Diversity in cry genes of Bacillus thuringiensis

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INTRODUCTION

The *Bacillus thuringiensis* Berliner research began when the Japanese bacteriologist, Ishiwata (1901) isolated the bacillus from diseased *Bombyx mori* larvae. He named it “sottokin”, which means “sudden death bacteria” in Japanese. A decade later, Ernst Berliner (1915) isolated a similar organism from diseased granary populations of *Ephestia kuehniella* Zell (Mediterranean flour moth) larvae obtained from Thueringen, Germany, and named the bacterium *Bacillus thuringiensis* (Bt). As Ishiwata did not formally describe the organism he found, Berliner is credited with naming it. Application of Bt was first reported by Husz in 1928 who isolated a Bt strain from *Ephestia* and tested it on European corn borer, *Ostrinia nubilalis* (Kumar et al., 1996). In 1960, stimulated by the growing concern over the use of chemical insecticides, a Bt strain was commercialized for the first time and marketed as “Thuricide”. Slowly, the first strain was replaced by a more potent strain (HD-1) isolated by Dulmage (1970).

In earlier days, it was believed that Bt was mostly active against lepidopterans, until Goldberg and Margalit (1977) isolated a strain *Bacillus thuringiensis israelensis* from a mosquito breeding pond in Negev desert, which proved highly toxic to mosquito and blackfly larvae. Later in 1983, Kreig and co-workers investigated a strain from dead mealworm pupae and designated as *Bacillus thuringiensis tenebrionis* which was found to be highly toxic to elm leaf beetle and Colorado potato beetle grubs. These findings ultimately led to more screening programs and now there are thousands of Bt isolates in various collections. The advancement
in genetic engineering and molecular biology in the early eighties led to the cloning of Bt crystal protein (*cry*) gene for the first time successfully (Wasano *et al.*, 2001). Initially several *cry* genes were expressed in plant colonizing microorganisms to target the stem and root dwelling insect pest. Today, the most efficient way to deliver *cry* genes seems to be the development of transgenic plants expressing them (Betz *et al.*, 2000; Kannaiyan, 2000; De Gosa *et al.*, 2001).

**CLASSIFICATION OF Bt SUBSPECIES**

Bt is a gram positive, spore forming bacteria that exists in a diverse locations, such as the soil, plant surfaces, insect cadavers and in grain storage dusts (de Maagd *et al.*, 1999). During the sporulation stage of its life cycle, Bt produces one or more proteinaceous crystal inclusions (Fig.1), possessing varying degrees of insecticidal activity (Bulla *et al.*, 1977). The presence of these parasporal crystals in the sporangia and their insecticidal activity has been the unique character that was available for differentiating Bt from its closely related species, *Bacillus cereus* (Claus and Berkeley, 1986 and Andrews *et al.*, 1987).

Helgason *et al.* (2000) observed a close similarity of the genomes of *Bacillus anthracis* strain to those of *Bacillus thuringiensis* and *Bacillus cereus* strains and suggested that they should be considered as belonging to one and the same species. These three species show differences in their phenotypes mostly due to plasmid borne genes.


In early days, Bt strains were classified into sub species based mainly on morphological and biochemical characters (Lynch and Baumann, 1985 and de Barjac and Franchon, 1990). In recent times, scientists used different methods for classification such as phage-typing (Ackermann et al. 1995), esterase pattern of vegetative cells (Norris, 1964), crystal serology (Lynch and Baumann, 1985), plasmid pattern (Lereclus et al., 1982), oligonucleotide probing (Prefontaine et al., 1987), and H -Flagellar serotyping (de Barjac and Frachon, 1990).

Although several methods were tried for classification, serotyping using’ H’ flagellar antigen remains the most widely used, simplest and practical method to classify Bt strains (de Barjac and Frachon, 1990). In 1993, Bouroque and co workers reported that there might be variation in the biochemical characters, plasmid patterns, shape, stability and the insecticidal activity of the Bt isolates even if placed within the same serotypes. A list of 80 Bt serotypes based on H serotyping is given in Table 1 (Lecadet et al., 1998).
Table 1. Serovars of *Bacillus thuringiensis* strains

<table>
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<th>S.No</th>
<th>Serovar</th>
<th>H.antigen</th>
<th>Toxicity*</th>
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</table>

* Toxic to L: lepidopteran larvae; D: dipteran larvae; C: coleopteran larvae (Based on Lecadet et al., 1998)


INSECTICIDAL CRYSTAL PROTEINS OF Bt

Cry protein synthesis

The insecticidal crystal proteins are formed during sporulation stage of the bacterium's life cycle. About 20 -30 per cent of the dry weight of the matured sporangium is accounted for these proteins (Lilley et al., 1980). Crystal formation can be observed microscopically during the later part of stage II (Somerville., 1971 and Mikkola et al., 1982). In Bt H-14, electron microscopic studies revealed the presence of nascent inclusions even at stage I (Abdel-Hameed et al., 1990). Once crystal formation is initiated, crystal protein is synthesised until the end of stage IV (Lecadet and Dedonder, 1971), although crystals may continue to enlarge until stage VI. Over 80 per cent of crystal proteins produced during sporulation is synthesised de novo from amino acids supplied by break down of proteins in the sporulation process (Monro, 1961). It is believed that all subunits of the crystals are synthesised simultaneously (Lecadet and Dedonder, 1971).

Structural features of Cry proteins

The crystal morphology in Bt is highly complex and it shows different forms like bipyramidal, cuboidal, spherical, squares and irregular (Chilcott and Wigley, 1994). These various forms of true crystals have been observed using phase contrast and electron microscopes (Huber and Luthy, 1981). Bipyramidal crystals synthesised in host cells are typically about 1.1 \( \mu \text{m} \) long and
0.511µm wide (Oeda et al., 1989). Bipyramidal crystals show a greater frequency of toxicity than all the other types. Most of the lepidopteran active isolates contain such inclusions (Attamthom et al., 1995); cuboidal crystals are active against lepidopteran and dipteran larvae or lepidopteran larvae alone (Yamamoto and Mclauzhtin, 1981); spherical and irregular crystals are mostly mosquitocidal, often active against certain coleopteran species (Krieg et al., 1983). Irregular crystals also include those with very little or no identified toxicity (Zelanzy et al., 1994). Based on Cry protein composition, the crystals have various forms. Most Cry1 type proteins form bipyramidal crystals, Cry2 type assume cuboidal form and the Cry3 proteins form flat rhomboidal crystals. The crystals formed by Bt israelensis are typically spherical. Many investigations carried out to study the chemical nature of the toxin showed that it is a glycoprotein. The toxicity of Cry proteins was affected by the glycosylation process (Bulla et al., 1977). Protein content ranges from 88 to 95 per cent and carbohydrates value range from 0.5 to 12 per cent have been reported. Further analysis carried out showed, crystals of Bt were found to contain 0.36 per cent silicon by weight and significant concentration of Ca, Fe and Mg as revealed by emission spectroscopy (Meenakshi et al., 1993 and Abdel-Hameed et al., 1991). The crystal toxin is insoluble in water or inorganic solvents, but soluble in alkaline solvents. Cry1 proteins are soluble at pH 9.5, while the Cry2 proteins are soluble at a pH of about 12. Similarly, Cry4A, Cry5B and Cyt toxins are soluble at pH 9.5, while the Cry4O toxin requires a pH of 12. The Cry3A toxin on the other hand, dissolves at pH's
below 4 and above 9.5. The crystals can also be dissolved at neutral pH in the presence of detergents and denaturing agents like urea, β-mercaptoethanol, OTT and SOS (Gill et al., 1992). The presence of cysteine residues at the C-terminal part of protoxin is attributed to the relative insolubility and the requirement of denaturing and reducing agents for further solubilization (Hofte and Whiteley, 1989). Separation of crystals from the spores and cell debris involves isopycnic centrifugation in sucrose or cesium chloride gradients (Fast, 1972), zonal gradient centrifugation using NaBr (Ang and Nickerson, 1978). In addition, relatively large scale and rapid purification of crystals from a broad range of Bt strains is possible by linear and discontinuous gradients using Renongrafin-76 (66.7% N-methylglucamine and 94% sodium salt of 3, 5 diacentamido 2, 4, 6, Tridobenzoic acid) (Sharpe et al., 1975).

Bt toxins usually termed protoxins are activated by the insect midgut proteases to yield the toxic fragment. Gill et al. (1992) reported that the larger protoxin of about 130-140 kDa undergoes proteolysis yielding a toxic fragment of 60-70 kDa derived from N-terminal half of the protoxin. Structurally the activated toxin can be divided into 3 structural regions.

I. N-terminal region, which is the toxic domain (amino acid sequence 1-279) consisting of several conserved hydrophobic regions.

II. A conserved C-terminal region (amino acid sequence 461-695) and


III. A variable region between these two region that contains most of the amino acid differences.

Hofte and Whiteley (1989) investigated sequences among a number of toxins and found five well conserved regions and designated as blocks from one to five. According to this it was predicted that the crystal toxin, consists of three domains namely

Domain I: Consists of a bundle of seven antiparallel $\alpha$-helices in which helix five is enriched by the remaining helices.

Domain II: Consists of three antiparallel $\beta$-Sheets.

Domain III: It has two twisted antiparallel $\beta$-sheets.

This has been confirmed by X-ray crystallographic studies (Li et al. 1991; Grochulski et al., 1995). The long hydrophobic and amphipathic helices of domain I suggest that this domain might be responsible for the formation of lytic pores in the intestinal epithelium of the target organism. The involvement of Domain II in receptor binding was supported by site directed mutagenesis and segment swapping experiments (Rajamohan et al., 1996; Jurat-Fuentes and Adang, 2001). The $\beta$-sandwich structure of domain III could play a number of roles. The main function consisting of maintaining the structural integrity of the toxin molecule, mostly it shields the molecule from proteases during proteolysis within the gut of the target pest (Mackinnon and Miller, 1989 and Li et al., 1991). The insecticidal bacterium


Bt synthesizes δ-endotoxin Cry proteins in two size classes, 135 and 70 kDa, and both form crystalline inclusions in cells after synthesis. Crystallization of 135-kDa proteins is due to intermolecular attraction of regions in the C-terminal half of the molecule, and the N-terminal half fails to crystallize when synthesized in vivo. In this crystallization process domain I of Cry3A plays a vital role (Park and Federici, 2001).

Mode of action of Cry protein

The Cry protein when ingested by the insect is first converted from the inactive form to active toxin protein by proteolysis in the alkaline mid gut region (Huber and Luthy, 1981; Lightwood et al., 2000), which then diffuses through peritrophic membrane and binds to high affinity receptors present on the midgut epithelium. When the activated toxin binds to the specific receptors (Hoffmann et al., 1988; Maclintosh et al., 1991), the interaction of receptor-toxin becomes irreversible and generates pores on the membrane. The formation of pore eventually disturbs the ionic gradient, further leading to the swelling of microvilli and destruction of epithelial membrane, leading to the cell death.

CLASSIFICATION OF cry GENES

The crystal proteins of Bt have been studied for their insecticidal properties and their high natural levels of production. The increasingly rapid characterization of new crystal protein genes, triggered by an effort to discover proteins with new insecticidal properties have made the classification of cry genes
more difficult. Earlier scientists used arbitrary designations like icp (McClenden et al., 1985), Bta (Sanchis et al., 1989), P1 and P2 (Donovan et al., 1988), type B and type C (Hofte et al., 1988) and 4.5 Kb and 6.6 Kb (Kronstad and Whiteley, 1986). The first attempt to systematically classify Bt crystal genes were undertaken by Hofte and Whiteley (1989). The systematic designations followed by Hofte and Whiteley were as follows. The principal toxicity spectrum was denoted by Roman letters (from I to IV) grouped under different classes. Within the class major and minor amino acid differences were denoted by upper and lower case letter, respectively (eg: cryIAa). Since, Bt genes encoding cytolytic proteins were totally unrelated to cry genes, they were designated as cyt genes. In general the term ‘cry’ (first given by Heid et al., 1982) was followed to designate the genes encoding crystal proteins and Cry for toxins.

**cry 1 Class**

The cry1 genes code for lepidopteran specific bipyramidal crystal proteins having molecular range of proteins having molecular range of 130 -140 kDa. Upon proteolytic cleavage they yield 60 -70 kDa core toxin fragment. The Cry1 proteins have related amino acid sequence; they display greater than 55 per cent identity (Hofte and Whiteley, 1989). The cry 1 gene class contains subclasses of genes (cry1 A to K). The carboxy terminal half of the all cry I encoded proteins are highly conserved, whereas the N terminal end is more variable. N-terminal variable region

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represents the active fragment whereas the conserved C-terminal region is involved mostly in crystallization.

**cry 2 Class**

The cry2 class of genes encode about 65 - 71 kDa proteins which form cuboidal inclusions during sporulation. It has three subclasses (cry2A, cry2B, cry2C). The Cry1 proteins share about 80 - 90 per cent amino acid sequence homology, but are dissimilar to the other Cry proteins, except in the first, N-terminal conserved domain. The Cry2 protein are toxic to either lepidopteran or lepidopteran and dipteran larvae (Widner and Whiteley, 1989).

**cry 3 Class**

It encodes 73 kDa coleopteran specific proteins (CryIII) (Herrnstadt et al., 1987). There are 5 sub classes (CryIII A to E) in this group of genes. Cry3 A and Cry3 B share about 67 per cent amino acid sequence identity and are homologous to the amino terminal half of the Cry1 protoxins. Cry3 D shows limited homology to other Cry3 toxins. The Cry3C encode proteins which form bipyramidal crystals unlike other Cry3 crystals which form rhomboidal proteins (McPherson et al., 1988).

**cry 4 Class**

This class of genes is composed of a mixed group of dipteran -specific crystal protein genes. This class contains 4 sub classes namely cry4A, cry4B, cry4C and cry4D encoding polypeptides with predicted molecular mass of 135, 128, 74 and
72kDa respectively. The Cry4C and Cry4D are naturally occurring truncated versions. The protoxins are protelytically activated to a toxic fragment of undetermined molecular weight (Chilcott and Ellar, 1988; Chungjatupomchai et al., 1988).

**Cyt genes**

*Cyt* genes encode a 27 kDa polypeptide which shows no homology to other crystal protein genes. These genes are cytolytic or haemolytic for a variety of invertebrate and vertebrate cells (Thomas and Ellar, 1983).

**Other cry genes**

The *cry 5* class of genes encode a protein having molecular weight of about 80 kDa. They show toxicity towards coleopteran and lepidopteran larvae. The *cry 6* class on the other hand are reported to exhibit activity against nematodes (Feitelson et al., 1992).

Apart from these well-characterized crystal proteins, there are several proteins which do not seem to be active against any of the insects tested (Martin and Travers, 1989).

**Crickmore classification of Bt genes**

When using Hofte and Whiteley system of classification it had certain limitations like


i. Toxins showing homology have different insecticidal spectrum.

Eg: CryllA is toxic to both lepidopterans and dipterans, whereas CrylIB is toxic to lepidopteran larvae only. But they are grouped together.

ii. Since, it is mainly function based, bioassay of new toxin against target organism is a must.

Recently, in 1998 under the chairmanship of Neil Crickmore, a standing committee of the Bacillus Genetic Stock Centre (BGSC) was instituted in assigning names to new cry and Cyt genes. Based on the recommendations of the committee Crickmore et al. (1998) revised the nomenclature for the cry and crt genes. The Table 2 shows a list of holotype toxins. In this newly revised system, a broad definition was given to Cry Protein: a parasporal inclusion (crystal) protein from Bt that exhibits some experimentally verifiable toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein. Similarly Cyt protein denotes a parasporal inclusion (Crystal) protein from Bt that exhibit haemolytic activity, or any protein that has obvious sequence similarity to a known Cyt protein.


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Hardy, A. G. and Quinlan, R. 1986. *Bacillus thuringiensis* and control of plant pests. *MIRCEN*, 2: 87-90


To organize the wealth of data produced by genome sequencing efforts, a new nomenclatural paradigm is emerging, exemplified by the internationally recognized cytochrome P 450 superfamily nomenclature system (White et al., 1998). The revised nomenclature is based mainly on the above system both in conceptual basis and in nomenclature format.

Some of the important features of revised nomenclature are:

I. The cry genes whose products share < 45% amino acid sequence homology are characterized by different Arabic numbers, designated as primary ranks (eg. cry1, cry2 etc.).

II. The cry genes of the same primary rank whose products show < 78% amino acid sequence homology are differentiated by secondary ranks by using uppercase letters (eg. cry1A, cry1B etc.).

III. The cry genes having same primary and secondary ranks whose products share less than 95% amino acid sequence homology receive separate tertiary rank, designated by lowercase letters (eg. cry1Aa, cry1Ab etc.).

IV. The cry genes whose products are different in amino acid sequence, but are more than 95% identical to each other are given separate quaternary ranks by another Arabic number (eg. cry1Aa1, cry1Aa2 etc.).

Under the revised system almost all cry genes retained the names they received under Hofte and Whiteley (1989) after a substitution


of Arabic for Roman numerals. There are few notable exceptions *cry*1G becomes *cry*9A, *cry*1IIc becomes *cry*7Aa, *cry*1ID becomes *cry*3C, *cry*1VC becomes *cry*10A, *cry*1VD becomes *cry*11A, *crt* becomes *cry*1A and *crtB* becomes *cry*2A. The known Cry and Cyt proteins now fall into 30 sets including Cyt1, Cyt2 and Cry1 through Cry28.

**LOCALIZATION AND REGULATION OF cry GENES**

The majority of *cry* genes are localized in the self replicating plasmids, having a size ranging from 140 to 150 MDa (Lereclus *et al.*, 1989; Whiteley and Schnepf, 1986). The role of plasmids were evident, after the cloning of the toxin structural genes were used as probes to determine their localization in most of the known strains (Lereclus *et al.*, 1989; Whiteley and Schnepf, 1986). Further investigation showed that in some strains (Bt var. *entomocidus*, 8t var. *aizawal*) *cry* genes are localized on the chromosome (Udayasuriyan *et al.*, 1996; Sekar *et al.*, 1987). The involvement of transposons as a part of *cry* genes composite structure was first demonstrated by a variety of approaches (Kronstad and Whiteley, 1984; Lereclus *et al.*, 1984). The *cry*4A gene, in the dipteran active Bt *israelensis* was flanked by 2 repeated sequences in opposite orientation (80urgouin *et al.*, 1988). In most of the *cry*2 family of crystal protein genes, they are present as a part of operon. Widner and Whiteley (1989), Wu *et al.* (1991) reported that *cry*2Aa and *cry*2Ac are distal part of operon containing two more open reading frames.

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17
The synthesis of crystal proteins in Bt is controlled by a variety of mechanisms occurring at the transcriptional, post transcriptional and post translational levels. The development of sporulation is controlled by the successive activation of sigma factors which binds to the core RNA polymerase to direct the transcription from sporulation specific promoters (Moran, 1993). The cry1Aa gene is a typical example of a sporulation dependent cry gene expressions. At post transcriptional level the stability of the cry mRNA is an important factor for high level of toxin production in Bt. The half life of cry mRNA is about 10 min, which is almost five fold greater than the half-life of an average bacterial mRNA (Glatron and Rapoport, 1972). One of the possible reasons for cry mRNA stability is due to the presence of a stem loop structure at 3' end which acts as a positive retroregulator by preventing 3' 5' exoribonuclease activity on cry mRNA.

CLONING OF cry GENES

The main objective of cloning cry gene using recombinant DNA methodologies is to improve Bt strains available for better production and to improve the toxicity. The first ever report on cloning of Bt crystal protein gene was reported in 1981. Schenpf and Whiteley (1981) cloned a crystal protein gene from Bt subsp. kurstaki and expressed in E. coli. The recombinant strains of E. coli synthesised a 130 kDa protein that showed positive reaction with antibody raised against a peptide of the same size from Bt crystals. In 1993, Kalman et al., cloned a new cry1C gene


(cry1Cb) from *Bt gallariae*, based on distinct electrophoretic mobility of PCR products amplified using specific primers. Chak et al. (1994) placed a cry1C gene, along with the alpha-amylase promoter from *B. subtilis* in a *B. thuringiensis*-derived cloning vector, generating a pair of recombinant plasmids pSB 744 and pSB 745 and expressed in *Bt subsp kurstaki cry-B* and HD73. Shin et al. (1995) cloned two cryV type genes, cryV1 and cryV465, from *Bt kurstaki* HD-1 and *Bt subsp entomocidus* BP 465 respectively and determined their nucleotide sequence. The cloned CryV1 protein was toxic to *Plutella xylostella* Lin. and *Bombyx mori*, whereas the CryV465 protein showed toxicity towards *Plutella xylostella* only.

Delecluse et al. (1995) cloned a mosquitocidal gene, designated cry11 B, encoding a 81 kDa crystal protein in *Bt subsp jegathesan*. The sequence of Cry11 B protein showed high homology towards Cry11A toxin (Cry1VD) from *Bt subsp israelensis*. A novel mosquitocidal protein gene, *cry20Aa* was cloned from *Bt subsp fukuokaensis* (H-3a; 3d: 3e). The gene product was naturally truncated and had a molecular weight of 86 kDa. The amino acid comparisons showed cry20Aa to be an entirely different protein (Lee and Gill, 1997). Two new crystal protein genes, *cry19A* and Orf2, were isolated from *Bt subsp jegathesan* encoding a 74.7 kDa protein and 60 kDa protein respectively. The orf2 amino acid sequence is very much similar to carboxy terminus of Cry4 proteins (Rosso and Delecluse, 1997). Sasaki et al (1997) cloned a cry2A-type gene from *Bt
serovar sotto SKWO1 -10.2-06 and designated as cry2 (SKW). The cloned protein was toxic to Bombyx marp. The Cry11a was active on both Plutella xylostella and Bombyx man. A new Cry11d protein was toxic to Plute/a xylos/e/a as Cry11a but less active on Bombyx man (Choi et al., 2000). The Cry2Aa of a new Bt strain (47-8) was effective against Helicoverpa armigera, cotton bollworm (Lenin et al., 2001).

EXPRESSION OF cry GENES IN RECOMBINANT BACTERIA

For many years conjugation (Gonzalez J.M. Jr and Carlton; 1992) and transduction (Lecadet et al., 1992) have been used to transfer recombinant plasmids. At present a variety of E.coli -Bt shuttle vectors have been constructed to facilitate the introduction of cloned cry genes in Bt. Some of these plasmids employ replicons derived from other Gram positive bacteria (eg: PBC16, PC194) while others employ replicons isolated from native Bt plasmids (Chak et al., 1994). In addition to these shuttle vectors, integrational vectors have been used to insert cloned cry genes into resident plasmids (Lereclus et al., 1992; Adams et al., 1994) or into the chromosome (Kalman et al., 1995), by homologous recombination. In several cases, the cloned cry gene transfer into Bt host strain has resulted in improved spectrum for toxicity (Chak et al., 1994; Lereclus et al, 1992; Kalman et al, 1995).

Heterologous promoters may also be used to improve the expression of certain cry genes, including promoters for


B. subtilis a.-amylase gene (Chak et al., 1994), cry3Aa (Baum and Malvar, 1995) and cry3Bb (Agaisse and Lereclus, 1995). The unique feature of these cry genes, the cry3Aa and cry3Bb gene is that it is sporulation-independent and may be induced or depressed during stationary phase, probably by transition-phase regulators. These sporulation-independent promoters may be useful in improving the production of sporulation-dependent Cry proteins. Heterologous recombination may be used, not only to integrate cry genes into a resident plasmid, or into the chromosome, but also to disrupt genes of interest.

In recent times, enhancing the stability of crystal proteins under field conditions has had a great attention. To combat this, scientists went for DNA manipulations and expressed the cloned cry genes in other microorganisms preferably plant associating microbes. The first report of such an expression was established, when Monsanto scientists successfully expressed a cry1Ab gene into a root colonizing bacterium Pseudomonas fluorescens. After that numerous attempts were made to introduce cry genes into a variety of organisms, for improved environmental delivery of Cry proteins (Table 3)
Table 3. Transgenic microbes as delivery agents for Cry proteins

<table>
<thead>
<tr>
<th>S.No</th>
<th>Cry proteins</th>
<th>Microbial host</th>
<th>Advantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cry1Ac</td>
<td><em>P. fluorescens</em></td>
<td>Foliar resistance</td>
<td>Cell Cap2 – product of mycogen crop since 1987</td>
</tr>
<tr>
<td>2.</td>
<td>Cry3A</td>
<td><em>Rhizobium leguminosarum</em></td>
<td>Control of root feeding insects of legumes</td>
<td>Skok et al. (1990)</td>
</tr>
<tr>
<td>3.</td>
<td>Cry1Ac</td>
<td><em>P. cepacia</em></td>
<td>Control of <em>Maduca sexta</em> &amp; <em>Heliothis virescens</em> Tobacco</td>
<td>Stock et al. (1990)</td>
</tr>
<tr>
<td>4.</td>
<td>Cry4B</td>
<td><em>Caulobacter crescentus</em></td>
<td>Control of mosquito larvae in aquatic environment</td>
<td>Thanabal et al. (1992)</td>
</tr>
<tr>
<td>5.</td>
<td>CryIVD</td>
<td><em>Agmenellum quadruplicatum PR-6</em> (Cyanobacterium)</td>
<td>Control mosquito larvae</td>
<td>Murphy and Stevens Jr. (1992)</td>
</tr>
<tr>
<td>6.</td>
<td>Cry1Aa</td>
<td><em>Bacillus megaterium</em></td>
<td>Prolonged protection of cotton plants against <em>Helicoverpa armigera</em></td>
<td>Bora et al. (1992)</td>
</tr>
<tr>
<td>7.</td>
<td>Cry1Ac</td>
<td><em>Clavibacter xyli</em></td>
<td>Control of internally feeding insects</td>
<td>Incide R product of crop genesis international, Lampel et al. (1994)</td>
</tr>
<tr>
<td>8.</td>
<td>Cry2A</td>
<td><em>Bacillus cereus</em></td>
<td>Control of <em>Heliothis virescens</em> on <em>Tomato</em></td>
<td>Moar et al. (1994)</td>
</tr>
<tr>
<td>9.</td>
<td>Cry1Aa</td>
<td><em>Azospirillum lipoferum</em></td>
<td>Control of hidden pest was aimed but transgene was not stable</td>
<td>Udayasuriyan et al. (1995)</td>
</tr>
<tr>
<td>11.</td>
<td>Cry1Ae</td>
<td><em>P. fluorescens</em></td>
<td>Control sugarcane borer <em>Eldana saccharina</em></td>
<td>Herrera et al. (1997)</td>
</tr>
</tbody>
</table>


INSECT RESISTANCE TO CRY PROTEINS

The increasing use of chemical insecticides led to the development of resistance and resurgence among insect populations. This led to the switching over of chemical insecticides to Bt-based products and bioformulations. In 1985, McGaughey reported that Indian meal moth populations from grain storage bins treated with Bt formulation showed high LC50 values relative to populations in untreated bins. Later resistance to Bt insecticides was reported from field population Plutella xylostella (Tabashnik et al., 1990). Recently de Maagd et al. (1999) showed that Bt toxins are not an exception for insects to develop resistance. Development of resistance to CrylIA and Cry1Ac proteins have been reported in Colorado potato beetle and in Plutella xylostella (Wierenga et al., 1996 and Tang et al., 1999). Globally the development of resistance to P. xylostella towards Bt subsp kurstaki has been reported from Florida (Shelton et al., 1993), Japan (Hama et al., 1992), The Philippines (Ferre et al., 1991) and China. Recently laboratory studies reveal 76 fold increase in resistance towards Cry1Ac α-endotoxin for Helicoverpa armigera (Hubner) by the end of the 10th generation (Kranthi et al., 2000). Due to the escalation of resistance to Bt α-endotoxins in recent times, scientists were forced to undertake studies for understanding the mechanism involved in resistance development in insects. A variety of studies conducted on different resistant populations shows that the primary reason of resistance
lies in the lowering of the affinity of the toxin to the brush border membrane (Hoffman et al., 1988 and Ferre et al., 1991). In contrast, studies made by Gould et al. (1992) and Tabashnik (1994) demonstrated cross resistance to Bt toxins. The reasons for this cross resistance can be attributed to proteolysis of protoxins or decreased solubilization of crystals in the midgut of the larvae. The inheritance of resistance to Bt toxins is also studied (Chaufux et al., 1997, Hama et al., 1992, Ramirez et al., 1995 and Bentur et al., 2000b).

SEARCH FOR NEW Bt PROTEINS

The insecticidal proteins of Bt are characterized by their non-toxicity to mammals. Bt is a major source for transfer of genes into plant genomes to impart insect resistance. A few kind of insecticidal crystal protein genes (cry1Ab and cry 1 Ac) of Bt are now widely used to develop insect resistant transgenic crops. Despite an earlier view that insects would not develop resistance to Bt toxins, now it is realized that insect resistance to Bt toxins can evolve under situations of continuous exposure and or selection pressure. This has caused a great concern for continuous use of transgenic plants with a single kind of Bt toxin. Due to differential binding specificity to the insect midgut membrane vesicle, a new Bt toxin may be effective in avoiding or at least delaying the development of insect resistance by alternate or combinatorial use.

integrate the use of a wide range of crop-protection agents, e.g., synthetic, naturally-occurring and behaviour modifying chemicals together with biorational, microbial and cultural methods.

REFERENCES


Commercial Bt-based bioinsecticides, are usually the formulations of spores and crystalline inclusions that are released upon lysis of Bt during its stationary phase of growth. The products are applied at 10-50 g or about 10-20 molecules per acre. The molecular potency of Bt toxins is higher compared to that of other pesticides, 300 times higher than synthetic pyrethroids or 80,000 times higher than organophosphates (Feitelson et al., 1992). Most of the Bt product is based on subsp. Kurstaki strain HD-1. It is effective on over 200 crops and against more than 55 lepidopteran species, whereas subsp. tenebrionis and/or san diego are effective against coleopteran insects, such as Colorada potato beetle (Table 5).

Bt products account for 90-95% of total biopesticide market. About 75% of Bt market is in North America and Far East (Lambert and Peferoen, 1992). Efforts are needed to expand the use of Bt in other parts of the world in order that the use of hazardous chemical pesticides is reduced. There is great need for higher potency Bt toxins to have effective control over less susceptible and/or hidden pests such as H. armigera. and S. litura (Hardy and Quinlon, 1986; Perlak et al., 1990; Whitlock et al., 1991). Loss due to a single polyhagous pest (H. armigera) alone is estimated at about Rs. 1000 crores per year in India (Singh, 1996). Variation of a single amino acid in the Bt toxin can remarkably influence the level of toxicity (Udayasuriyan et al., 1994; Rajamohan et al., 1995). The discovery rate of new Bt toxin is more because of its genome diversity. Therefore, it is reasonable to search for new Bt toxins which are more effective against insect
OUR studies on screening of indigenous Bt strains for toxicity (Fig. 2) and molecular characterization of new Bt strains revealed difference in nature and composition of Bt toxins, between native and standard Bt strains. Hence, cloning of toxin genes from new Bt strains and their characterization are essential to expand the use of Bt and for the management of resistance development in insects.

**CONCLUSION**

Due to the various constraints that has emerged with the use of chemical pesticides such as, development of resistance and resurgence in insects, residual toxicity, secondary outbreak of minor insect pests and biological magnification in the ecosystem, scientists are now looking for new avenues to control or manage insect pests. One of the earliest solutions that was answered to the above problems was the use of biological agents in pest control. Among the various options that are available as on date, use of soil bacterium, the Bt has a great potential for use as a biopesticide.

In environmental impact terms, the narrow spectrum of biopesticides such as Bt has been seen as an advantage. However, this has proved to be a double-edged sword in that, in commercial terms, a broader spectrum of activity is usually required (Cannon, 1993). In practice, these demands are not mutually exclusive, and the recent explosion of research into Bt biology has yielded novel isolates (and an increasing number of *cry* genes) with

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**Table 5 Natural and genetically modified B.t products registered for agricultural use**

<table>
<thead>
<tr>
<th>BT STRAIN</th>
<th>COMPANY</th>
<th>PRODUCT</th>
<th>TARGET INSECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(a) Natural</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kurstaki HD-1</td>
<td>Abbot, USA</td>
<td>Biobit, Dipel, Foray</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>kurstaki HD-1</td>
<td>Thermo Trilogy, USA</td>
<td>Javelin, Steward, Thuriode</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>kurstaki</td>
<td>Abbot</td>
<td>Bactospeine, Futura</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>kurstaki</td>
<td>Thermo Trilogy</td>
<td>Able, Costar</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>aizawai</td>
<td>Abbot</td>
<td>Florbac, Xentari</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>teinebrio</td>
<td>Abbot</td>
<td>Novodar</td>
<td>Coleoptera</td>
</tr>
<tr>
<td>teinebrio</td>
<td>Thermal Trilogy</td>
<td>Trident</td>
<td>Coleoptera</td>
</tr>
<tr>
<td>kurstaki</td>
<td>BioDalia, Israel</td>
<td>Bio-Ti</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>kurstaki</td>
<td>Rimi, Israel</td>
<td>Bitnyon (granular feeding baits)</td>
<td>Lepidoptera, Trachedra amydraula</td>
</tr>
<tr>
<td><strong>(b) Genetically modified</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aizawai recipient</td>
<td>Thermo Trilogy</td>
<td>Agree, Design</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>kurstaki donor</td>
<td>Ecogen, USA</td>
<td>Condor, Cutlass (transconjugant), CRYMAX, Leptino</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>kurstaki recipient</td>
<td>Ecogen</td>
<td>Leptinox (recombinant)</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>kurstaki</td>
<td>Ecogen</td>
<td>Raven (recombinant)</td>
<td>Lepidoptera, Coleptera</td>
</tr>
<tr>
<td>kurstaki</td>
<td>Mycogen, USA</td>
<td>MVP</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>d-endotoxin</td>
<td>Mycogen, USA</td>
<td>MATTCH</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>MTRACK</td>
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<td>Coleptera</td>
</tr>
<tr>
<td>fluorescens</td>
<td>CellCap ®</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Based on Navon (2000)