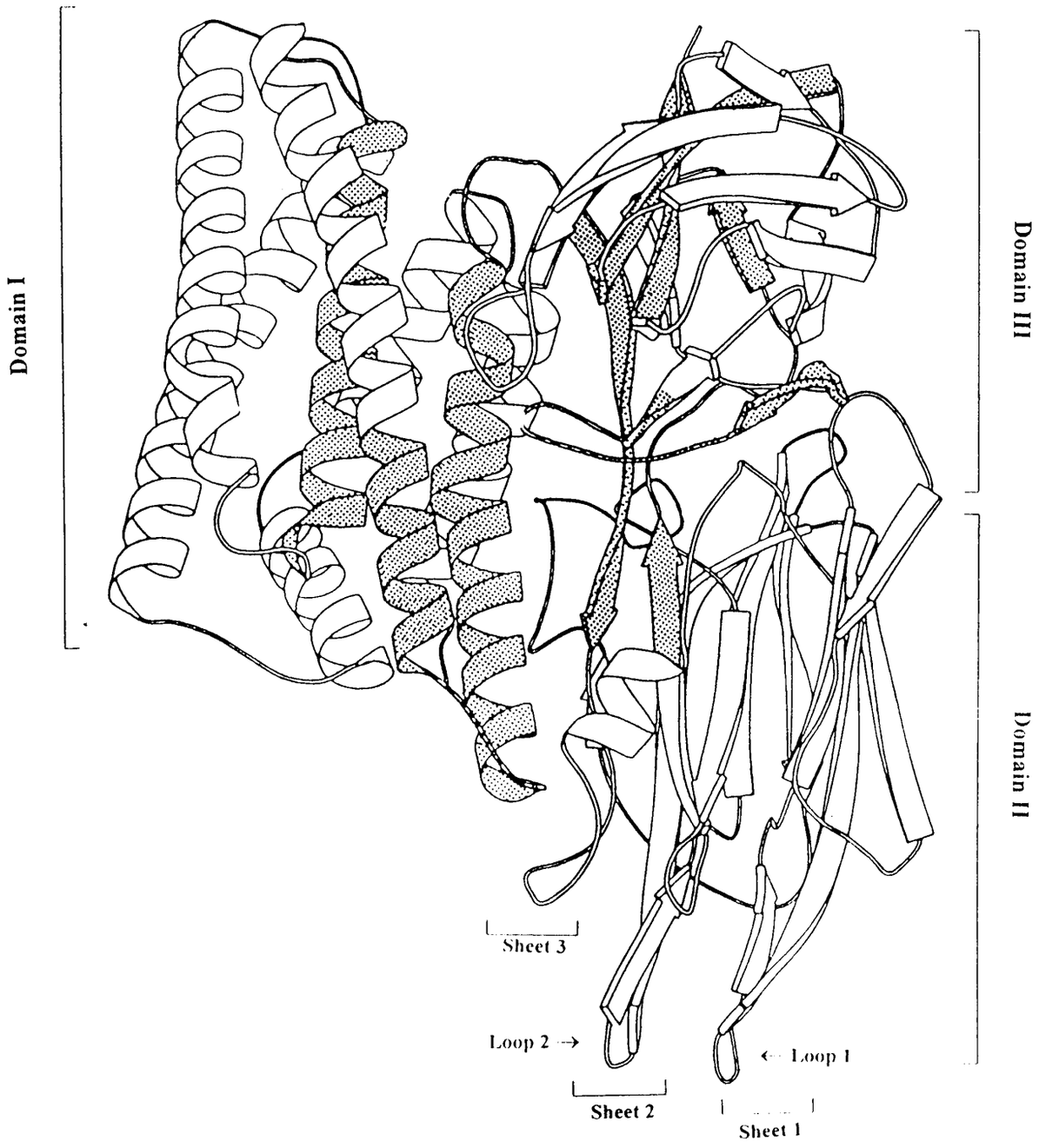

Figure 3 Line drawing of the three-dimensional structure of *Coleoptera* active Cry3A ICP from *Bacillus thuringiensis* var *tenebrionis*. Domains I, II and III are labeled and the arrows show the position of the loops. Reproduced by permission of Dr DJ Ellar. The original drawing was taken from Reference [112].

the formation of an ion channel. The second domain goes from amino acid 291 to 500 and is connected to domain I by a long linker, which extends from the end of the α7 helix to the first β-strand of sheet 3. Domain 2 comprises three β-sheets, arranged in a typical 'Greek-Key' topology [159]. Analyses derived from the three-dimensional structures, as well as from site-directed mutagenesis experiments, reveal that this domain is the one responsible for receptor-binding [146,166]. The third domain of Cry molecules is formed by a beta sandwich structure containing an Arg-rich sheet which is buried into the domain. The particular role of the third domain in the mechanism of action of δ-endotoxins is still unclear [39,49,71,128]. It is supposed to be essential to toxin stability and ion-channel function despite the fact that some toxins do not use this domain to carry out their cytolytic activity [89]. In addition, Domain 3 seems to be closely related to the receptor recognition specificity [103].

Little is known about the details of the ICP mechanism of action, but undoubtedly it occurs as a multi-step process

[102]. Seven critical stages, some of them from *in vitro* evidence obtained using an artificial membrane system and purified toxins, have been described: solubilization of the crystal, proteolytic activation, toxin-receptor recognition, structural and energetic conformational changes of the toxin molecule, membrane insertion, channel formation, ionic disequilibrium, and cell death. The target tissue is the larval midgut, which is an elaborate cell matrix adapted during evolution to the specific ecological conditions for different species. Basically it is composed of two major cell types: a columnar cell, with a microvillate apical membrane, which plays its role in nutrient uptake; and a goblet cell, characterized by a large vacuolar cavity permanently linked to the gut lumen through a complex valve [102,103].

When the parasporal body is ingested by the insect larvae, it is solubilized due to the extreme pH of the larval midgut (highly alkaline in *Lepidoptera*, highly acidic in *Coleoptera* larvae). Crystal solubility may be the limiting step of the biocidal process depending upon the different ICP composition [13]. Protoxins are processed by digestive proteases, most of them after beta-23, releasing the toxic protease-resistant core. Other protease cleavage sites have been also identified in important structural positions: after amino acid 118 in Cry3a [36], 145 in Cry2A [127], 230 in Cry4A [7] and 204 in Cry4B [7]. The peptides, resulting from excision of the C-terminal region of the proteins, possibly remain associated with the rest of the toxin molecule. Cry11A is apparently a unique exception in the generally-assumed activation mechanism, since the 65-kDa protoxin is cleaved into two fragments of 30 kDa and 35 kDa. Differential processing by the proteases within the larval midgut can determine the specificity of a given *Bt*. toxin [78]. Once an active toxin is generated in the insect midgut, it pierces the peritrophic membrane [24] and binds the highly specific receptors found on the brush border membrane of the epithelium columnar cells. This fact has been demonstrated by *in vitro* kinetic binding and competition studies, which have shown a great complexity of Cry protein-binding mechanisms. These studies have also demonstrated a positive correlation between toxicity of ICPs and their binding constants [53,87,180]; however, there have been controversial reports denying this correlation [63,70,194]. It was observed that some Cry1 toxins, such as Cry1Ab and Cry1Ac, bind to the epithelium cell membrane of non-susceptible insects [193]. Toxin association becomes irreversible shortly after binding by membrane insertion, which is directly correlated with the ICPs' toxicity. A drastic decrease in toxicity was observed for mutated toxins which had lost their capacity for irreversible binding, probably due to deficient pore formation and function [199].

A remarkable variety of lepidopteran midgut molecules (MW ranging from 63 to 210 kDa) with affinity for Cry proteins, have been identified using solid-phase blot assays [131,152,177,183]. These proteins are candidates to be the Cry-protein receptors in the midgut epithelium. Two principal Cry-binding proteins were characterized in *Manduca sexta* midgut: a Cry1Ac-affinity N-aminopeptidase (120 kDa) and a Cry1Ab-affinity cadherin-like (210 kDa). Both are high molecular weight glycosylated proteins [101,155] whose genes have been cloned. In other insects the pattern of Cry-binding proteins is even more complex, with several proteins binding to one toxin [88]. A 144-kDa binding protein for the Cry3A toxin was the first putative receptor reported for Coleopteran insects [20]. Immediately after binding to a specific receptor, *Bt*. toxins insert rapidly and irreversibly into the plasma membrane of the target cell. The next steps involve the pore formation. Based on the current concept of membrane channel architecture, the long hydrophobic helices of the pore-forming domain are likely to be the components of the pore. The conversion from a water-soluble to a membrane form needs a large change in conformation; it seems that these proteins become membrane-inserted via a molten globule intermediate which would relieve the energy barrier of the conversion. The triggering event for such a transition is not clear for the Cry toxins. Similar to other bacterial toxins, some studies support the notion that pH and toxin-receptor interactions are the critical factors for membrane insertion [40,64]. It is not known yet whether the toxin itself or a multimeric transmembrane complex is necessary to form the ion channel. On the basis of structural predictions some authors proposed a general model for pore formation [56,57]. In this model, a hexameric toxin pore was lined on the grounds of geometrical considerations of the estimated hydrophobic phase area and the internal pore radius: 0.6 nm [86]. The selectivity of Cry toxin-induced pores was reviewed by Knowles and Dow [103] who provided evidence that the pore is permeable to small ions—mainly K^+—and molecules [165].

The mechanism by which the Cry toxin-induced pore causes the observed damage to the insect gut cells proposes a cation leakage in the apical membrane of the columnar cell, resulting in depolarization and a consequent efflux of H^+ ions [81,82]. The rise in cytoplasmatic pH and membrane depolarization would probably lead to closure of gap junctions connecting goblet cells to damaged columnar cells. Gap junctions are normally involved in K^+ recirculation between both kinds of cells, allowing an efficient uptake of nutrients by the columnar cells [149]. This phenomenon causes a dysfunction of the the 'K^+ pump' system in the apical membrane of the goblet cell which compromises its transport capacity because of deficiency of K^+ ions. As a result of all these events the columnar cells would swell and burst by a colloid-osmotic process and the goblet cells would tend to shrink [56,57]. This may be due to the possibility that osmotic pressure exerted by the intracellular molecules is exceeded by the polyanionic matrix filling the vacuolar cavity [55]. Finally, the insect stops feeding and dies by starvation or septicemia.

Non-Cry toxic factors of *B. thuringiensis*

In addition to Cry δ-endotoxins, *Bt* is able to produce other toxic compounds: phosphatidylinositol-specific phospholipase C (PIPLC, α-exotoxin or 'heat-labile' exotoxin), thuringiensin (β-exotoxin or 'heat-stable' exotoxin) and the 'louse factor' [193].

The α- and β-exotoxins are active against a variety of invertebrates. α-Exotoxin—PIPLC—degrades the cell membrane phospholipid phosphatidylinositol, as well as

lysophosphatidylinositol, glycosilphosphatidylinositol and *myo*-inositol-1,2 cyclic-phosphate [96]. As a research reagent, PIPLC from *Bt*. has been used widely in cell biology for studies on the functional characterization of glycosilphosphatidylinositol-anchored proteins or GPI-anchored proteins [104]. These proteins are present on the surface of all eukaryotic cells from protozoa to mammals , involving a variety of important cellular functions. Since bacterial PIPLC solubilizes GPI-anchored proteins from the cell surface, it is used as a valuable analytical tool for biochemical and cell biology studies of diseases caused by prions which comprise GPI-anchored proteins. To this group of diseases belong scrapie—a transmissible neurodegenerative disease, Creutzfeldt–Jacob disease, Gerstman–Sträussler syndrome, human kuru and bovine spongiform encephalopathy, mad cow disease [104].

The β-exotoxin—thuringiensin—is a nucleotide composed of adenine, glucose and allaric acid. This low molecular weight heat-stable non-selective insecticidal toxin is produced by many *Bt*. strains [60]. In the target cells the toxicity of this compound is due to inhibition of RNA biosynthesis by competition with ATP for its binding sites. It is believed that the phosphate group on allaric acid is essential for activity [61]. The β-exotoxin is used commercially as an insect-controlling agent in several countries [99]. However, as the δ-endotoxins are usually used for more selective pest management programs, the levels of β-exotoxin in the commercial *Bt*. bioinsecticide preparations must be controlled. Some methodologies for detecting and monitoring the β-exotoxin production have been proposed based on ELISA [19] or HPLC assays [31].

Cyt proteins are produced by many *Bt*. strains. These toxins are membrane pore-forming proteins, without significant selectivity in their action mechanism. They are lethal to dipteran larvae and have shown broadly cytolytic and hemolytic activity *in vitro*. CytA is present in parasporal inclusions of *Bt*. subsp *israelensis* together with Cry4, Cry10 and Cry11 δ-endotoxins, which act synergically with CytA [111]. CytB is the only mosquitocidal protein of *Bt*. subsp *kyushuensis* and shares 39% amino acid sequence identity with CytA [105]. Recently, the three-dimensional structure of this protein has been reported. CytB has a single domain of α/β architecture, with two outer layers of α-helices and a β-sheet between; it is supposed that its homologue CytA would show a similar folding pattern [111].

Recent studies [59] have led to the discovery of a novel proteinaceous toxin in two *Bt*. isolates. The protein was named Vip3A and the deduced amino acid sequences of two homologues (Vip3Aa and Vip3Ab) have been reported. However, no homology was found with the previously known insecticidal proteins; thus Vip3A represents a completely novel class of toxins. It is expressed during the vegetative stage of growth starting at mid-log phase, as well as during sporulation, and shows biological activity in several lepidopteran species. *Vip3A* gene homologues seem to be present in about 15% of the screened *Bt*. isolates.

Bioinsecticidal preparations that include *Bt*. spores supplementing the ICPs are usually more toxic to the target insects than those prepared from isolated crystals. Normal germination and outgrowth in the insect hemolymph leads to a septicemia which contributes to larval mortality in certain insects. The spores shortened the time of death of susceptible insect larvae and the larval death is characterized by a blackening of the larva due the oxidative degradation of hemolymph [98]. The described effect is not highly specific and its magnitude depends upon the resistance character of the larvae [98].

Other toxic factors have been described, eg immune response inhibitors and proteases [163]. Some investigations on the involvement of live *Bt*. cells in the pathogenesis mechanism of this bacterium have indicated that flagellar proteins may contribute to the development of the infection during its early stages (for review, see Moens *et al*, 1996 [122]). *Bt*. culture supernatant provides additional enhancement of larvicidal activity, which seems to be related to toxic factors of a yet unknown nature [93].

Biodiversity of *B. thuringiensis*. Approaches for worldwide screening

Microbial biodiversity has been already recognized as a major resource for biotechnological products and processes (for a review, see Bull *et al*, 1992 [30]). The planning and optimization of screening programs of industrial microorganisms must indeed be considered as a sequence of unit stages and as a phase of the bioprocess operation [29]. All the work already performed with *cry* genes is a logical consequence of biodiversity studies on this bacterium.

Bacillus thuringiensis is a very polymorphic bacterial species, and a great number of strains has been isolated. Worldwide distribution of many different *Bt*. strains is a subject of continuous investigation by many public and corporate research institutions [38,62,114,132]. The International Entomopathogenic Bacillus Collection (IEBC) at Institut Pasteur, France, is the world reference center for this species, although many important *Bt*. culture collections have been organized at other institutions [62]. Advances in the development of new *Bt*.-based biopesticides and in the genetic manipulations of their genes for plant biotechnology depend on the variety of available strains with different insecticidal spectra and their corresponding *cry* genes. For these reasons there has been an intense interest in the isolation and analysis of new strains as well as in performing extensive screening programs of existing culture collections for novel biocidal activities.

One of the major difficulties in this work is the repeated isolation and rescreening of the same strains and genes, which increases the number of strains to be evaluated. The classification based on flagellar antigens is of little utility when searching for new biocidal specificities, and the characterization of a given set of strains by direct bioassays turns out to be a long and difficult procedure [35]. Because researchers are mainly interested in δ-endotoxins as the major toxic factors of *Bt*., several screening systems for detecting their genes in new isolates have been proposed (Table 1). These systems allow the subsequent selection of promising strains on the basis of their biological activity. To date, the reported methodologies allow the determination of the ICP genes harbored by a given strain by means of: (1) direct detection of the gene using DNA hybridization or PCR; (2) detection of the *cry* gene products

Table 1 Different methods for screening and classification of *B. thuringiensis* isolates based on δ-endotoxins and *cry* genes

Screening method	Reference	Comments
DNA probes	[108]	Discriminates by Southern blot 3 *cry1A* gene subclasses.
Polyclonal Abs	[151]	ELISAs using polyclonal antibodies against Cry1 & Cry2 proteins.
DNA probes	[142]	Dried agarose gels hybridization with 5 oligonucleotide probes.
Monoclonal Abs	[91]	A set of monoclonal antibodies.
DNA probes	[186]	Southern blot using 4 *cry* gene fragments as DNA probes.
DNA probes	[115]	Hybridization with oligonucleotide probes and CNBr mapping.
DNA fingerprinting	[121]	
Numeric taxonomy	[14]	Numeric analysis of several biochemical traits.
PCR	[35]	Single reaction with 12 oligoprimers; discriminates *cry1Aa, cry1Ab, cry1Ac, cry3A, cry3B, cry4A* & *cry4B.*
PCR	[23]	Two consecutive reactions with 7 oligoprimers; discriminates *cry1Aa, cry1Ab* & *cry1Ac*.
PCR	[100]	Single reaction with 10 oligoprimers; discriminates *cryAa, cry1Ab, cry1Ac, cry1B, cry1C, cry1D, cry1E* & *cry1F*.
PCR-RFLP	[73]	Single *cry5*-specific reaction with 2 oligoprimers and subsequent RFLP of the PCR products using *Kpn*I.
Polyconal Abs & PCR	[37]	Prescreening with an ELISA using polyconal antibodies against Cryl proteins. Two reactions with 13 oligoprimers; discriminates *cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C* & *cry1D.*
PCR	[38]	The system designed by Kalman was used.
DNA probes	[160]	DNA dot-blot hybridization using PCR-generated probes specific to *cry1* & *cry5*.
PCR-RFLP	[109]	Single reaction with 4 oligoprimers and subsequent RFLP of the PCR products using *Pst*I and *Xba*I; discriminates *cry1Aa, cry1Ab, cry1Ac, cry1B, cry1Ca, cry1Cb, cry1D, cry1E, cry1F, cry1G, cry3A, cry3B1, cry3B2, cry3C, cry4A, cry4B, cry4C* & *cry5*.
DNA probes	[44]	Non-radioactive colony hybridization with digoxigenin-labelled PCR-generated probes. Discriminates *cry1A, cry3A* and *cry4A*, as well as detects flagellin B and phospholipase C.
DNA probes	[59]	Colony hybridization using a fragment of the *vip3Aa* gene. Detects putative homologues of this gene.

by an immunoassay system; (3) combination of both techniques; and (4) indirect inference from physiological data.

Southern blot tests for *cry* gene homologues [108] and immunoassays (ie ELISAs) for ICPs reactive with several monoclonal or polyclonal antibodies [91,185] were the first molecular screening techniques used to characterize the novel isolates. The use of monoclonal antibodies for the screening of *Bt*. isolates—without establishing a defined procedure—was proposed by Huber-Lucac *et al* in 1986 [95]; polyclonal antisera were first employed by Samasanti *et al* in the same year [144,151]. The main advantage of the immunochemical methods is that they allow the direct detection of gene expression. Cross-reactivity was regarded as the major limitation of these systems; the assays based on monoclonal antibodies are particularly expensive. Recently, we have proposed the introduction of negative immunoadsorbtion steps in the anti-Cry IgG purification procedure as a way to overcome the cross-reactivity problem. This improvement allowed us to obtain subclass-specific polyclonal antibodies [143], which are the basis of our immunochemical screening system [144].

The DNA probe hybridization approaches including Southern blot [35] and dried-agarose hybridization [142] were the first employed for direct detection of *cry* genes; however, they are too expensive and time-consuming to be used in massive screening programs. More recently [44,59,160] simplified DNA-hybridization techniques (ie dot blot and colony hybridization) have been used as screening methods (Table 1). To date, the polymerase chain reaction (PCR)—a procedure for rapid determination of the presence or absence of specific DNA sequences by selective amplification [150]—has been reported as the most efficient and reliable screening technique. Electrophoretic profiles of the amplified products allow detection of specific *cry* genes in known and novel *Bt*. isolates. After the first report [84], other PCR systems for large-scale routine testing have been proposed (Table 1). They are inexpensive, sensitive, easy to perform, and may be used for extensive screening programs because of their rapidity. The 'multiplex PCR' methods are based upon the simultaneous amplification of several target sequences by a set of primers in a single reaction mix. In the PCR-RFLP (Restriction Fragment Length Polymorphism) methods [73,109], the amplified DNA is digested with restriction endonucleases to give a specific electrophoretic profile; this technique seems to have the best performance for the detection of novel *cry* genes.

The proposed 'molecular systematic' methods also permit the study of genetic diversity among *Bacillus thuringiensis* strains through the diversity of *cry* genes. These genes are present on plasmids in most of the isolated strains, but others with chromosomally located *cry* genes were also found. The variety of plasmid arrays observed in related *B. thuringiensis* strains can be explained as a result of conjugation-like plasmid-exchange mechanisms, transposition or homologous recombination [89,142]. This situation results in a great diversity of strains producing two, three or even more δ-endotoxins. Differences in their genetic background, implying adaptation to specific ecological niches and living conditions have been observed. Using specific primers, the screening of *cry1Aa, cry1Ab, cry1Ac, cry1C, cry1D* and *cry5* genes from 225 *Bacillus thuringiensis* iso-

lates from Taiwan were identified and their geographical distribution observed [38]. Strains with profile *cry1Aa, cry1Ab, cry1Ac*-type genes were found in isolates from mountainous areas (3000 m above sea level), where the weather is cold and dry. The *cry1C, cry1D*-type genes were found in isolates from hot and humid areas. In contrast the *cry1Aa*- and *cry1Ac*-type genes were observed in isolates from all screened areas. More extensive studies are necessary to clearly establish the relationship between the genetic background of *B. thuringiensis* isolates and the environmental factors. It is advisable to perform such studies in regions with biodiversity, including the so called megadiversity regions of the planet: Amazonia, Indonesia and Madagascar [30].

Heterologous expression of *cry* genes in transgenic organisms

Expression in bacteria

The particularities of the *cry* genes expression process in *Bt*. have been reviewed elsewhere [4]; we will emphasize in our discussion the heterologous production of Cry toxins. Considering that these proteins are biological pesticides of great economic importance, it has been desirable to study their expression in organisms to develop alternative delivery systems, more suitable for field application. This research has allowed the production of biopesticides which, when released in insect pest-infested areas, would facilitate the ingestion of lethal doses by the susceptible larvae.

Despite the advantages of the biodegradability, selectivity, and safety for humans and the environment, the use of *Bt*.-based insecticides has several limitations. These include short-time field persistence, mainly because of the susceptibility of the toxin to environmental conditions such as ultraviolet radiation from sunlight, and in some cases, the inaccessibility of the target pests inside plant stems and leaves to the toxin (stem borer, leaf mining). As a solution, heterologous expression of *cry* genes has been studied in many microorganisms. Some examples of the microbial hosts expressing *cry* genes reported to date can be found in Table 2.

Generally, *Escherichia coli* is an effective expression system for *cry* genes, because the parameters of the biosynthetic processes are better understood in this bacterium. There are a lot of reports in which the cloning and expression of novel *Bt*. toxin genes have been performed mainly to attempt their characterization [21,28,72,118,153,158]. The use of *E. coli* as an expression host allows the selective production of Cry proteins with particular biocidal specificity for their individual study. In addition, it can also be used as an effective vehicle for industrial production of crystal proteins, functionally improved by genetic and protein engineering [13,22,32,50]. Ge *et al* [72] have optimized the culture conditions for suitable hyperexpression in *E. coli* of the gene *cry1Ac*73 encoding the Cry1Ac insecticidal crystal protein. P*tac* promoter was found to have the best performance for the production of protoxins to high levels on pBR322 derivatives (pKK223–3) in *E. coli* host JM103. The expression in *E. coli* of the truncated *cry1A* gene versions encoding the ICP toxic fragments has also been achieved [182]; however, the observed levels (0.76% of total proteins) were significantly lower when compared with those obtained in systems where the full-length polypeptide is synthesized.

δ-Endotoxins are environmentally labile, which is why the most successful systems to produce them as bioinsecticides are those in which they remain encapsulated and protected from degradation. The ICPs are highly susceptible to various factors, such as ultraviolet light, heat, presence of tannins and humidity cycles. Hence, expression of *cry* genes in organisms that make the toxins more stable in the environment was proposed as a way to overcome this problem. Introduction of the *cry1Ab* gene into *Pseudomonas fluorescens* was one of the earliest attempts with this purpose [130]. The first genetically engineered bioinsecticide was developed by the Mycogen Corporation (San Diego, CA, USA) by the use of recombinant *P. fluorescens*-generated artificial capsules as vehicles for stabilizing and delivering *Bt*. proteins in the field. This bacterium is sensitive to tetracycline, and is not pathogenic to either plants or animals. The value of the encapsulation system has been successfully tested in agricultural practice.

More recently, the expression of *cry* genes in *B. subtilis* and *B. thuringiensis*, which are unable to initiate sporulation, constitutes an alternative to improve the stability of ICP in insecticidal formulations. When the *cry3A* gene was expressed in *B. subtilis* and *B. thuringiensis* spoOA$^-$ strains, large amounts of the toxin were produced and accumulated in both cases to form a crystal inclusion that remains encapsulated within ghost cells [3,110]. It is important to note that there are some reported *B. subtilis* spoOA$^-$ mutants that do not produce proteases [27], and this defect in a *Bt*. Spo$^-$ background could be beneficial for the production of heterologous proteins [110]. Similar studies were done expressing *cry1Aa* gene in *B. thuringiensis* SigE$^-$ and SigK$^-$ mutant strains [26]. The genes *sigE* and *sigK* encode sporulation sigma factors σ^{35} and σ^{28}, blocking the sporulation at early and late stages, respectively. A transcriptional *cry1Aa-lacZ* fusion was expressed efficiently in a SigK$^-$ strain under the control of Btl promoter, which is recognized by the σ^{35} factor produced by this mutant. The toxin remained encapsulated within the mother cell wall [26]. However, the persistence of crystals encapsulated within the *Bt*. ghost cells has not been field-tested yet.

The use of plant-associated microorganisms carrying *Bt. cry* genes is another choice to improve the ICP stability under field conditions. In the case of the cotton leaf-colonizing *Bacillus megaterium* [164], the *Cry1Aa* gene was introduced by conjugal transfer into *B. megaterium* strain RS1. The transcipient microorganism—*B. megaterium* RS1 43—survived in the cotton phyllosphere for a longer period than the donor. In a previous study, an ICP gene from *Bt*. subsp *kurstaki* HD-1 was cloned into the plant-colonizing *Pseudomonas cepacia* strain 526 to control tobacco hornworm [169]. In this case it was found that only 1% of the *Pseudomonas* cells retained the toxin gene 3 days after application on axenically-grown tobacco plants. Some natural vascular-colonizing microorganisms such as *Clavibacter xyli* subsp *cynodontis*—a xylem-inhabiting endophyte bacterium—are efficient delivery systems for biopesticides [175]. An engineered strain carrying a δ-

Table 2 Examples of the expression of *cry* genes in transgenic microorganisms*

Transformed organism	Gene	References
Bacillus subtilis	*cytA*, *cry4* – subclass not specified	[173, 189]
168-11 (sporogenic mutant)		
OJ87 (asporogenic mutant)		
OA34 (asporogenic mutant)		
Bacillus subtilis	*cry1A* – subclass not specified	[43]
Bacillus subtilis	*cry1A* – subclass not specified	[161, 162]
	cry2A – subclass not specified	[162]
	cytA	[198]
	cry4D	[173]
Bacillus subtilis spoOA mutant	*cry3A*	[3]
Bacillus subtilis	*cry4* – subclass not specified	[188, 198]
Bacillus megaterium	*cry1Aa*	[164]
	cry1A – subclass not specified	[162]
	cry2A – subclass not specified	[162]
	cry4D	[54]
Bacillus sphaericus 2362	*cytA*	[15]
	cry4D	[174]
Bacillus thuringiensis spoOA mutant	*cry3A*	[110]
Bacillus thuringiensis sigE and *sigK* mutants	*cry1Aa*	[26]
Pseudomonas fluorescens	*cry1Ab*	[130]
Pseudomonas fluorescens 701E1	*cry3* – subclass not specified	[118]
Pseudomonas fluorescens Ps 3732–3-7 & 112–12	*cry1A* – subclass not specified	[74]
Pseudomonas fluorescens	*cry1Ac*	[84]
Pseudomonas cepacia	*cry1A* – subclass not specified	[169]
Synechocystis sp PCC6803	*cry4B*	[41]
	cytA	
Synechococcus sp PCC7942	*cry4B*	[167]
*Baculovirus***	*cry4D*	[135]
Caulobacter crescentus	*cry4B*	[172]
Ancylobacter aquaticus	*cry4B*	[197]
Bradyrhizobium	*cry4C*	[126]
Rhizobium fredii and *Rhizobium meliloti*	*cry4B*	[77]
Agmenelum quadruplicatum	*cry4B*	[8]
PR-6	*cry4D*	[124]
Clavibacter xyli subsp *cynodontis*	*cry1Ac*	[175]
Saccharomyces cerevisiae	*cry1Aa*	[190]
Pichia pastoris	*cry1Ab****	Ayra C. (unpublished)

*The reports of *E. coli* expressing *cry* genes were omitted.
**In this case it refers to *cry4D* gene from *Bacillus thuringiensis* subsp *morrisoni* PG-14 inserted in a Baculovirus vector.
***Typical and modified versions.

endotoxin gene was used to colonize several important crop plants [106] including corn (*Zea mays* L). Turner *et al* tested in greenhouse and field experiments a prototype version of *C. xyli* subsp *cynodontis* expressing a *cry* gene (MDR1.3) for the control of the European corn borer (*Ostrinia nubilalis*) [175]. The recombinant strain of *C. xyli* containing the chromosomally inserted *cry* gene eventually shed the gene when grown *in vitro* or *in planta*. It was found that the dominant segregant class of strain MDR1.3 had lost the entire integrated plasmid from the chromosome. The other segregant class that was detected had retained at least one copy of the cassette. Finally, the authors report that segregant colonies made up less than 15% of the total *C. xyli* colonies isolated from corn at the end of the growing season. The continual loss of the δ-endotoxin gene reduces the chances of activity against non-target insects in the unlikely event that the recombinant endophyte escapes from corn to a suitable perennial host.

Other plant-associated organisms have also been used as suitable hosts for the production of high amounts of Cry proteins. Contrary to the considerable degradation observed for some *cry* gene products expressed in *E. coli*, the anti-dipteran toxin Cry4B appeared to be very stable in the plant symbiotic *Rhizobia*, reaching expression levels between 2 and 5% [77].

Cyanobacteria—the most extensive group of photosynthetic bacteria—represent a considerable portion of the mosquito larvae diet. These bacteria have a wide range of temperature tolerance and simple nutritional requirements [134,148], which is the reason why some scientists have focused their research in the expression of anti-diptera *cry* genes in several aquatic microorganisms such as *Cyanobacteria* sp, *Caulobacter* sp and *Synechococcus* sp (see Table 2). Unfortunately, in all cases the expression levels and toxicity have been very low [8,41,51]. Even when the *cry11A* gene from *B. thuringiensis* subsp *israelensis* was introduced into *Agmenellum quadruplicatum* cell extracts they showed low toxicity despite the high expression levels of the protein [124].

Expression in yeast

Expression of the lepidopteran-active insecticidal *cry1Aa* gene in *Saccharomyces cerevisiae* has been reported recently [190]. The 80-bp 5′-noncoding region of the wild-

type toxin gene was removed and replaced by a synthetic fragment to establish the consensus sequence around the first ATG codon observed in most yeast genes. As there are three upstream ATG codons in the *cry1Aa* gene that are out of frame with the coding sequence, their removal was required for efficient translation from the ATG initiation codon, since 5′-proximal AUG codons are used as the initiation site for translation for almost all yeast mRNA. A 135-kDa protein product was detected by Western blot analysis and the expression levels increased up to 1.3% of total soluble proteins while with the wild-type gene, including the 80 bp of 5′-noncoding region, no recombinant protein was detected [190]. In a previous study another insecticidal protein—the binary toxin of *B. sphaericus* Bsp2—was expressed in the methylotrophic yeast *Pichia pastoris* to develop a mosquito biolarvicide [168]. The authors modified the two wild-type genes in order to obtain higher levels of the binary toxin product in *Pichia* cells. Codon usage was changed to the homologous codons frequently found in highly expressed *P. pastoris* genes, which implied an increase in the G+C content to 40–55% in the foreign gene.

Currently, we are involved in the development of *P. pastoris* expression systems for extensively modified versions of the truncated *cry1Ab* gene encoding the ICP toxic fragment from *B. thuringiensis* subsp *kurstaki* HD-1. As the G+C content of the entomotoxin genes from *B. thuringiensis* and *B. sphaericus* are nearly similar, expression levels of the modified *cry* gene version are expected to be high. Also, some changes in the gene sequences were designed following some general recommendations reported elsewhere in order to improve their expression in eukaryotic cells [139]. We expect that the modified *cry* genes would be efficiently expressed in this microorganism since plant genes express well [18,80,85,147]. In this case we would be able to assess the possible use of yeast as a model system for monitoring the effectiveness of sequence changes before initiating plant transformation experiments. The most important aspects concerning the expression of *cry* genes in eukaryotic hosts are analyzed below.

Expression in plants

The introduction of heterologous genes into plant genomes and their expression was achieved in 1983 by L Herrera-Estrella *et al* [83]; their work became the starting point for further plant genetic engineering developments. Since then there has been great progress in tissue culture, genetic transformation and molecular biology of plants. The most widely used method of plant transformation is the infection with genetically-modified strains of *Agrobacterium tumefaciens* [48,94]. Initially, many crop plants such as the major monocot species could not be transformed by this technology. For such cases, alternative methods were proposed [157], ie polyethyleneglycol-mediated transfer [176], microinjection [47], protoplast and intact cell electroporation [9,10,67,68,113,141], and particle bombardment or gene gun [154]. To date, many advances in *Agrobacterium*-mediated transformation of recalcitrant plants have been reported [58,75,92,145], and many research groups are increasing their efforts in this direction.

Recombinant DNA technology has the potential to complement the efforts of plant breeders. As we recently described [48], genetic pools of many crop plant species seem to lack the genes for resistance to their major insect pests. In these cases the production of pest-resistant cultivars by classical breeding is not possible and the transgenic approach using plant expression systems containing *cry* genes is a real alternative for obtaining 'self-pesticide' elite varieties resistant to insect attack. Thus, the generation of transgenic plants resistant to insect pest attack remains as one of the most important targets in plant genetic engineering [48].

The field use of transgenic self-pesticide crop plants expressing *Bt*. toxins may contribute to a reduction in the use of chemical pesticides, which are non-selective and environmentally dangerous. Additionally, transgenic plants cause continuous persistence of the toxin in the field, a distinct disadvantage when compared with the *Bt*.-based biopesticides. Cry-expressing transgenic varieties of several crops have been obtained. However, the self-pesticide spectrum is still limited: only lepidopteran and coleopteran larvae of defined species have been described.

The first crop plants were transformed with genetic constructions containing full-length and truncated *cry1* genes [66,177]. Plants with complete protoxin-encoding *cry* genes integrated into their genomes showed extremely low levels of δ-endotoxin; the levels of recombinant protein were much higher when truncated gene versions—encoding only the active fragments of the protoxins—were transferred to the plants. When a truncated version of *cry1Ab* gene was used, the heterologous toxin level detected in leaves of transgenic tobacco plants represented up to 0.001% (0.07% in exceptional clones) of total leaf protein; no reliable detection was reported when full-length genes were used [140,161].

The use of more efficient expression systems did not provide a remarkable increase in toxin yields. The use of stronger promoters, the introduction of consensus sequences flanking the translation initiation ATG codon and the use of untranslated leader sequences from plant viruses as translational enhancers, only slightly increased the Cry protein levels in transgenic plants. It was clearly observed that the achievement of optimal expression levels is impossible when unmodified (wild) *cry* genes are transferred into the nuclear plant genome. Despite this fact, the transgenic plants showed a remarkable entomocidal activity against target insects [52,181]. Although low-expressing plants have been protected against some insects, higher expression levels are required for an efficient control of agronomically important pests. Thus, it was necessary to find a suitable solution to developing the idea of using *cry* genes in field pest control through their expression in transgenic plants.

In 1991, Murray *et al* [125] reported the results obtained from a series of experiments on electroporated plant protoplast and transgenic plants. These studies allowed them to correlate the observed low expression level of *cry* genes in plant cells with the instability of their transcripts. The analysis of the nucleotide sequences of *Bt*. *cry* genes showed the putative causes of the instability of their transcripts in plants:

(1) The existence of polyadenylation sequences in plants. In higher plants, polyadenylation involves RNA cleavage in a defined site, *cis*-regulated by a series of consensus sequences. These sequences are normally present in *cry* genes and this may be the reason why truncated and degraded *cry* RNA forms are found.
(2) The presence of RNA polymerase II premature transcription-termination signals.
(3) The presence of hairpin-favoring sequences, probably forming complex RNA secondary structures.
(4) Some sequence stretches are similar to plant consensus splicing signals.
(5) *cry* genes are richer in A and T than native plant genes.

The analysis of *cry* gene sequences revealed that the A+T content represents about 60% of the total nucleotide content of the protein-encoding region; this value is 10% higher than that of typical plant genes. In plants, the A+T-rich DNA segments are found usually in intergenic regions and regulatory sequences. This consideration presumes that an elevated A+T content in the coding regions of *Bt. cry* genes could contribute to their low expression levels in transgenic plants. On the other hand, codon usage is very different in plants and native *cry* genes.

All these aspects were taken into account for modifying the nucleotide sequence of *cry* genes. Perlak and Adang [1,2,138–140] reported the design of partially- and totally-modified *cry* genes, by directed mutagenesis and DNA chemical synthesis, respectively. The main purpose was to change at maximal extension the sequences acting as RNA-destabilizing signals, without any alteration in the amino acid sequence of the gene product. At the same time, it was necessary to design modified *cry* genes with lower A+T content (similar to that of the plant genes). For example, the synthetic *cry3A* gene constructed by Adang *et al* [1] contains 55% A+T. In addition, the designed modified genes have a plant-optimized codon usage to ensure a better performance during the translational steps of the gene-expression process.

Today, there are different crop varieties transformed with genetic constructions containing synthetic and partially-modified *cry1A* genes encoding lepidopteran-active toxins, and the respective expression levels obtained have been up to 100-fold higher than those observed with non-modified *cry* genes [138–140] (see Table 3). Recently, a novel methodology was reported for generating plants expressing the *cry* genes at high level without the need of extensive sequence modifications [116]. The procedure is based upon the gene-gun (biolistic) plant transformation technology, allowing the delivery of genetic vectors to the plastids. Due to the prokaryotic characteristics of the plastid gene-expression systems, the wild-type *cry* gene inserted into the plastid genome (plant plastome) expressed very high levels (1–5% total proteins) of the ICP. This is the only method allowing the production of full-length Cry protoxins in transgenic plants, but the lack of reliable plastid-transformation methodologies in many crop species is a serious limitation for its extensive use.

Resistance development

The development of insect resistance to *Bt*. Cry proteins has been extensively reviewed elsewhere [65,117]. Several insect species (eg *Heliothis virescens*, *Leptinotarsa decemlineata*, *Plodia interpunctella* and *Plutella xylostella*) have been selected for resistance to *Bt*. toxins under laboratory conditions. To date, only one insect species (*Plutella xylostella*) has developed high-level resistance to a δ-endotoxin (Cry1Ab protein) under field conditions [65]. Apparently, resistance involves the modification of the Cry1Ab-binding site in the insect midgut brush border membrane, while the binding sites for Cry1B and Cry1C seem to remain unaltered. Inheritance of this trait in several populations of these insects has also been studied. However, resistance-development mechanisms in other species involve more complex phenomena (ie cross-resistance to several toxins, resistance to one toxin and a simultaneous rise of susceptibility to another, resistance by altered proteolytic processing, etc) [65].

Resistance management is defined as a set of coordinated actions in order to delay or prevent adaptation of pest species. The susceptibility of a given species to a particular pesticide may be viewed as a renewable genetic resource. In the case of self-pesticide transgenic plants and *Bt*.-based products, there have been several controversies as to how to maintain the susceptibility of target insects and to minimize the risks of their adaptation to the Cry toxins [5,6,65,97,179]. Presently, different experimental developments are being evaluated according to their potential to reduce the risks of raising resistant insect populations. The use of biopesticides containing hybrid toxins [22] and of plants expressing more than one Cry protein [178,182,184] has been proposed. The development of reliable resistance management strategies is required to maintain Cry proteins as effective environmentally-friendly biocontrol agents [170,171,191,192].

Concluding remarks

Bacillus thuringiensis is the most prominent biological agent for selective control of pest insects. During the last decades, *Bt*.-based formulations became more widespread, initiating the replacement of some harmful chemical pesticides. The understanding of ICP toxicity biochemistry together with the progress in molecular biology have provided the possibility for screening and characterizing a large number of isolates. The wide diversity of this bacterium provides promise for the isolation of new strains with novel biocidal specificities and the development on this basis of new industrial biopesticide formulations. These facts have stimulated basic and applied research in several directions, using conventional as well as recombinant procedures. These investigations included the improvement of *Bt*.-based formulations, industrial processes and stability of the applied products under field conditions.

The limited field stability of ICPs is a serious problem regarding the commercial use of *Bt*. preparations. These practical difficulties together with the need for studying the toxicity of the individual Cry proteins were the reasons to search for other bacterial hosts. ICP encapsulation systems

Table 3 Autopesticide transgenic plants expressing *B. thuringiensis* toxins

Crop	Gene	Expression levels	Signal system	Reference
Tobacco	t*cry1Ab*	2 ng mg^{-1} total proteins	Promoter:2′ManS Terminator: 3′ T7 S.M.:NPTII	[178]
Tobacco	t*cry1Ab*	6 ng mg^{-1} total proteins	Promoter:35S CaMV Terminator:tNOS S.M.:NPTII(Kan)	[17]
Tobacco	t*cry1Ab*	1 ng mg^{-1} total proteins	Promoter:35S CaMV Leader sequence:AMV Terminator:t35S CaMV S.M.:NPTII(Kan)	[34]
Tobacco	t*crylAb*	Not determined	Promoter:35S CaMV Terminator:tNOS S.M.:NPTII(Kan)	[123]
Tobacco (chloroplasts	w*cry1C*	50 000 ng mg^{-1} total proteins	Promoter:Prrn Terminator:rps16 S.M.:(aadA)Spt/Str	[116]
Tobacco Tomato	PM*cry1Ab* PM*cry1Ac* TM*cry1Ab* TM*cry1Ac*	200 ng mg^{-1} total proteins 600–2000 ng mg^{-1} total proteins	Promoter:35S CaMV Double enhancer Terminator:tNOS S.M.:NPTII(Kan)	[139]
Tobacco Tomato	t*cry1Ab* t*cry1Ca*	Not determined	Promoter:35S cAMV Terminator:tNOS S.M.:NPTII(Kan)	[22] [179]
Tomato	t*cry1Ab*	Approximately 1–2 ng mg^{-1} total proteins	Promoter:35S CaMV Terminator:tNOS S.M.:NPTII(Kan)	[66]
Potato	TM*cry3A*	250 ng mg^{-1} total proteins	Promoter:MAC Terminator:tManS S.M.:NPTII(Kan)	[1] [138]
Potato	PM*cry3A*	1000 ng mg^{-1} total proteins	Promoter:35S CaMV Double enhancer Terminator: Not reported S.M.:Not reported	[140]
Broccoli	TM*cry1Ab*	Not determined	Promoter:35S CaMV Terminator:tNOS S.M.:NPTII(Kan)	[120]
Maize	TM*cry1Ab*	12 750 ng mg^{-1} total proteins	Promoter:35S CaMV Terminator:t35S CaMV S.M.:bar(Basta)	[107]
Rice	TM*cry1Ab*	84 ng mg^{-1} total proteins	Promoter:35S CaMV Terminator:t35S CaMV S.M.:AphlV(Hygr)	[195]
Sugarcane	t*cry1Ab*	2 ng mg^{-1} total proteins	Promoter:35S CaMV Terminator:tNOS S.M.:Gus	[11] [12]
Soy bean	t*cry1Ab*	1–3 ng mg^{-1} total proteins	Promoter:35S CaMV Leader sequence: AMV Terminator:Orf25TDNA S.M.:hpn(Hygr)	[137]

(Continued)

were created in order to increase the persistence and stability of the toxins under field conditions; very promising is the expression of *cry* genes in some plant-associated microorganisms. Such hosts can confer pest-protection to their 'partners' without genetically engineering the plant itself. One of the most important aspects of this approach is its potential use when the pest damages the internal plant tissues, where the larvae are difficult to reach with conventional application methods. The first transgenic plants resistant to insect attack were produced in 1987; to date, *cry* genes have been engineered into many plant species. Originally, the main limitation for the field use of transgenic plants was the low expression levels of native *cry* genes in the plant cells. This problem has been solved successfully by two different methodologies: extensive modification of the *cry* gene sequence for optimizing its nuclear expression, and transformation of plastids by plasmid vectors containing *cry* genes.

The relationship between the genetic pool of *cry* genes and microbial diversity of the host bacterium permit the

Table 3 Continued

Crop	Gene	Expression levels	Signal system	Reference
Cabbage	t*cry1Ab* t*cry1B*	Not determined	Promoter:35S CaMV Terminator:tNOS S.M.:NPTII(Kan)	[184] [185] [119]
Sweet potato	*cry3A*	Not determined	Promoter:35S CaMV Terminator:tNOS S.M.:NPTII(Kan)	[69]
Alfalfa	PM*cry3A*	Not determined	Promoter:35S CaMV Terminator:tNOS S.M.:NPTII(Kan)	[196]

Legend:
S.M. – selection marker.
Prefix:
t – truncated gene.
w – wild gene.
PM – partially modified gene.
TM – totally modified gene.
Signal system:
2′ManS: 2′ promoter fragment of TR DNA. AMV: Leader sequence from the alfalfa mosaic virus coat protein gene.
3′T7: Polyadenylation site of the TL DNA gene 7.
35S CaMV: Cauliflower Mosaic Virus 35S promoter.
aad(Spt/Str): Spectinomycin-Streptomycin resistance gene.
aphiV(Hygr): Hygromycin resistance gene.
bar(Basta): Basta resistance gene.
GUS: β-Glucuronidase gene.
hpn(Hygr): Hygromycin resistance gene.
NPTII(Kan): Neomycin phosphotransferase II.
orf25 TDNA: T DNA poliadenylation site.
t35S CaMV: Cauliflower Mosaic Virus 35S transcription terminator.
tNOS: Nopalin Sinthetase transcriptional terminator.

screening and classification of *Bacillus thuringiensis* isolates using the advantages of molecular systematics. The rich *Bacillus thuringiensis* biodiversity and the wide arsenal of its insecticidal compounds promote this bacterium as the most important biological control agent to date and a promising base for the development of a worldwide environmentally-safe pesticide industry.

Acknowledgments

We thank MS Orlando L Pardo (CIGB Vaccine Division, Cuba), BS Ariel F Martínez (Havana University, Cuba) and Dr Reynol Farrera (CINVESTAV, Mexico) for style corrections and help with the English version. We are very grateful to Dr Viviana Falcón and the CIGB electronmicroscopy team for the electromicrographs of sporulating *Bt*. cells, as well as to Elfos Scientiae publishers (Havana, Cuba) for authorizing us to reproduce these figures. Special acknowledgments are due to Dr Donald H Dean (Ohio State University, USA) for unpublished data on the classification of Cry proteins, Dr David J Ellar (University of Cambridge, UK) for drawing of the Cry3A three-dimensional structure, and to Dr Marguerite-M Lecadet (Institute Pasteur, France) for the information on *B. thuringiensis* strain serotyping. Our work is partially supported by the Cuban Council of State (Grant 3031/234–95). Roberto I Vázquez-Padrón is a grantee of the International Foundation for Science (Grant F/2560–1).

References

1 Adang JM, MS Brody, G Cardineau, N Eagan, RT Roush, CK Shewmaker, A Jones, JV Oakes and K McBride. 1993. The reconstruction and expression of a *Bacillus thuringiensis cryIIIA* gene in protoplasts and potato plants. Plant Mol Biol 21: 1131–1145.
2 Adang MJ, TA Rocheleau, DJ Merlo and EE Murray. 1990. Synthetic insecticidal crystal protein gene. EPO Patent Appl 0359472 A2.
3 Agaisse H and D Lereclus. Expression in *Bacillus subtilis* of the *Bacillus thuringiensis cryIIIA* toxin gene is not dependent on a sporulation-specific sigma factor and is increased in SpoOA mutant. J Bacteriol 176: 4734–4741.
4 Agaisse H and D Lerecluse. 1996. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? J Bacteriol 177: 6027–6032.
5 Alstad DN and DA Androw. 1996. Response to AR Ives: Evolution of insect resistance to *Bacillus thuringiensis*-transformed plants. Science 273: 1413.
6 Alstad DN and DA Androw. 1995. Managing the evolution of insect resistance to transgenic plants. Science 268: 1894–1896.
7 Angsuthanasombat C, N Crickmore and DJ Ellar. 1993. Effect on toxicity eliminating a cleavage site in a predicted interhelical loop in *Bacillus thuringiensis* CryIVB delta-endotoxin. FEMS Microbiol Lett 111: 255–262
8 Angsuthansombat C and S Panyim. 1989. Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadriplicatum* PR-6. Appl Environ Microbiol 55: 2428–2430.
9 Arencibia A, P Molina, C Gutierrez, V Greenidge, E Menéndez, GA de la Riva and G. Selman-Housein. 1992. Regeneration of transgenic sugarcane (*Saccharum officinarum* L) plants from intact meristematic tissue transformed by electroporation. Biotecnol Aplic 9: 156–165.
10 Arencibia A, P Molina, GA de la Riva and G Selman-Housein. 1995. Production of transgenic sugarcane (*Saccharum officinarum* L) plants by intact cell electroporation. Plant Cell Rep 14: 305–309.
11 Arencibia A, R Vázquez, D Prieto, E Carmona, P Tellez, A Coego, L

Hernandez, GA de la Riva and G Selman-Housein. 1996. Transgenic sugarcane (*Saccharum officinarum* L) plants are tolerant to stem borer (*Diatraea saccharalis* F) attack despite the low expression levels of *cryIA(b)* gene from *Bacillus thuringiensis* var *kurstaki* HD-1. Biotecnol Aplic 13: 130–131.

12 Arencibia A, R Vázquez, D Prieto, ER Carmona, P Téllez, A Coego, L Hernández, GA de la Riva and G Selman-Housein. 1997.Transgenic sugarcane plants resistant to stem borer attack. Mol Breeding 3: 247–255.

13 Aronson AI, D Wu and C Zhang. 1995. Mutageneseis of specificity and toxicity regions of a *Bacillus thuringiensis* protoxin gene. J Bacteriol 177: 4059–4065.

14 Bagci H, SR Shareef and K Ozdamar. 1991. Application of numerical taxonomy in classification of varieties of *Bacillus thuringiensis*. Doka Turk Biyol Derg 15: 70–81.

15 Bar E, J Lieman-Hurwitz, E Rahamim, A Keynan and N Sandler. 1991. Cloning and expression of *Bacillus thuringiensis* δ-endotoxins DNA in *Bacillus sphaericus*. J Invertebr Pathol 57: 149–158.

16 Barloy F, A Delécluse, L Nicolas and M Lecadet. 1996. Cloning and expression of the first anaerobic toxin gene from *Clostridium bifermentas* subsp *malaysia*, encoding a new mosquitocidal protein with homologies to *Bacillus thuringiensis* delta-endotoxins. J Bacteriol 178: 3099–3105.

17 Barton K, HR Whiteley and NS Yang. 1987. *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to Lepidopteran insects. Plant Physiol 85: 1103–1109.

18 Baunsgaard L, K Venema, KB Axelsen, JM Villalba, A Welling, B Wollenweber and MG Palmgren. 1996. Modified plant plasma membrane H^+-ATPase with improved transport coupling efficiency identified by mutant selection in yeast. Plant J 10: 451–458.

19 Bekheit HKM, AD Lucas, SJ Gee, RO Harrison and BD Hammock. 1993. Development of an ELISA for the β-exotoxin of *Bacillus thuringiensis*. J Agric Food Chem 41: 1530–1536.

20 Belfiore CJ, RK Vadlamudy, YA Osman and LA Bulla. 1994. A specific binding protein from *Tenebrio monitor* for the insecticidal toxin of *Bacillus thuringiensis* subs *tenebrionis*. Biochem Biophys Res Commun 200: 359–364.

21 Ben-Dov E, S Boussiba and A Zaritsky. 1995. Mosquito larvicidal activity of *Escherichia coli* with combination of genes from *Bacillus thuringiensis* subsp *israelensis*. J Bacteriol 177: 2851–2857.

22 Bosch D, B Schipper, H Van der Kleij, RA De Maagd and WJ Stiekema. 1994. Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. Bio/technology 12: 915–918.

23 Bourque SN, JR Valéro, J Mercier, C Lavoie and RC Levesque. 1993. Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*. Appl Environ Microbiol 59: 523–527.

24 Bravo A, S Jansens and M Peferoen. 1992. Immunocytochimical localization of *Bacillus thuringiensis* insecticidal Crystal proteins in intoxicated insects. J Invertebr Pathol 60: 237–253.

25 Bravo A, A Lorence and R Quintero. 1995. Biopesticides compatible with environment: *Bacillus thuringiensis*, a unique model. Biocontrol 1: 41–55.

26 Bravo A, H Agaisse, S Salamitou and D Lereclus. 1996. Analysis of CryIA(a) expression in *sigE* and *sigK* mutants of *Bacillus thuringiensis*. Mol Gen Genet 250: 734–741.

27 Brehm SP, SP Staal and JA Hoch. 1973. Phenotype of pleiotropic-negative sporulation mutants of *Bacillus subtilis*. J Bacteriol 115: 1063–1070.

28 Brizzard BL, HE Schnepf and JW Kronstad. 1991. Expression of the *cryIB* crystal protein gene of *Bacillus thuringiensis*. Mol Gen Genet 231: 59–64.

29 Bull AT. 1992. Isolation and screening of industrially important organisms. In: Recent Advances in Industrial Applications of Biotechnology (Vardar-Sukan F and SS Sukan, eds), pp 1–17, Kluwer Academic, Dordrecht, The Netherlands.

30 Bull AT, M Goodfellow and JH Slater. 1992. Biodiversity as a source of innovation in biotechnology. Ann Rev Microbiol 46: 219–252.

31 Campbell DP, DE Dieball and JM Brackett. 1987. Rapid HPLC assay for the β-exotoxin of *Bacillus thuringiensis*. J Agric Food Chem 35: 156–158.

32 Caramori T, AM Albertini and A Galizzi. 1991. *In vivo* generation of hybrids between two *Bacillus thuringiensis* insect-toxin-encoding genes. Gene 98: 37–44.

33 Carlson CR, DA Caugant and A-B Kolsto. 1994. Genotypic diversity among *Bacillus thuringiensis* and *Bacillus cereus* strains. Appl Environ Microbiol 60: 1719–1725.

34 Carozzi NB, GW Warren, N Desai, SM Jayne, R Lotstein, DA Rice, S Evola and MG Koziel. 1992. Expression of a chimeric CaMV 35S *Bacillus thuringiensis* insecticidal protein gene in transgenic tobacco. Plant Mol Biol 20: 539–548.

35 Carozzi NB, VC Kramer, GW Warren, S Evola and MG Koziel. 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl Environ Microbiol 57: 3057–3061.

36 Carroll J, J Li and DJ Ellar. 1989. Proteolytic processing of a coleopteran-specific delta-endotoxin produced by *Bacillus thuringiensis* var *tenebrionis*. Biochem J 261: 99–105.

37 Ceron J, L Covarrubias, R Quintero, A Ortiz, M Ortiz, E Aranda, L Lina and A Bravo. 1994. PCR analysis of the *cryI* insecticidal crystal family genes from *Bacillus thuringiensis*. Appl Environ Microbiol 60: 353–356.

38 Chak KF, DC Chao, MY Tseng, SS Kao, SJ Tuan and TY Feng. 1994. Determination and distribution of *cry*-type genes of *Bacillus thuringiensis* isolates from Taiwan. Appl Environ Microbiol 60: 2415–2420.

39 Chen JX, MK Lee and DH Dean. 1993. Site-directed mutations in a highly conserved-region of *Bacillus thuringiensis* delta-endotoxin affect inhibition of short circuit current across *Bombyx mori* midguts. Proc Natl Acad Sci USA 90: 9041–9045.

40 Choma CT and H Kaplan. 1990. Folding and unfolding of the protoxin from *Bacillus thuringiensis*: evidence that the toxin moiety is active in active conformation. Biochemistry 29: 10971–10977.

41 Chungjatupornchai W. 1990. Expression of the mosquitocidal protein genes of *Bacillus thuringiensis* subsp *israelensis* and the herbicide resistance gene bar in *Synechocystis* PCC6803. Curr Microbiol 21: 283–288.

42 Crickmore N, DR Zeigler, JS Feitelson, E Schnepf, D Lambert, J Lereclus, J Baum and DH Dean. 1995. Revision of the nomenclature for the *Bacillus thuringiensis cry* genes. 28th Annual Meeting of the Society for the Invertebrate Pathology, 16–21 July 1995, Ithaca, NY, USA.

43 Crissman JW, SC Causey, L Thorne and TJ Pollock. 1989. Accumulation of the insecticidal crystal protein of *Bacillus thuringiensis* subsp *kurstaki* in post-exponential-phase *Bacillus subtilis*. Appl Environ Microbiol 55: 2302–2307.

44 Damgaard PH, HD Larsen, BM Hansen, J Bresciani and K Jogensen. 1996. Enterotoxin-producing strains of isolates from food. Lett Appl Microbiol 23: 146–150.

45 De Barjac H. 1981. Identification of H-serotypes of *Bacillus thuringiensis*. In: Microbial Control of Pests and Plants Diseases (Burges HD, ed), pp 35–43, Academic Press, NY, USA.

46 De Barjac H and E Franchon. 1990 Classification of B.t. strains. Entomophaga 35: 233–240.

47 de la Pena A, H Lorz and J Schell. 1987. Transgenic rye plants obtained by injecting DNA into young floral tillers. Nature 325: 274–276.

48 De la Riva GA and M Adang. 1996. Expression of *Bacillus thuringiensis* δ-endotoxin genes in transgenic plants. Biotecnol Aplic 13: 251–260.

49 De Maagd RA, H Van der Klei, LP Bakker, WJ Stiekema and D Bosch. 1996. Different domain of *Bacillus thuringiensis* delta-endotoxin can bind to insect midgut membrane proteins on ligand blots. Appl Environ Microbiol 62: 2753–2757.

50 De Maagd RA, MSG Kwa, H Van der Kleij, T Yamamoto, B Schipper, JM Vlak, WJ Stiekema and D Bosch. 1996. Domain III substitution in *Bacillus thuringiensis* δ-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Appl Environ Microbiol 62: 1537–1543.

51 De Marsac MT, F de la Torre and J Szulmajster. 1987. Expression of the larvicidal gene of *Bacillus sphaericus* 1593M in the cyanobacterium *Anacystis nidulans* R2. Mol Gen Genet 209: 396–398.

52 Delannay X, BJ La Vallee, RK Proksch, RL Fuchs, SR Sims, JT Greenplate, PG Marrone, RB Dodson, JJ Augustine, JG Layton and DA Fischhoff. 1989. Field performance of transgenic tomato plants

expressing the *B. thuringiensis* var *kurstaki* insect control protein. Bio/technology 7: 1265–1269.
53 Delnolf P, S Jansens, M Perferoen, D Degeele and J Van de Rie. 1993. Two different *Bacillus thuringiensis* delta-endotoxin receptors in the midgut brush border membrane of the European corn borer, *Ostrinia nubilalis*. Appl Environ Microbiol 59: 1828–1837.
54 Donovan WP, CC Dankocsik and MP Gilbert. 1988. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp *israelensis*. J Bacteriol 170: 4732–4738.
55 Dow JAT, BJ Gupta, TA Hall and WR Harvey. 1984. X-ray microanalysis of elements in frozen hydrated sections of an electrogenic K^+ transport system: the posterior midgut of tobacco hornworn (*Manduca sexta*) *in vivo* and *in vitro*. J Membr Biol 77: 223–241.
56 Endo Y and J Nishiitsutsuji-Uwo. 1980. Mode of action of delta-endotoxin from *Bacillus thuringiensis*: histopathological changes in the silkworn midgut. J Invertebr Pathol 36: 90–103.
57 Endo Y and J Nishiitsutsuji-Uwo. 1981. Mode of action of delta-endotoxin from *Bacillus thuringiensis*: ultrastructural changes of midgut epithelium of Pieris, Lymrn and Ephestia larvae. Appl Ent Zool 16: 231–241.
58 Enríquez-Obregón GA, RI Vázquez-Padrón, D Prieto-Samsónov, M Pérez and G Selman-Housein. 1997. Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using antioxidant compounds. Biotecnol Aplic 14: 169–174.
59 Estruch JJ, GW Warren, MA Mullins, GJ Nye, JA Craig and MG Koziel. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc Natl Acad Sci USA 93: 5389–5394.
60 Farkas J, K Sebesta, K Horska, Z Samek, L Dolejs and F Sorm. 1969. The structure of exotoxin of *Bacillus thuringiensis* var *gelechiae*. Collect Czech Chem Commun 38: 1118–1120.
61 Farkas J, K Sebesta, K Horska, Z Samek, L Dolejs and F Sorm. 1976 Structure of thuringiensin, the thermostable exotoxin from *Bacillus thuringiensis*. Collect Czech Chem Commun 42: 909–996.
62 Feiltelson JS, J Payne and L Kim. 1992. *Bacillus thuringiensis*: insects and beyond. Bio/technology 10: 271–275.
63 Feldmann F, A Dullemans and C Waalwijk. 1995. Binding of CryIVD toxin from *Bacillus thuringiensis* subssp *israelenses* to larval dipteran midgut proteins. Appl Environ Microbiol 61: 2601–2605.
64 Feng Q and WJ Beckel. 1994. pH-induced conformational transitions of CryIA(a), CryIa(c) and CryIIIA delta-endotoxin in *Bacillus thuringiensis*. Biochemisty 33: 8521–8526.
65 Ferré J, B Estriche, Y Bel and J Van Rie. 1995. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis* insecticidal crystal proteins. FEMS Microbiol Lett 132: 1–7.
66 Fischhoff DA, KS Bowdish, FJ Perlak, PG Marrone, SM McCormick, JG Niedermeyer, DA Dean, K Kusano-Kretsmer, EJ Mayer, DE Rochester, SG Rogers and RT Fraley. 1987. Insect tolerant transgenic tomato plants. Bio/technology 5: 809–812.
67 Fromm M, L Taylor and V Walbot. 1985. Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82: 5824–5828.
68 Fromm M, L Taylor and V Walbot. 1986. Stable transformation of maize after gene transfer by electroporation. Nature 319: 791–793.
69 García R, J Mena, A López, J Rodríguez, Z Zaldua, D Somonte, E Pimentel, M García, M Gómez, GA de la Riva, R Morán and G Selman-Housein. 1995. Transgenic sweet potato plants resistant to pests. Field Performance. Adv Mod Biotech 3: II.72.
70 Garczynski SF, JW Crim and MJ Adahg. 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* delta-endotoxin by protein blot analysis. Appl Environ Microbiol 57: 2816–2820.
71 Ge AZ, D Rivers, R Milne and DH Dean. 1991. Functional domains of *Bacillus thuringiensis* insecticidal crystal proteins. J Biol Chem 266: 17954–17958.
72 Ge AZ, RM Pfister and DH Dean. 1990. Hyperexpression of a *Bacillus thuringiensis* delta-endotoxin-encoding gene in *Escherichia coli*: properties of the product. Gene 93: 49–54.
73 Gleave AP, R Williams and RJ Hedjes. 1993. Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B. thuringiensis* subsp *kurstaki*. Appl Environ Microbiol 59: 1683–1687.
74 Graham TL. 1988. Genetic engineering of soil microorganisms for pest control. Agric Ecosyst Environ 24: 317–323.
75 Graves A and S Goldman. 1986. The transformation of *Zea mays* seedling with *Agrobacterium tumefaciens*: detection of T-DNA specified enzymes activities. Plant Mol Biol 7: 43–50.
76 Grochulski P, L Masson, S Borisova, M Puztai-Carey, M Schawarts, R Brusseau and M Cygler. 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. J Mol Biol 254: 447–464.
77 Guerchicoff A, CP Rubinstein and RA Ugalde. 1996. Introduction and expression of an anti-dipteran toxin gene from *Bacillus thuringiensis* in nodulating Rhizobia. Cell Mol Biol 42: 729–735.
78 Haider MZ, B Knowles and DJ Ellar. 1986. Specificity of *Bacillus thuringiensis* var *colmeri* insecticidal delta-endotoxin is determinated by differential proteolytic processing by larval gut proteases. Eur J Biochem 156: 531–540.
79 Haider MZ and DJ Ellar. 1987. Characterization of the toxicity and cytopathic specifity of a cloned *Bacillus thuringiensis* crystal protein using insect tissue culture. Mol Microbiol 1: 59–66.
80 Hamilton AJ, M Bugayen and D Griersen. 1991. Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. Proc Natl Acad Sci USA 88: 7434–7437.
81 Harvey WR, M Cioffi and MG Wolfersberguer. 1986. Transport physiology of the lepidopteran midgut in relation to the action of B.t delta-endotoxin. In: Fundamental and Applied Aspects of Invertebrate Pathology (Vlan JM, D Peters and RA Samson, eds), pp 11–14, Grafish bedrijf Ponsen and Loijer, Wageningen, The Netherlands.
82 Harvey WR, DN Crawford, NS Eisen, V Fernandes, D Spaeth and MG Wolfersberguer. 1987. The potassium impermeable apical membrane of insect epithelia: a target for the development of safe insecticides. Mem Inst Oswaldo Cruz 82: 29–34.
83 Herrera Estrella L, A Depicker, M Van Montagu and J Schell. 1983. Chimeric genes are transferred and expressed in plant cells using a Ti plasmid-derived vector. Nature 303: 209–213
84 Herrera G, S Snyman and J Thompson. 1994. Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugarcane borer, *Eldana saccharina*. Appl Environ Microbiol 60: 682–690.
85 Hirt H. 1996. In and out of the plant cell cycle. Plant Mol Biol 31: 459–464.
86 Hodgman TC and DJ Ellar. 1990. Models for the structure and functions of the *Bacillus thuringiensis* delta-endotoxin determined by compilational analysis. DNA Sequence 1: 97–106.
87 Hofmann C, H Vanderbruggen, H Höfte, J Van Rie, S Jansens and H Van Mellaert. 1988. Specificity of *Bacillus thuringiensis* delta-endotoxin is correlated with the presence of high-affinity binding sites in the brush border membrane of the target insect midguts. Proc Natl Acad Sci USA 85: 7844–7848.
88 Hofmann C, P Lüthy, R Hötter and V Pliska. 1988. Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of cabbage butterfly (*Pieris brassicae*). Environ J Biochem 173: 85–91.
89 Höfte H and H Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 53: 242–255.
90 Höfte H, H de Greve, J Seurinck, S Jansens, C Mahillon, J Vandekerckhove, H Vanderbruggen, M Van Montagu, M Zabeau and M Vaeck. 1986. Structural and functional analysis of a cloned delta-endotoxin of *Bacillus thuringiensis* var *berliner* 1715. Eur J Biochem 161: 273–280.
91 Höfte H, J Van Rie, S Jansens, A Van Houtven, H Vanderbruggen and M Vaeck. 1988. Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis*. Appl Environ Microbiol 54: 2010–2017.
92 Hooykaas G, P Hooykaas and R Schilperoot. 1984. Expression of Ti plasmid genes into monocotyledonous plants infected with *Agrobacterium tumefaciens*. Nature 311: 763–764.
93 Hori H, S Asano and K Ogiwara. 1996. Fractionation and some features of synergistic compound which enhances the insecticidal activity of δ-endotoxin of *Bacillus thuringiensis*. Appl Ent Zool 31: 29–35.
94 Horsch RB, JE Fry, NL Hoffman, M Wallroth, D Eichholtz, SG Rogers and RT Fraley. 1985. A simple and general method for transferring genes into plants. Science 227: 1229–1231.
95 Huber-Lucac M, F Jaquet, P Lüthy, R Hütter and DG Braun. 1986.

Characterization of monoclonal antibodies to a crystal protein of *Bacillus thuringiensis* subsp *kurstaki*. Infect Immun 54: 228–232.
96 Ikezawa H. 1991. Bacterial PIPLCs—unique properties and usefulness in studies of GPI anchors. Cell Biol Int Rep 15: 1115–1131.
97 Ives AR. 1996 Evolution of insect resistance to *Bacillus thuringiensis*-transformed plants. Science 273: 1412–1413.
98 Johnson DE and WH McGaughey. 1996. Contribution of *Bacillus thuringiensis* spores to toxicity of purified Cry proteins towards indianmeal moth larvae. Curr Microbiol 33: 54–59.
99 Jong JZ, DY Hsiun, WT Wu and YM Tzeng. 1995. Fed-batch culture of *Bacillus thuringiensis* for thuringiensin production in a tower-type bioreactor. Biotech Bioengin 48: 207–213.
100 Kalman S, KL Kiehne, JL Libs and T Yamamoto. 1993. Cloning of a novel *cryIC*-type gene from a strain of *Bacillus thuringiensis* subsp *galleriae*. Appl Environ Microbiol 59: 1131–1137.
101 Knight PJK, N Crickmore and DJ Ellar. 1994. The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopectidase N. Mol Microbiol II: 429–436.
102 Knowles B. 1994. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin. Adv Insect Physiol 24: 275–3076.
103 Knowles HB and AT Dow. 1993. The crystal delta-endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. BioEssays 15: 462–469.
104 Kobayashi T, H Tamura, R Taguchi, S Udaka and H Ikezawa. 1996. High-level expression of *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C by the *Bacillus brevis* host-vector system. Jpn J Med Sci Biol 49: 103–112.
105 Koni PA and DJ Ellar. 1993. Cloning and characterization of a novel *Bacillus thuringiensis* cytolitic delta-endotoxin. J Mol Biol 229: 319–327.
106 Kotska SJ, PW Reeser and DP Miller. 1988. Abstr Annu Meet Am Phytopathol Soc, abstr No. 222.
107 Koziel GM, GL Beland, C Bowman, NB Carozzi, R Crenshaw, L Crossland, J Dawson, N Desai, M Hill, S Kadwell, K Launis, K Lewis, D Maddox, K McPherson, M Meghji, E Merlin, R Rhodes, GW Warren, M Wright and S Evola. 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/technology 11: 194–200.
108 Kronstad JW and HR Whiteley. 1986. Three classes of homologous *Bacillus thuringiensis* crystal-protein genes. Gene 43: 29–40.
109 Kuo WS and KF Chak. 1996. Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. Appl Environ Microbiol 62: 1369–1377.
110 Lereclus D, H Agaisse, M Gominet and J Chaufaux. 1995. Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis* SpoOA mutant. Bio/technology 13: 67–71.
111 Li J, PA Koni and DJ Ellar. 1996. Structure of the mosquitocidal delta-endotoxin CytB from *Bacillus thuringiensis* and implications for membrane pore formation. J Mol Biol 257: 129–152.
112 Li J, J Carroll and DJ Ellar. 1991. Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5A resolution. Nature 353: 815–821.
113 Lörz H, B Baker and J Scell. 1985. Gene transfer to cereal cells mediated by protoplast transformation. Mol Gen Genet 199: 473–497.
114 Martin PAW and RS Travers. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl Environ Microbiol 55: 2437–2442.
115 Masson L, M Bossé, G Préfontaine, L Péloquin, PCK Lau and R Brousseau. 1990. Characterization of parasporal crystal toxins of *Bacillus thuringiensis* ssp-*kurstaki* strains HD-1 and NRD-12: use of oligonucleotide probes and cyanogen bromide mapping. In: American Chemical Society Symposium Series, 198th National Meeting (Hickle LA and WL Fitch, eds), pp 432–1989, Miami Beach, FL, USA.
116 McBride KE, Z Svab, DJ Schaaf, PS Hogan, DM Stalker and P Maliga. 1995. Amplification of a chimeric *Bacillus thuringiensis* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Bio/technology 13: 362–365.
117 McGaughey WH and ME Whalon. 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. Science 258: 1451–1455.
118 McPherson SA, FJ Perlak, RL Fuchs, PG Marrone, B Lavrik and DA Fischhoff. 1988. Characterization of the coleopteran-specific protein gene of *Bacillus thuringiensis* var *tenebrionis*. Bio/technology 6: 61–66.
119 Menéndez E, GA de la Riva, E Borrego, R Armas and G Selman-Housein. 1995. *Agrobacterium* mediated transformation for the production of transgenic cabbage (*Brassica oleracea*) cv Hercules plants. Biotecnol Aplic 13: 142
120 Metz T, RT Roush, JD Tang, AM Shelton and ED Earle. 1995. Transgenic broccoli expressing a *Bacillus thuringiensis* insecticidal crystal protein: Implications for plant resistance management strategies. Mol Breeding 1: 309–317.
121 Miteva V, A Abadjieva and R Grigorova. 1991. Differentiation among strains and serotypes of *Bacillus thuringiensis* by M13 DNA fingerprinting. J Gen Microbiol 137: 593–600.
122 Moens S and J Vanderleyden. 1996. Functions of bacterial flagella. Crit Rev Microbiol 22: 67–100.
123 Molina P, B Goyenechea, R Morán, L Romero, GA de la Riva and S Pérez. 1991. Actividal larvicida en plantas transgénicas de tabaco. Biotecnol Aplic 8: 191–198.
124 Murphy RC and SE Stephens Jr. 1992. Cloning and expression of the *cryIVD* gene of *Bacillus thuringiensis* subsp *israelensis* in the cyanobacterium *Agmenellum quadriplicatum* PR-6 and its resulting larvicidal activity. Appl Environ Microbiol 58: 1650–1655.
125 Murray EE, TR Rochleau, M Eberle, C Stock, V Sekar and M Adang. 1991. Analysis of unstable transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts. Plant Mol Biol 16: 1035–1060.
126 Nambiar P, S Ma and B Iyer. 1990. Limiting and insect infestation of nitrogen-fixing root nodule of the *Pigeon pea* (Cajanus cajan) by engineering the expression of an entomocidal gene in its root nodules. Appl Environ Microbiol 56: 2866–2869.
127 Nicholls CN, W Ahnmad and DJ Ellar. 1989. Evidence for two diferent types of dual specifity insecticidal P2 toxins in *Bacillus thuringiensis* subspecies. J Bacteriol 171: 5141–5147.
128 Nishimoto T, H Yoshisue, K Ihara, H Sakai and T Komano. 1994. Functional analysis of block 5, one of the highly conserved amino acid sequences in the 130-kDa CryIVA protein produced by *Bacillus thuringiensis* subsp *israelensis*. FEBS Lett 348: 259–254.
129 O'Donnell AG, HJH McFie and JR Norris. 1980. An investigation of the relation between *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* using pyrolisis gas-liquid chromatography. J Gen Microbiol 119: 189–194.
130 Obukowicz MG, FJ Perlack, K Kusanokretzmer, EJ Meyer and LS Watrud. 1986. Integration of δ-endotoxin gene of *Bacillus thuringiensis* into the cromosome of root colonizing strain of pseudomonads using Tn5. Gene 45: 327–331.
131 Oddou P, H Hartmann, F Radecke and M Geiser. 1993. Immunologically unrelated *Heliotis* sp and *Spodoptera* sp midgut membrane-proteins bind *Bacillus thuringiensis* CryIA(b) delta-endotoxin. Eur J Biochem 212: 145–150.
132 Ohba M and K Aizawa. 1986. Distribution of *Bacillus thuringiensis* strains in soils of Japan. J Invertebr Pathol 47: 277–282.
133 Ohba M, K Ueda and K Aizawa. 1992. Serotyping of *Bacillus thuringiensis* environmental isolates by extracellular heat-stable somatic antigens. Can J Microbiol 38: 694–695.
134 Ordúz S, N Restrepo, MM Patiño and W Rojas. 1995. Transfer of toxin genes to alternate bacterial hosts for mosquito control. Mem Inst Oswaldo Cruz 90: 97–100.
135 Pang Y, R Frutos and BA Federici. 1992. Synthesis and toxicity of full length and truncated bacterial CryIVD mosquitocidal proteins expressed in lepidopteran cells using a baculovirus vector. J Gen Virol 73: 89–101.
136 Parker MW and P Pattus. 1993. Rendering a membrane protein soluble in water: a common packing motif in bacterial protein toxins. TIBS 18: 390–395.
137 Parrot WA, JN All, MJ Adang, HR Bailey, HR Boerma and CN Stewart. 1994. Recovery and evaluation of a soybean plants transgenic for a *Bacillus thuringiensis* var *kurstaki* insecticidal gene. In vitro Cell Dev Biol 30P: 144–149.
138 Perlak FJ, RW Deaton, TA Armstrong, RL Fuchs, SR Sims, JT Greenplate and DA Fischhoff. 1990. Insect resistant cotton plants. Bio/technology 8: 939–943.
139 Perlak FJ, RL Fuchs, DA Dean, SL McPherson and DA Fischhoff. 1991. Modification of the coding sequence enhances plant expression

of insect control protein genes. Proc Natl Acad Sci USA 88: 3324–3328.
140 Perlak FJ, TB Stone, YM Muskopf, LJ Petersen, GB Parker, SL McPherson, J Wyman, S Love, G Reed, G Biever and DA Fischhoff. 1993. Genetically improved potatoes: protection from damage by colorado potato beetles. Plant Mol Biol 22: 313–321.
141 Potrikus I. 1989. Gene transfer to cereals: an assessment. Trends Biotech 8: 33–38.
142 Prefontaine G, P Fast, PCK Lau, MA Hefford, Z Hanna and P Brousseau. 1987. Use of oligonucleotide probes to study the relatedness of delta-endotoxin genes among *Bacillus thuringiensis* subspecies and strains. Appl Environ Microbiol 53: 2808–2814.
143 Prieto D, J González, A Martínez, C Ayra-Pardo, GA de la Riva and RI Vázquez. 1996. Biodiversidad de *Bacillus thuringiensis* en Cuba: metodología para la detección de genes de proteínas insecticidas en los aislamientos nacionales. In: Proceedings of the First Workshop on Culture Collections (Instituto Finlay, ed), p 12, Havana, Cuba.
144 Prieto D, R Vázquez, J González and GA de la Riva. 1995. Obtainment of subclass-specific polyclonal antibodies against *Bacillus thuringiensis* δ-endotoxins by selective immunopurification. Adv Mod Biotech 3: II.74.
145 Rainieri D, P Bottino, M Gordon and E Nester. 1990. Agrobacterium mediated transformation of rice (*Oriva sativa* L). Bio/technology 8: 33–38.
146 Rajamohan F, JA Cotrill, F Gould and DH Dean. 1996. Role of domain II, loop 2 residues of *Bacillus thuringiensis* CryIAb delta-endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliotis virescens*. J Biol Chem 271: 2390–2396.
147 Rentsch D, B Hirner, E Schmelzer and WB Frommer. 1996. Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. Plant Cell 8: 1437–1446.
148 Rippka R, J Deruelles, JD Waterbury, M Hernan and RY Stanier. 1979. Genetic assignments, strain histories and properties of pure culture of cyanobacteria. J Gen Microbiol 111: 1–61.
149 Sacchi VF, P Parenti, GM Hanozet, B Giordana, P Lüthy and MG Wolfersberger. 1986. *Bacillus thuringiensis* inhibits K^+ gradient dependent amino acid transport across the brush border membrane of *Pietris brassicae* midgut cell. FEBS Lett 204: 213–218.
150 Saiki RK, F Gaeertner and A Wong. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491.
151 Samasanti W, A Tojo and K Aizawa. 1986. Insecticidal activity of bipiramidal and cuboidal inclusions of δ-endotoxins and distribution of their antigens among various strains of *Bacillus thuringiensis*. Agric Biol Chem 50: 1731–1735.
152 Sanchis V and DJ Ellar. 1993. Identification and partial purification of a *Bacillus thuringiensis* CryIC delta-endotoxin binding protein from *Spodoptera littoralis* gut membranes. FEBS Lett 3: 264–268.
153 Sanchis V, D Lereclus, G Menou, J Chaufaux, S Guo and M Lecadet. 1989. Nucleotide sequence and analysis of the N-terminal coding region of the *Spodoptera*-active δ-endotoxin gene of *Bacillus thuringiensis* var *aizawai* 7.29. Mol Microbiol 3: 229–238.
154 Sanford J. 1988. The biolistic process. Trends Biotechnol 6: 299–302.
155 Sangadala S, FS Walters, LH English and MJ Adang. 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and Rb^+K^+ efflux *in vitro*. J Biol Chem 269: 10088–10092.
156 Schnepf E and HR Whiteley. 1981. Cloning and expression of *B. thuringiensis* crystal protein gene in *E. coli*. Proc Natl Acad Sci USA 84: 7036–7040.
157 Shillito R, M Saul, J Paszkowski, M Müller and I Potrikus. 1985. High efficiency direct transfer to plants. Bio/technology 3: 1099–1103.
158 Shimizu M, K Oshie, K Nakamura, Y Takada, K Oeda and H Ohkawa. 1988. Cloning and expression in *E. coli* of the 135-kDa insecticidal protein gene from *Bacillus thuringiensis* subsp *aizawai* IPL7. Agric Biol Chem 52: 1565–1573.
159 Shimizu T, DG Vassylyev, S Kido, Y Doi and K Morikawa. 1994. Crystal structure of vitelline membrane outer layer protein I (VMI-I): a folding motif with homologous Greek key structures related by an internal three-fold symetry. EMBO J 13: 1003–1010.
160 Shin BS, SH Park, SK Choi, BT Koo, ST Lee and JI Kim. 1995. Distribution of *cryV*-type insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV*-type genes from *Bacillus thuringiensis* subsp *kurstaki* and *Bacillus thuringiensis* subsp *entomocidus*. Appl Environ Microbiol 61: 2402–2407.
161 Shivakumar AG, GJ Gunling, TA Benson, D Casuto, F Miller and BB Spear. 1986. Vegetative expression of the δ-endotoxin genes of *Bacillus thuringiensis* subsp *kurstaki* in *Bacillus subtilis*. J Bacteriol 166: 194–204.
162 Shivakumar AG, RI Vanags, DR Wilcox, L Katz, PS Vary and JL Fox. 1989. Gene dosage effect on the expression of the delta-endotoxin genes of *Bacillus thuringiensis* subsp *Kurstaki* in *Bacillus subtilis* and *Bacillus megaterium*. Gene 79: 21–31.
163 Siden I, G Dalhmmar, B Telander, HG Boman and H Somerville. 1979. Virulence factors in *Bacillus thuringiensis*: purification and properties of a protein inhibitor of immunity in insects. J Gen Microbiol 114: 45–52.
164 Singh Bora R, MG Murty, R Shenbagarathai and V Sekar. 1994. Introduction of a lepidopteran specific insecticidal crystal protein gene of *Bacillus thuringiensis* subsp *kurstaki* by conjugal transfer into a *Bacillus megaterium* strain that persists in the cotton phyllosphere. Appl Environ Microbiol 60: 214–222.
165 Slatin SL, CK Abrams and L English. 1990. Delta-endotoxins form cation selective channel in planar lipid bilayer. Biochem Biophys Res Commun 169: 765–772.
166 Smith GP and DJ Ellar. 1994. Mutagenesis of two surface-exposed loops of the *Bacillus thuringiensis* CryIC delta-endotoxin affects insecticidal specificity. Biochem J 302: 611–616.
167 Soltes-Rak E, DJ Kushner, DD Williams and JR Coleman. 1993. Effect of promoter modification on mosquitocidal *cryIVB* gene expression on *Synechococcus* sp strain PCC7942. Appl Environ Microbiol 59: 2404–2410.
168 Sreekrishna K, WD Prevatt, GP Thill, GR Davis, P Koutz, KA Barr and SA Hopkins. 1993. Production of *Bacillus* entomotoxins in methylotrophic yeast. EPO Patent Appl 586892A1.
169 Stock CA, TJ McLoughlin, JA Klein and MJ Adang. 1990. Expression of *Bacillus thuringiensis* crystal protein gene in *Pseudomonas cepacia* 526. Can J Microbiol 36: 879–884.
170 Tabashnik BE. 1994. Evolution of resistance to *Bacillus thuringiensis*. Ann Rev Entomol 39: 47–79.
171 Tabashnik BE, N Fison and MW Johnson. 1991. Managing resistance to *Bacillus thuringiensis*: lessons from the diamondback moth (Lepidoptera: Plutellidae). J Econ Entomol 84: 49–55.
172 Thanabalu T, J Hindley, S Brenner, C Oei and C Berry. 1992. Expression of the mosquitocidal toxins of *Bacillus thuringiensis* subsp *israelensis* by recombinant *Caulobacter crescentus*, a vehicle for biological control of aquatic insect larvae. Appl Environ Microbiol 58: 905–910.
173 Thorne L, F Garduno, T Thompson, D Decker, M Zounes, M Wild, AM Walfield and T Pollock. 1986. Structural similarity between the Lepidoptera- and Diptera-specific insecticidal toxin genes of *Bacillus thuringiensis* subsp *kurstaki* and *israelensis*. J Bacteriol 166: 801–811.
174 Trissicook M, S Pontuwatana, A Bhumiratana and W Panbangred. 1990. Molecular cloning of the 130 kilodalton mosquitocidal delta-endotoxin gene from *Bacillus thuringiensis* subsp *israelensis* in *Bacillus sphaericus*. Appl Environ Microbiol 56: 1710–1716.
175 Turner JT, JS Lampel, RS Stearman, GW Sundin, P Gunyuslu and JJ Anderson. 1991. Stability of the δ-endotoxin gene from *Bacillus thuringiensis* subsp *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp *cynodontis*. Appl Environ Microbiol 57: 3522–3528.
176 Uchimiya H, T Fushimi, H Hashimoto, H Harada, K Syono and Y Sugawara. 1986. Expression of a foreign gene in callus derived from DNA-treated protoplasts of rice (*Oryza sativa* L). Mol Gen Genet 204: 204–207.
177 Vadlamudi RK, TH Ji and LA Bulla. 1993. A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp *berliner*. J Biol Chem 268: 12334–12340.
178 Vaeck M, A Reynaerts, H Höfte, S Jansens, M De Beukeleer, C Dean, M Zabeau, M Van Montagu and J Leemans. 1987. Transgenic plants protected from insect attack. Nature 328: 33–37.
179 van der Salm T, D Bosh, G Honee, L Feng, E Munsterman, P Bakker, WJ Stiekema and B Visser. 1994. Insect resistance of transgenic plants that express modified *Bacillus thuringiensis cry1Ab* and *cry1Ac* genes: a resistance management strategy. Plant Mol Biol 26: 51–59.

180 Van Rie J, S Jansen, H Höfte, D Degheele and H Van Mellaert. 1989. Specificity of *Bacillus thuringiensis* delta-endotoxin: importance of specific receptors on the brush border membrane of the midgut of target insect. Eur J Biochem 186: 239–247.
181 Vázquez R, D Prieto, GA de la Riva and G Selman-Housein. 1996. Development of an immunoradiometric assay for quantitative determination of CryIAb protein in transgenic sugarcane plants. J Immunol Meth 196: 33–39.
182 Vázquez R, D Prieto, MI Olóriz, GA de la Riva and G Selman-Housein. 1995. Heterologous expression of recombinant δ-endotoxins from *Bacillus thuringiensis* in *E. coli*: comparative study in three transcriptional systems. Rev Lat-Amer Microbiol 37: 237–244.
183 Vázquez RI, D Prieto, A Martínez and GA de la Riva. 1996. P210k: the putative binding protein for *Bacillus thuringiensis* CryIA(c) δ-endotoxin in the midgut brush-border membrane of the lepidopteran *Diatraea saccharalis*. Biotecnol Aplic 13: 117.
184 Vázquez RI, D Prieto, J González and GA de la Riva. 1995. Development of combinatorial genetic constructions containing truncated versions of *cryIA(b)* and *cryIB* genes from *Bacillus thuringiensis* for further production of autopesticide cabbage plants. Adv Mod Biotech 3: II.73.
185 Vazquez RI, J Gonzalez, D Prieto, GA de la Riva and G Selman-Housein. 1996. Obtención de plantas transgénicas de col (*Brassica oleraceae* var Hercules 31) con resistencia putativa al ataque de *Plutella xylostella*. Abstract of III International Coloquium on Plant Biotechnology. Plant Biotechnology Institute, Las Villas Central University, June 23–26, p 87, Santa Clara, Cuba.
186 Visser B. 1989. A screening for the presence of four different crystal protein gene types in 25 *Bacillus thuringiensis* strains. FEMS Microbiol Lett 58: 121–124.
187 Von Tersch MA, SL Slatin, CA Kuleza and LH English. 1994. Membrane-permeabilizing activities of *Bacillus thuringiensis* coleopteran-active toxin CryIIIB2 and CryIIIB2 domain I peptide. Appl Environ Microbiol 60: 3711–3717.
188 Ward ES and DJ Ellar. 1988. Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp *israelensis* which encode 130-kilodalton mosquitocidal proteins. J Bacteriol 170: 727–735.
189 Ward ES, AR Ridley, DJ Ellar and JA Todd. 1996. *Bacillus thuringiensis* var *israelensis* δ-endotoxin. Cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. J Mol Biol 191: 13–22.
190 Watanabe K, M Toyoda, H Hashimoto, K Nakagawa, K Nakamura, Y Yabusaki, A Tanimura and Y Saito. 1996. Expression of 135-kDa insecticidal protein gene from *Bacillus thuringiensis* in the yeast *Saccharomyces cerevisiae*. Biosci Biotech Biochem 60: 1483–1485.
191 Whalon ME and DL Norris. 1996. Resistance management for transgenic *Bacillus thuringiensis* plants. Biotech Dev Monitor 29: 8–12.
192 Whalon ME, DL Miller, RM Hollingworth, EJ Grafius and JR Miller. 1993. Selection of a Colorado beetle (Coleoptera: Chrysomelidae) strain resistant to *Bacillus thuringiensis*. J Econ Entomol 86: 226–233.
193 Whiteley HR and HE Schnepf. 1986. The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. Ann Rev Microbiol 40: 549–576.
194 Wolfersberguer MG. 1990. The toxicity of two *Bacillus thuringiensis* delta-endotoxins to Gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. Experientia 46:475–477
195 Wünn J, A Klöti, G Burkhardt, GCG Biswas, K Launis, VA Iglesias and I Potrykus. 1996. Transgenic Indica rice breeding line IR58 expressing a synthetic *cry1Ab* gene from *Bacillus thuringiensis* provides effective insect pest control. Bio/technology 14: 171–176.
196 Yamamoto T and GK Powell. 1993. *Bacillus thuringiensis* crystal proteins: recent advances in understanding its insecticidal activity. In: Advanced Engineered Pesticides (Leo Kim, ed), Marcel Dekker, NY, USA.
197 Yap WH, T Thanabalu and AG Porter. 1994. Expression of mosquitocidal toxin genes in a gas-vacuolated strain of *Ancylobacter aquaticus*. Appl Environ Microbiol 60: 4199–4202.
198 Yoshida K, K Sen, H Sakai and T Komano. 1989. Expression of two 130-kDa protein genes of *Bacillus thuringiensis* var *israelensis* in *Bacillus subtilis*. Agric Biol Chem 53: 3033–3035.
199 Xue Jun Chen, A Curtiss, E Alcantara and D Dean. 1995. Mutations in domain I of *Bacillus thuringiensis* δ-endotoxin Cry1A. Reduce the irreversible binding of toxin manduca sexta brush border membrane vesicles. J Biol Chem 270: 6412–6419.