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Bacillus thuringiensis
A genomics and proteomics perspective

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Key words: *Bacillus thuringiensis,* cadherin receptors, cry toxins, parasporal crystals, sporulation, toxin-receptor interaction

Abbreviations: AC, adenylyl cyclase; AFLP, amplified fragment length polymorphism; BC, *Bacillus cereus* group; Bt, *Bacillus thuringiensis*; ORF, open reading frame; PKA, protein kinase A; TBR, toxin-binding region

Bacillus thuringiensis (Bt) is a unique bacterium in that it shares a common place with a number of chemical compounds which are used commercially to control insects important to agriculture and public health. Although other bacteria, including *B. popilliae* and *B. sphaericus*, are used as microbial insecticides, their spectrum of insecticidal activity is quite limited compared to Bt. Importantly, Bt is safe for humans and is the most widely used environmentally compatible biopesticide worldwide. Furthermore, insecticidal Bt genes have been incorporated into several major crops, rendering them insect resistant, and thus providing a model for genetic engineering in agriculture.

This review highlights what the authors consider the most relevant issues and topics pertaining to the genomics and proteomics of Bt. At least one of the authors (L.A.B.) has spent most of his professional life studying different aspects of this bacterium with the goal in mind of determining the mechanism(s) by which it kills insects. The other authors have a much shorter experience with Bt but their intellect and personal insight have greatly enriched our understanding of what makes Bt distinctive in the microbial world. Obviously, there is personal interest and bias reflected in this article notwithstanding oversight of a number of published studies. This review contains some material not published elsewhere although several ideas and concepts were developed from a broad base of scientific literature up to 2010.

Background and History

The concept and practice of utilizing microorganisms to control insect pests is not new.[1,2] Pest control strategies in prehistoric times are mentioned in the writings of ancient Egyptian and Chinese scholars. One story relates the practice by gardeners of several Egyptian pharaohs of maintaining bacterial collections for use against insects that attacked and ravaged the gardens surrounding their houses and tomb chapels. Later, in the third century, maladies of insects, most likely occasioned by bacteria, viruses and fungi, were observed. Indeed, Aristotle[3] described in his writings insect diseases such as foulbrood of the honey bee (*Apis millifera*). Louis Pasteur studied silkworm diseases and differentiated pebrine and flacherie diseases of the silkworm *Bombyx mori.* Also, Kirby[4] and Bassi[5] made significant contributions to the area of insect pathology, and they, along with Pasteur are considered among the pioneers of infectious disease and pathogenic microbiology.

The era of Bt had its beginning when, in 1901, a Japanese scientist named Shigetane Ishiwata isolated a bacterium from dead silkworm larvae while he was investigating the cause of the so-called "sotto disease" (sudden-collapse disease). The disease was responsible for the loss of large numbers of silkworms in Japan and the surrounding region. Ishiwata named the bacterium *Bacillus sotto.*[6] A few years thereafter, in 1911, a German scientist Ernst Berliner isolated a related strain from dead Mediterranean flour moth larvae he found in a flour mill in the German state of Thuringia. He appropriately named the organism *Bacillus thuringiensis.* Berliner studied the bacterium and found inclusion bodies or "Restkorper" alongside the endospore.[7,8] The year was 1915. Mattes[9] in 1927 again observed the same inclusion bodies in Bt but it was not until much later (25 years) that insecticidal activity was attributed to these highly refractile bodies now referred to as "parasporal crystals," a phrase coined by Christopher Hannay in 1953.[10] Once the significance of the parasporal crystals was realized by Thomas Angus, he promptly demonstrated in the same year the insecticidal activity of the inclusion bodies.[11] And, together with Philip Fitz-James, Hannay in 1955 discovered that the toxic parasporal crystals are composed of protein.[12]

The first commercial insecticide based on Bt, Sporine, was produced in France in 1938 and used primarily to control flour moths. In the United States, Bt was first manufactured commercially in 1958 and, by 1961, Bt-based bioinsecticides were being registered by the US Environmental Protection Agency. Since 1996, insect-resistant transgenic crops, known as Bt crops, have expanded around the globe and are proving to be quite efficient and helpful in reducing the use of chemical insecticides.[13,14] Latest estimates indicate that more than 50% of the cotton and 40% of the corn planted in the US are genetically engineered to produce Bt insecticidal toxins. The current global market for pesticides (herbicides, insecticides, fungicides, nematicides and fumigants) is valued at $25.3 billion. Biopesticides represent only 2.5% of

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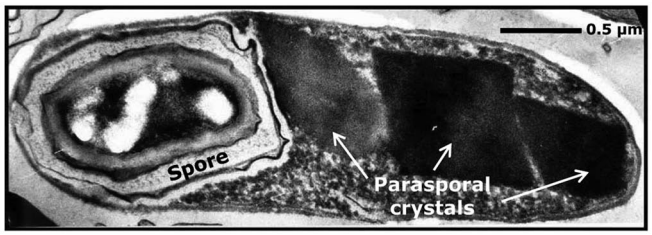

Figure 1. Transmission electron micrograph of a sporulated cell of *Bacillus thuringiensis* subsp. *morrisoni* strain C18 (x 44,000). Strain C18 was isolated from dead cotton bollworm larvae. The strain is unique in that it harbors 15 *cry* genes. The host range of C18 includes three major insect orders: Lepidoptera, Diptera and Coleoptera as well as nematodes. The parasporal crystals of C18 vary in shape and size (arrows), and represent ~50% of the cell dry weight.

this market but their share is expected to increase to about 4.2%, or more than $1 billion, in 2010.

Interestingly, some strains of Bt produce non-insecticidal proteins that crystallize into irregular-shaped parasporal inclusions.[15] Inclusions of one isolate treated with protease were toxic to human cancer cells, including leukemic T (MOLT-4) and cervical cancer cells (HeLa).[16] Cytotoxicity was dose-dependent. Another non-insecticidal protein, parasporin, also showed strong cytotoxic activity against MOLT-4 and HeLa cells.[17]

Life Cycle of *Bacillus thuringiensis*

The life cycle of Bt is characterized by two phases which include vegetative cell division and spore development, otherwise referred to as the sporulation cycle.[18] The vegetative cell is rod-shaped (2–5 µm long and about 1.0 µm wide) and divides into two uniform daughter cells by formation of a division septum initiated midway along the plasma membrane. Sporulation, on the other hand, involves asymmetric cell division and is characterized by seven stages[19] which include (stage I) axial filament formation, (stage II) forespore septum formation, (stage III) engulfment, first appearance of parasporal crystals and formation of a forespore, (stages IV to VI) formation of exosporium, primordial cell wall, cortex and spore coats accompanied by transformation of the spore nucleoid and (stage VII) spore maturation and sporangial lysis. **Figure 1** portrays a fully sporulated cell of Bt in which there are several parasporal crystals lying along side the endospore. The production of crystal proteins by Bt during sporulation is a unique genetically regulated biological phenomenon that, probably, relieves stress physically by offsetting water loss during spore formation and affords an additional survival advantage by exerting lethal action against host insects. In turn, the toxic action provides sufficient host nutrients to allow germination of the dormant bacterial spore and its return to vegetative growth.

Classification and Taxonomy of *Bacillus thuringiensis*

As indicated above, vegetative cells of Bt are characterized as large stout rods that are straight or slightly curved with rounded ends. They usually occur in pairs or short chains. Bt is Gram-positive, non-capsulated and motile with peritrichous flagella. Classification of Bt strains has been accomplished by H serotyping, the immunological reaction to the bacterial flagellar antigen.[20] The *hag* gene encodes flagellin, which is responsible for eliciting the immunological reaction in H serotyping. Specific flagellin amino acid sequences have been correlated to specific Bt H serotypes and at least 69 H serotypes and 82 serological varieties (serovars) of Bt have been characterized.[21] H serotyping, however, is limited in its capability to distinguish strains from the same H serotype or from the same serovar.[22] Due to its economic importance, it has become necessary to develop alternative tools for classification and grouping of Bt strains and isolates. Accordingly, several screening programs have been established to isolate novel Bt strains with unique insecticidal properties. As a result, numerous Bt strains with activity against lepidopteran, dipteran and coleopteran insects have been isolated. Additionally, Bt strains active against insects belonging to the orders Hymenoptera, Homoptera, Orthoptera and Mallophaga as well as nematodes, mites and protozoa have been isolated.

The placement of Bt as a separate species within the genus Bacillus has been controversial since the publication of *The Genus Bacillus* in 1973,[23] and *Bergey's Manual of Determinative Bacteriology* in 1974.[24] The genus Bacillus is one of the most diverse genera in the class Bacilli and includes aerobic and facultatively anaerobic, rod-shaped, Gram-positive spore-forming bacteria with G + C contents ranging from 32–69%.[25] Based on phylogenetic heterogeneity, eight genera in the class Bacilli have been proposed: Bacillus, Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus, Salibacillus and Gracilibacillus.[26-30] Many species of these genera are of practical importance because they produce antibiotics and peptides with anti-microbial, anti-viral and anti-tumor activities. They also synthesize thermostable enzymes and molecules that can suppress soil-borne phytopathogenic organisms.[31-35]

In the monograph *The Genus Bacillus,* Gordon et al.[23] considered Bt a variety of *B. cereus* (Bc) along with *B. anthracis* (Ba) and *B. mycoides* (Bm). Certainly, Bt, Ba and Bc share many common phenotypic and genotypic properties to the extent that the three species have been placed under one group called the *Bacillus cereus* (BC) group. Ba is the causative agent of anthrax, an acute and often lethal disease in humans and animals. Bc is an opportunistic human pathogen and may cause food poisoning, eye infections and periodontal disease, among other ailments. Bt possesses a variety of special features including its (1) ability to live in the environment free and independent from other Gram-positive spore-forming bacilli, (2) production of entomocidal parasporal crystal proteins and (3) survival in a unique environmental niche in the midgut and hemocoel of insects. In addition to Ba, Bc, Bt and Bm, there are two other highly related species *B. pseudomycoides* (Bpm) and *B. weihenstephanensi* (Bw) in the BC group.

Curiously, Bt has been alleged to be an opportunistic pathogen in animals and human.[36-38] Two Bt strains, Bt 97-27 (subsp. *konkukian*) and Bt Al Hakam (isolated in Iraq by a United Nations Special Commission), were initially designated as human pathogens. Bt 97-27 was first isolated from necrotic

tissue in a twenty-eight year old male hospital patient.[36] The designation of strain 97-27 as Bt was based on biochemical tests and the appearance of inclusion bodies.[36] However, a second isolate from the same patient lacked inclusion bodies. Sequence analysis showed no insecticidal (*cry*) genes present on the 97-27 chromosome or a lone single plasmid pBT9727.[37] Sequence analysis of the Al Hakam genome also revealed no chromosome-encoded or plasmid-encoded ORFs with significant similarity to any insecticidal genes. Interestingly, pBT9727 of strain 97-27 and the pXO2 plasmid of Ba have almost identical replication proteins and a highly conserved origin of replication. However, the pXO2 region encoding a poly-c-D-glutamic acid capsule was replaced on pBT9727 with genetic mobile elements, suggesting that pBT9727 may have evolved from pXO2 to perform other functions in strain 97-27. Phylogenetic lineage placement using amplified fragment length polymorphism analysis (AFLP)[39] and comparative sequence analysis[40] indicate that strain 97-27 is more related to Bc and Ba than to Bt. To the authors' knowledge, no other studies have been reported that characterize Bt as an opportunistic pathogen of humans or warm-blooded animals.

Genome sequences of a number of strains in the BC group have been completed: Bc (ATCC 14579 and 10987 and strain E33L), Bw-KBAB4, Ba-Ames, Ba-Ames Ancestor and Ba-Sterne. Sequencing and annotation of the genome of Bt subsp. *kurstaki* is near completion in the Bulla laboratory. Comparison of all the genome sequences, including that of subspecies *kurstaki,* reveals enormous similarity in terms of nucleotide sequence identity and gene and operon organization, a combination not observed heretofore among different bacterial species. The primary distinguishing features of Bt are its virulence and pathogenicity factors, represented by the insecticidal genes located on the chromosome and several plasmids. Those of Ba are its tripartite toxin and capsule encoded by plasmids only.[41]

Analysis of 16S and 23S rDNA nucleotide sequences, a powerful technology for bacterial identification, likewise indicates that species belonging to the BC group have almost identical sequence similarity.[42] Molecular techniques such as multi-locus enzyme electrophoresis,[43,44] AFLP analysis[45] and multi-locus sequence typing[46] have helped resolve some of the issues related to BC group classification. Analysis of various Bc and Bt strains reveal very high diversity in multi-locus genotypes, indicating that Bc and Bt exhibit a low degree of clonality and that exchange of genetic material can occur frequently in their natural environments.[43] It has been suggested that Ba, Bc and Bt should be classified as one species,[43] or that Ba be considered in the lineage of Bc.[44] Pulsed-field gel electrophoresis and dendrographic analysis corroborate the latter idea[44] by showing that Ba isolates fall into the same cluster but are distinct from Bc and Bt.[47] Additionally, the use of the gene *gyrB* (a house-keeping gene encoding the B subunit of DNA gyrase, topoisomerase type I) has proven very useful to distinguish members of the BC group.[48-51]

We have constructed a phylogenetic tree, based on sequence alignment of the 16S rRNA of 20 strains in the BC group using the Bootstrapped neighbor-joining method (**Fig. 2**). Bootstrap volumes are reported on the branches. The tree displays division of the 20 strains into two major groups. Group A includes all Ba, Bc and Bpm strains and Group B includes Bw strain KBAB4 and Bm strain SDANFMO448. There are two clusters in Group A, each marked with a Roman numeral. Cluster I includes Ba strains Sterne CDC684 and Ames Ancestor A0248; Bc strains AH187 and AH 820 and ATCC 10987 and 14579; Bpm strain CIP 5259; and Bt serovar *konkukian* and Bt Al-Hakm. Bc AH820 and serovar *konkukian* in Cluster 1, both alleged human pathogens, are more closely related whereas Bpm strain CIP 5259 and Bc ATCC 14579, neither of which are pathogenic, are the most divergent in the cluster. Cluster II includes Bt serovars *tenebrionis, morrisoni, kurstaki, sotto, israelensis* and *berliner* and Bc G9842. We infer from the dendogram that both Bt serovar *konkukian* and the Al-Hakm strain group rather well with the Ba and Bc strains, but not with the Bt serovars, as previously suggested.[39,40]

Expression and Regulation of Insecticidal Genes

One of the most dramatic aspects of Bt sporulation is the formation of parasporal crystals. The insecticidal toxins (Cry toxins) of Bt, oftentimes referred to as δ-endotoxins after Heimpel,[52] are somewhat specific to certain insects. The family of genes coding for these toxins is the *cry* gene family.[53,54] A common characteristic of *cry* genes is that they are expressed during the stationary phase of growth. Cry proteins, the end-products of *cry* gene expression, constitute 20–30% of the cell dry weight and generally accumulate in the mother cell, beginning in stage III of sporulation and continuing through stage VII.[18]

The term δ-endotoxin, relative to Bt Cry toxin, is a misnomer. Heimpel[52] named the parasporal crystalline protein of Bt as such because it forms inside the cell and because it is fourth in the order of other toxic components discovered in the bacterium. However, endotoxins are associated with the lipo-polysaccharide moiety of the complete "O" somatic antigen complex found in the outer membrane of various Gram-negative bacteria and are an important factor in their ability to cause disease.[55] Based on its mode of action, a Cry toxin is a "simple" toxin, which is defined as a monomer or oligomer of a toxic simple protein.[56] A Cry toxin exists as a toxic monomer capable of oligomerization. The parasporal crystals of Bt are oligomers composed of polypeptide protoxin subunits. The protoxin is the immediate atoxic precursor of a Cry toxin, i.e., upon activation of the protoxin, an insecticidal Cry toxin is generated.[57,58] Interestingly, the processing of protoxin to toxin is different among the respective toxin groups, depending on host specificity, i.e., toxins that kill moths, beetles or mosquitoes. For example, Cry1A and Cry4 toxins (~65 kDA) that primarily kill moths and mosquitoes, respectively, are the products of protoxins in the molecular weight range of 125–135 kDa whereas Cry3 toxins (~68 kDa) that kill beetles are conversion products of 72-kDa protoxins. The mechanism of Cry toxin action is discussed later in this review.

The high level of Cry protein synthesis appears to be coordinately controlled by a variety of mechanisms occurring at the transcriptional, posttranscriptional and posttranslational levels. Typically, initiation of the sporulation process in Bt is controlled by successive activation of sigma factors that bind the

Figure 2. Bootstrapped neighbor-joining tree of 20 strains belonging to different species of the BC group. The tree was generated based on nucleotide sequence alignment of 16S rRNA. Bootstrap volumes are reported on the branches. The 20 strains are divided into two main groups. Group A includes all Ba, Bc and Bpm strains and Group B includes Bw strain KBAB4 and Bm strain SDANFMO448. There are two clusters within Group A, each marked with Roman numerals I and II. Cluster I includes Ba strains Sterne CDC684 and Ames Ancestor A0248; Bc strains AH187 and AH 820 and ATCC 10987 and 14579; Bpm strain CIP 5259; and Bt serovar *konkukian* and Bt Al-Hakm. Bpm strain CIP 5259 and Bc ATCC 14579 are the most divergent in this cluster. Cluster II includes Bt serovars *tenebrionis, morrisoni, kurstaki, sotto, israelensis* and *berliner* and Bc G9842. The horizontal bar represents 0.02% differences in nucleotide similarities.

core RNA polymerase and direct transcription utilizing sporulation-specific promoters.[59] There are five sporulation-specific transcription factors, σ^H, σ^F, σ^E, σ^G and σ^K, which are tightly regulated and appear in fixed order during sporulation. The σ^H factor is active in the pre-divisional cell; σ^E and σ^K are active in the mother cell; and σ^F and σ^G are active in the forespore. Several *cry* gene promoters have been identified and their sequences determined.[60-64] The *cry1Aa* gene, for example, is expressed via two overlapping promoters (BtI and BtII).[65] Transcription from the downstream promoter, BtI, is active between stages II and VI of sporulation and employs σ^E. Later transcription is facilitated by the upstream promoter, BtII, which is active from stage VI through the end of sporulation, and employs σ^K. The sequential use of the two promoters most likely ensures synthesis of the protoxin throughout the sporulation cycle. Some *cry* genes, however, appear to be expressed independently of sporulation. For instance, *cry3A* gene expression is enhanced and prolonged in mutant strains unable to initiate sporulation.[66] Also, De Souze et al.[67] and Malvar et al.[68] reported that the *cry3A* gene is expressed during vegetative growth. The *cry3A* promoter, recognized by σ^A, the sigma factor of vegetative cells, is activated at the end of exponential vegetative growth and remains active through stage III of sporulation.

An additional regulatory mechanism for expression of some *cry* genes at the transcriptional level involves a regulatory protein that binds with different affinities within an inverted repeat and a potential bend region lying 200–300 base pairs upstream of different *cry1* promoters.[69] This regulatory protein was identified as the E2 subunit of pyruvate dehydrogenase. A chimera consisting of a mutated upstream region and the coding sequence of β-galactosidase resulted in decreased binding of the E2 subunit and reduction in β-galactosidase synthesis. Apparently,

involvement of the E2 subunit of pyruvate dehydrogenase in *cry* gene regulation implicates a connection between catabolic activity and Cry toxin synthesis.[69]

Regulation of *cry* gene expression at the posttranscriptional level may depend on mRNA stability. The half-life of *cry* mRNA is approximately ten minutes, which is at least five-fold greater than the half-life of an average bacterial mRNA.[70] The presence of putative transcriptional terminators—acting as positive retroregulators—at the 3'-end of many *cry* genes could contribute to mRNA stability.[71] In other words, the transcriptional terminator could increase *cry* mRNA stability by protecting it from exonuclease degradation, beginning at the 3'-end. Certainly, the processing activities of 3'-5' exoribonucleases are impaired by 3' stem-loop structures.

Agaisse and Lereclus[72] identified a perfect Shine-Dalgarno (SD) sequence (GAAAGGAGG) in Bt required for stabilization of *cry3A* mRNAs. The sequence mapped at a position between -125 and -117 in the 5' untranslated region and the authors called the sequence the stabilizing Shine-Dalgarno (STAB-SD) motif. It is quite possible that stability of the mRNA resulted from interaction of the 3'-end of 16S rRNA of the 30S ribosomal subunit and STAB-SD. The binding of a 30S ribosomal subunit to this sequence probably protects the mRNA from 5'-3' ribonuclease activity, resulting in stable transcripts. Potential STAB-SD sequences also have been identified in similar positions upstream of the *cry3Ba* and *cry7Aa* genes[73,74] and upstream of *cry1Ic* in the intergenic region between the *cry1Ac* and *cry1Ic* genes.[75]

It may be that formation of stable parasporal crystals, comprised of Cry proteins, represents a posttranslational regulatory mechanism that protects the toxins from premature proteolytic degradation. The presence of a cysteine-rich region in the C-terminal half of the protoxin also may contribute to a stable crystal structure through formation of disulfide bonds. Those toxins that lack the cysteine-rich C-terminal region, e.g., truncated Cry toxins, may circumvent adversity by forming intermolecular salt bridges and hydrophobic interactions that can stabilize crystal structure. Several investigators have reported that certain parasporal crystals require additional proteins to stabilize and crystallize the cognate Cry toxins.[76-78] These accessory proteins may act as chaperons to stabilize nascent protoxin molecules, facilitating crystallization.

One interesting feature of *cry* genes is their high degree of plasticity. This particular characteristic may contribute to the versatility of Cry toxins as it relates to their insect host range. The most likely explanation for such genetic plasticity is the presence of numerous transposons and insertion elements that flank the *cry* genes. Indeed, these transposable elements may facilitate gene multiplication and evolution of new toxins.[79] Furthermore, the fact that *cry* genes are carried on transmissible plasmids increases the likelihood of horizontal gene transfer among different Bt strains, which leads to the creation of new strains with different sets of Cry toxins.[80,81]

The organization and clustering of *cry* genes in operons has been demonstrated in numerous Bt strains.[82] Specifically, *cry1Ac, cry1F, cry1I, cry2Aa, cry2Ac, cry9Ca* and *cry11Aa* are constituents of operons. Some of the operons include at least another upstream reading frame such as *orf1* or *p19*. A second orf (*orf2* or *p20*) resides in *cry2* and *cry11* operons. Why are the different *cry* genes organized in operons? One explanation is that such organization facilitates differential gene expression, rendering parasporal crystals containing different Cry toxins, or different crystals, each of which contains a specific individual toxin. The nature of and the molar concentration of toxins produced by a given subspecies of Bt reflect not only specificity to a target insect(s) but also the structural design of individual parasporal crystals. A consequence of this scenario could be synergism among the various Cry toxins.[75,83]

Another plausible explanation for the arrangement of *cry* genes in operons is enhancement of genetic recombination that might generate new toxins or combinations thereof, which, in turn, would impart novel host specificities. Also, conservation in the usage of sigma factors could be a potential outcome. Sharing polymerases with multiple copies of *cry* genes most likely would bring about competition for the appropriate transcription factors, ultimately affecting structural and physiological properties not only of the parasporal crystals but of the endospore itself. Aronson et al.[84] reported that a plasmid-cured acrystalliferous strain of Bt had normal spore coats whereas the parental strain was deficient in spore coat protein. The deficiency in spore coat protein most likely was due to a limited capacity of the organism to transcribe σ^K- and σ^E-dependent spore-coat genes.

Significantly, most of the *cry* genes organized in operons, e.g., *cry1Ic1, cry2Aa, cry2Ac* and *cry11A,* code for truncated toxins which lack the cysteine-rich C-terminal region necessary for proper folding and crystallization. In some cases, protoxins may act as molecular chaperons for the truncated toxins just as the P19 and P20 proteins presumably do for Cry2A and Cry11, respectively.[75-77]

Biochemistry and Functional Proteomics of Cry Toxins

Once a mature spore is formed, both the spore and parasporal crystal are released from the mother cell into the environment where they are readily available for larval consumption. The Cry toxin contained in the crystal is the virulence factor that truly distinguishes Bt from its genetic cousins Ba and Bc. And, it is the Cry toxin that establishes safe harbor for the bacterium in an insect carcass. Different parasporal crystals are made either of single or multiple Cry proteins. For example, the parasporal crystal of Bt subsp. *kurstaki* HD-73 contains Cry1Ac protein only, whereas the parasporal crystal of HD1 strain, which belongs to the same subspecies, is comprised of five different Cry toxins—Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab. Another feature of Cry toxins is that their precursor protoxins co-crystallize in various forms and shapes as evidenced by electron microscopy (see **Fig. 1**). Cry toxins are encoded by *cry* genes found mainly on large plasmids. However, the genes may be integrated into the chromosome. Since the cloning and sequencing of the first *cry* genes,[85,86] nucleotide sequences have been reported for more than 300 *cry* genes. Höfte and Whiteley[87] and Carlton[88] proposed a universal nomenclature and classification scheme for

Cry proteins and their genes based on host range. Later, another nomenclature format, based on amino acid sequence similarity, was proposed.[54] In the classification proposed by Crickmore et al.[54] the *cry* genes are divided into 51 groups and subgroups and the Cry toxins are separated into six major classes according to their insect host specificities and include: Group 1—lepidopteran (Cry1, Cry9 and Cry15); group 2—lepidopteran and dipteran (Cry2); group 3—coleopteran (Cry3, Cry7 and Cry8); group 4—dipteran (Cry4, Cry10, Cry11, Cry16, Cry17, Cry19 and Cry20); group 5—lepidopteran and coleopteran (Cry1I); and group 6—nematodes (Cry6). The Cry1I, Cry2, Cry3, Cry10 and Cry11 toxins (73–82 kDa) are unique because they appear to be natural truncations of the larger Cry1 and Cry4 proteins (130–140 kDa).

The three-dimensional structures of a number of Cry toxins have been published: Cry3Aa,[89] Cry1Aa,[90] Cry1Ac,[91] Cry2Aa,[92] Cry3Bb,[93] Cry4Ba,[94] Cry4Aa[95] and Cry8Ea1.[96] All Cry toxins contain three structural domains and share a high degree of topological similarity. Domain I is composed of a bundle of seven α-helices connected by loops. The α-helical bundle has a central amphipathic α helix that is well conserved among all the toxins described. Various mutations in Domain I appear to abolish toxicity but not binding to cellular receptors. Whether these mutations affect overall conformation of the toxin molecule, compromising toxicity, is not known. Domain II consists of three sets of antiparallel β-sheets, each terminating with a loop. The beta sheets are packed around a central hydrophobic core forming a so-called beta-prism structure. Domain III is a sandwich of two antiparallel β-sheets that form a "jelly-roll" topology. Results of site-directed mutagenesis and truncation analysis provide strong evidence for the involvement of Domains II and III in receptor binding and insecticidal activity.

Domain I purportedly functions to form ion channels in the cell membrane,[53] and the hydrophobic motifs within this domain are what effect toxicity. Upon contact with the cell membrane, the domain undergoes refolding to facilitate insertion of the toxin into the membrane as with other bacterial toxins.[97,98] Several articles have reported that hydrophobic α-4 and α-5 helices insert into the membrane and that this orientation is responsible for toxicity.[99,100] However, there is no in situ or in vivo evidence to support these claims. What function the other helices have is not clear although alanine substitutions of four highly conserved aromatic residues, Trp_{243}, Phe_{246}, Tyr_{249} and Phe_{264}, in α helix 7 of the mosquitocidal Cry4B toxin resulted in a dramatic decrease in toxicity against the mosquito *Stegomyia aegypti*.[101] In addition, a nearly complete loss in toxicity was found for Phe_{264}Ala/Tyr_{249}Ala double mutants. Further mutagenic analysis of the double mutants showed that upon replacement with other aromatic residues, particularly at Tyr_{249} and Phe_{264}, insecticidal activity was regained.[101] In other work,[102] deletion of 42 amino acid residues immediately upstream of α-helix 1 followed by replacement of Lys residues (Lys_{63} and Lys_{64}) by alanine and proline, respectively, enhanced the potency of Cry2A toxin. An explanation afforded by the authors is that removal of the 42-amino acid fragment exposes a formerly occluded region of the toxin which corresponds to the receptor-binding region of the Cry2 toxin.

Domain II is the most divergent domain among the Cry toxins and its replacement or switching with domains II and III of other toxins can affect host specificity.[103,104] The loops connecting strands of the antiparallel β-sheets are exposed at the apex of the domain and represent the least conserved regions amongst the Cry toxins. Interestingly, the apical loops of Cry1A, Cry2A, Cry3A, Cry4A and Cry5A toxins are highly variable in length. Cry5Aa toxin has the longest loop whereas Cry3Aa has the shortest one.[105] What impact loop length has on domain structure and function is not known. Certainly, the span of the loops contributes to the configuration of Domain II and, most likely, influences the interactions of all three domains as well as the binding of individual toxins to their cognate receptors. Boonserm et al.[94] have suggested that shorter loops are more likely to disturb the structure of the core β-sheets of Domain II and, consequently, interrupt the interaction of Domains I and II. Whatever their structural or functional roles, the loops appear to be key elements in receptor recognition, binding and specificity.

The contribution of the surface-exposed loops in Domain II to insect toxicity has been examined in a number of site-directed mutagenesis studies.[106-108] The involvement of loop 2 (specifically Arg_{368}-Ile_{375}) in toxicity of Cry1Ab toxin to the tobacco hornworm was indicated by single amino acid deletion or alanine substitution for Phe_{371} and Gly_{374}.[109] The relevance of Arg_{368} and Arg_{369} also was shown using the same approach. Alanine substitutions of loop 3 residues, Ser_{438}-Ser_{443}, in the Cry1Ab toxin showed reduced toxicity and binding in both the tobacco hornworm and tobacco budworm.[110] Likewise, deletion of residues Ala_{440}-Ala_{443} in loop 3 of Cry1Aa resulted in reduction in toxicity to the silkworm as well as to the tobacco hornworm. Other experiments with Cry3A toxin implicated loops 1 and 3 of Domain II in receptor binding whereas loop 2 double mutations had no effect on binding or toxicity.[111]

Domain II loops in the mosquitocidal toxins Cry4A, Cry4B, Cry11A and Cry11B are very important not only in receptor binding but in specificity as well. When loop 3 of Cry4B was made to mimic that of Cry4A by site-directed mutagenesis, Cry4B became toxic to *Culex pipiens* whereas the wild-type toxin was not.[112] Curiously, mutations in loops 1 and 2 of Cry4B eliminated toxicity to Aedes and Anopheles larvae but not to Culex.[112] In vitro competition binding assays with Cry11A toxin indicated that loops 2 and 3 were important for binding to *Aedes aegypti* brush-border membrane vesicles.[113] Peptides corresponding to loops α8, 1 and 3, but not loop 2, of the Cry11B toxin competed with toxin binding in Aedes midgut membranes and mutagenesis data suggested that loops α8, 1 and 3 are involved in toxicity.[114] Taken together, all of these results provide strong evidence that loop 3 is important to the toxin-receptor interaction.

Domain III has been correlated with receptor binding[115-117] and channel formation in the cell membrane.[118,119] It has been linked to toxicity as well. Aronson and co-workers[84] reported that single alanine substitutions of two serine residues at positions 503 and 504 in the Cry1Ac toxin significantly decreased binding affinity of the toxin and reduced toxicity to the tobacco hornworm. In vitro Domain III swapping in certain Cry1 toxins, has resulted in alterations in insect specificity.[120,121]

Examples of toxins that may have undergone domain swapping naturally are toxins with dual specificity, especially to moths and beetles, such as Cry1I.[75] Domain III swapping has been suggested as an evolutionary scheme[79,122] and that such activity may be responsible for the emergence of toxins with varying specificities. In any event, Domain III most assuredly is involved in both receptor binding and insecticidal action.

A significant number of studies on the functionality of the different domains and regions within the framework of Cry toxins point to the involvement of Domains II and III in receptor binding and toxin action. Numerous site-directed mutagenesis experiments indicate participation of the three surface-exposed loops (loops 1, 2 and 3) of domain II.[123-126] Putative binding sites also have been estimated by testing whether synthetic peptides can disrupt the toxin-receptor interaction.[107,127] Conclusions drawn from these studies were that loop α8 and loop 2 (Domain II) of Cry1Ab and loops 2 and 3 (Domain II) of Cry1Aa are the sites that participate in receptor binding.[107,128] Domain III also was linked to toxin action because mutations in this domain affected both toxicity and receptor binding.[84] Other reports show the dependence on specific sequences in Domains II and III for insecticidal activity.[129-131]

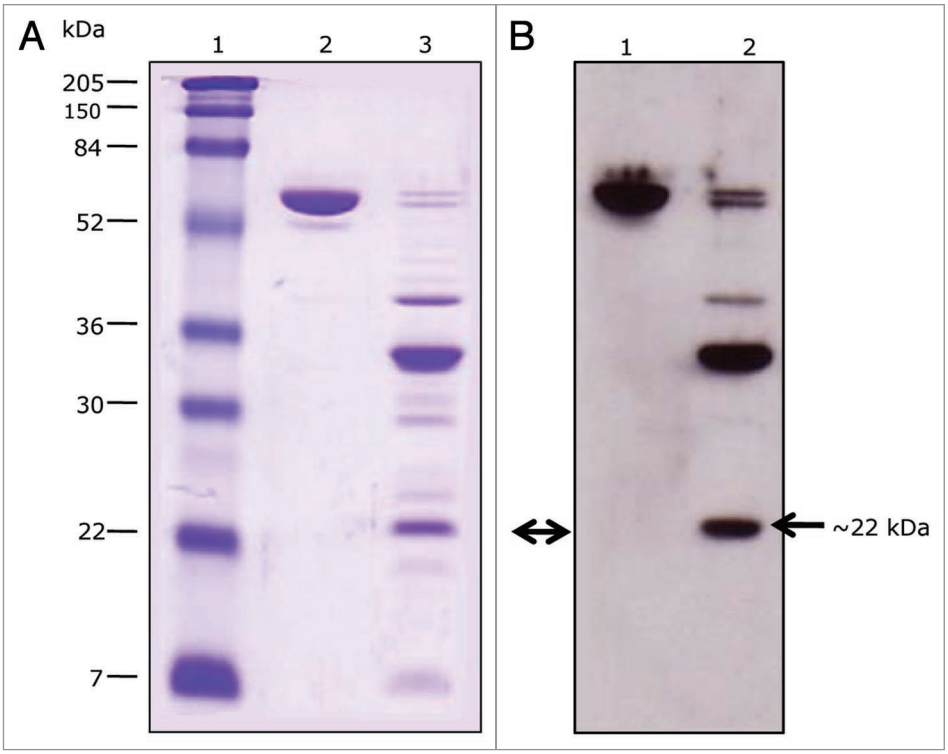

Figure 3. Cadherin receptor and Cry1Ab toxin binding analysis. (A) SDS-PAGE. Lane 1, molecular markers; lane 2, Cry1Ab toxin; lane 3, proteinase K-digested Cry1Ab. (B) Immunoligand blot. Cry1Ab toxin and its proteinase K proteolytic fragments were trans-blotted to a PVDF membrane and tested for their ability to bind the soluble toxin-binding region of BT-R_1 (TBR). Lane 1, Cry1AB toxin; lane 2, proteinase K digested Cry1Ab. The smallest binding fragment of Cry1Ab (~22 kDa, double arrow) was subjected to N-terminal amino acid sequence analysis. The N-terminal amino acid sequence is ^{442}NSSVSIIRAPMFSWIHR^{458}.

To circumvent complications inherent in site-directed mutagenesis, Natalya Griko and Mohamed Ibrahim (Bulla laboratory) utilized truncation analysis in an attempt to localize the receptor-binding region in the Cry1Ab toxin. The toxin was subjected to proteinase K and the resulting proteolytic fragments were exposed to the soluble toxin-binding region (TBR) of BT-R_1.[132] Immuno-ligand blot analysis revealed several fragments that bound the TBR (**Fig. 3**). The minimal binding fragment was ~22 kDa. The N-terminal amino acid sequence of this fragment is "NSSVSIIRAPMFSWIHR," which corresponds to amino acid residues 442–458 in loop 3 of Domain II (**Fig. 4**). Apparently then, loop 3 of Domain II and the N-terminal portion of Domain III are determinants in binding Cry1Ab to its corresponding receptor BT-R_1.

The relevance of Domain III for binding Cry1Ab toxin was demonstrated a number of years ago by de Maagd et al.,[133] who constructed special hybrids of Cry1Ab and Cry1C and examined their binding capabilities both in vivo and in vitro. Only one hybrid containing Domains I and II of Cry1C and Domain III of Cry1Ab bound to a 200-kDa protein in brush border membrane vesicles prepared from the beet armyworm. Hybrids devoid of Domain III showed no ability to bind the protein, establishing the relevance of Domain III of Cry1Ab in receptor binding. Similar kinds of hybrids derived from Cry1C and Cry1E[120] showed Domain III to be a major determinant of toxicity. Likewise, Domain II of several Cry toxins have been documented as necessary, but not sufficient, for toxin activity.[134] Thus, the receptor-binding region of Cry toxins can be narrowed to a region that includes loop 3 in Domain II and part of Domain III.

Domain I is the most conserved among Cry toxins. Domain II and the beginning of Domain III are the least-conserved. As mentioned above, the putative receptor-binding region in Cry1Ab is localized in loop 3 of Domain II and the immediate N-terminal region of Domain III. Based on immunoligand blot analysis (**Fig. 3**) and the mutation analysis of loop 3 of Domain II described above, the putative receptor-binding region most likely includes β10, loop 3 and β11-β16 (**Fig. 4**). Based on motif structural analysis of this region, two putative solvent-exposed regions (residues 432–449 and 480–493) were identified (shown in red in **Fig. 4**). Multiple sequence alignment of the putative receptor binding regions of Cry1Ab, Cry3Aa and Cry4Ba revealed that β10, loop 3 and β11 are the most divergent (**Fig. 4A**), supporting the assumption that this region has a major role in determining specificity.

Three-dimensional structure prediction of this particular region is represented in ribbon format in **Figure 4B**. Structurally,

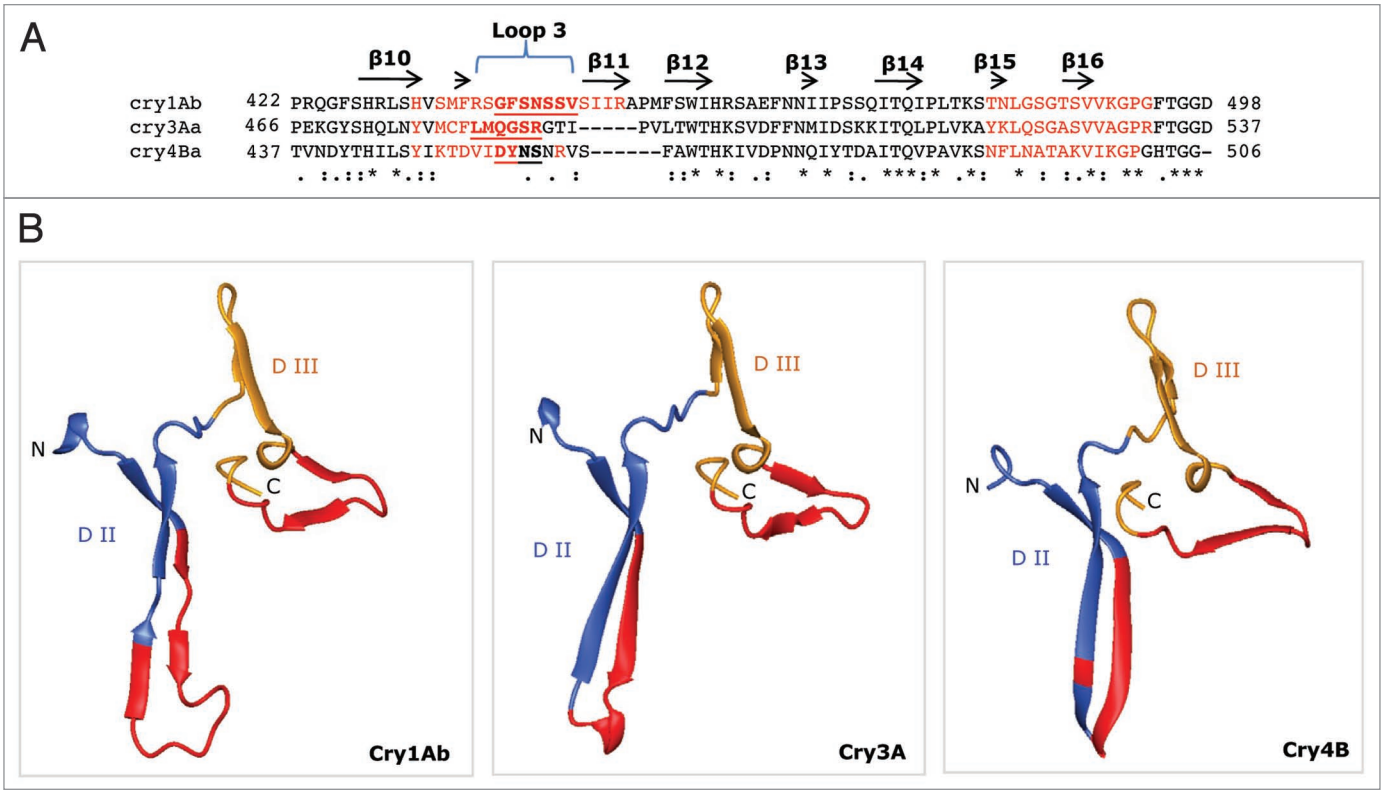

Figure 4. Proposed secondary structure and three-dimensional model for the putative receptor-binding regions of Cry1Ab, Cry3A and Cry4B. (A) Multiple sequence alignments and secondary structure prediction of the amino acid residues spanning β10, loop 3, β11-β16 (end of Domain II and beginning of Domain III) of Cry1Ab (residues 422–498); Cry3A (residues 466–537) and Cry4B (residues 437–506), using Pfam[181] and SAS[182] databases. The sequence in red represents solvent-exposed residues. Underlined, bold residues in the three Cry toxins constitute loop 3. The symbols (*), (:) and (.) beneath the alignments denote identical, similar and less similar amino acid residues, respectively. (B) Three-dimensional structure in ribbon format for the putative receptor-binding regions on Cry1Ab, Cry3A and Cry4B. The Cry3A and Cry4B structures are from crystal structures (PDB codes 1DLC and 1W99, respectively). The Cry1Ab structure was generated as a model by the SWISS-MODEL protein modeling server[173] using the crystal structure of the Cry1Aa toxin (88% sequence identity; PDB code 1CIY) as a template. Blue, red and golden shaded areas highlight predicted β-strands and loops.

Cry1Ab, Cry3A and Cry4B have very similar topologies, except that Cry3A and Cry4B have more extended β10 strands and shorter loops 3. Interestingly, multiple sequence alignment of the putative receptor-binding regions of Cry1Aa and Cry1Ab (very closely related toxins) revealed that loop 3 is the least conserved, whereas β10 and β11-β16 are the most conserved (data not shown).

Cadherins as Cognate Receptors for Cry Toxins

The receptor molecules that serve as targets for Cry toxins are cadherins and are localized in the midgut of insects. Cadherins belong to a large family of calcium-dependent transmembrane glycoproteins which are highly diverse and multi-functional. Several key functions include cell-cell adhesion, cell migration, regulation of tissue organization and morphogenesis.[135,136] Cadherins also are involved in signal transduction pathways and interact with other cell adhesion molecules through their ectodomain and with specific cytoplasmic proteins via their cytoplasmic domain.[136-139] During embryonic morphogenesis, the expression of multiple members of the cadherin family is spatio-temporally regulated and correlates with a variety of morphogenetic events that involve cell aggregation or disaggregation. The functional relevance of midgut-specific cadherins in insect larvae is manifested in their involvement in controlling cell growth, cell division and cell death through various signaling pathways.[140] Significantly, the concentration of the cadherin BT-R_1 in the midgut of the tobacco hornworm increases dramatically, along with accumulation of its mRNA, during larval growth and development of the insect.[141] The increasing number of cadherin molecules in developing larvae emphasizes their importance in maintaining epithelial organization and correlates directly to the susceptibility of the tobacco hornworm to Cry1Ab toxin.[142] BT-R_1 serves as a receptor for the Cry1Ab toxin of Bt and is coupled to programmed oncotic-like cell death,[143,144] which is triggered by binding of the toxin to a highly conserved structural motif in the receptor.[132] Apparently, the Cry1Ab toxin, like other Cry toxins, takes advantage of a natural phenomenon involving inborn death ligands that act to destroy the midgut epithelium and clear larvae of excessive tissue before entry to the pupal stage.

Cadherins serve as Cry toxin receptors on midgut epithelial cells in a variety of insects, including the tobacco hornworm,[145,146] tobacco budworm,[147,148] silkworm,[149,150] cotton

bollworm,[151] pink bollworm,[152] European corn borer,[153] western corn rootworm,[154] yellow mealworm beetle[155] and mosquito.[156] Recently, Mohamed Ibrahim and Natalya Griko (Bulla laboratory) characterized a cadherin molecule (BT-R_3) in *Anopheles gambiae,* the primary mosquito vector of malaria, and demonstrated that, once bound to the Cry4Ba toxin, death ensues in insect cells transfected with the BT-R_3 cDNA (Ibrahim M and Griko N, unpublished data).

Obviously, there is ample evidence to implicate cadherins as primary targets of Cry toxins within the insect gut. Gahan et al.[147] reported that resistance to the Cry1Ac toxin by the tobacco budworm is linked to a cadherin gene. Similarly, Morin et al.[152] showed that the pink bollworm has three different cadherin alleles, all linked to the emergence of a resistance phenotype in transgenic cotton expressing the Cry1Ac toxin. Mutations in genes encoding cadherin proteins also are tightly linked to resistance to the Cry1A toxin by the cotton bollworm.[157] Fabrick et al.[155] showed that knocking down TmCad1, a Cry3A-binding cadherin in the yellow mealworm, by injecting the insect with *Tmcad1*-specific double stranded RNA resulted in resistance to the toxin.

Cadherins are comprised of repeating calcium-binding cadherin repeats of approximately 110 amino acids in length. The ectodomain of cadherins can range from five cadherin repeats in classical cadherins[136,137] to as many as thirty-four.[158] Cry toxin-binding cadherins from different insect orders, share a structure composed of four domains: ectodomain (EC), membrane proximal extracellular domain (MPED), transmembrane domain (TM), and cytoplasmic domain (CYTO) (**Fig. 5**). The EC consists of 11–12 ectodomain modules, each of which is composed mainly of β-strands organized in β-barrel cadherin repeats connected one to another by interdomain linkers. The EC modules adjacent to the MPED contain the Cry toxin-binding region.[155,156,159,160] Toxin-binding regions (TBRs) have been identified for cadherins representative of three major insect orders: Lepidoptera (moths, skippers and butterflies),[132,149] Coleoptera (beetles)[155] and Diptera (mosquitoes and black flies, among others).[156] All of the TBRs are located within those EC modules positioned at or near the MPED, suggesting that this particular area of the cadherin molecule is critical not only for toxin binding but for mediating toxin action as well (**Fig. 5**).

Comparative sequence analysis of the toxin-binding motifs in the BT-Rs of moth (BT-R_1), mosquito (BT-R_3) and beetle (BT-R_4), combined with their secondary structures and three-dimensional folding predictions, reveal highly conserved structural features in all three molecules (**Fig. 6**). For consistency, we ascribe BT-R_4 to the cadherin of the yellow mealworm beetle, which was called Tmcad1 in the published article by Fabrick et al.[155] BT-R_2 is the Cry toxin receptor in the pink bollworm[161] but is not included for comparison in **Figure 6**. Inspection of the three BT-R sequences discloses two highly conserved stretches of amino acid residues within the N- and C-termini that flank EC11 of BT-R_3 and EC12 of BT-R_1 and BT-R_4. These sequences are highlighted in the black boxes (**Fig. 6A**) and represent signatures that mark the toxin-binding function in all three BT-Rs. Griko et al.[132] observed the same high level of conservation of these signature sequences in a number of lepidopteran insects.

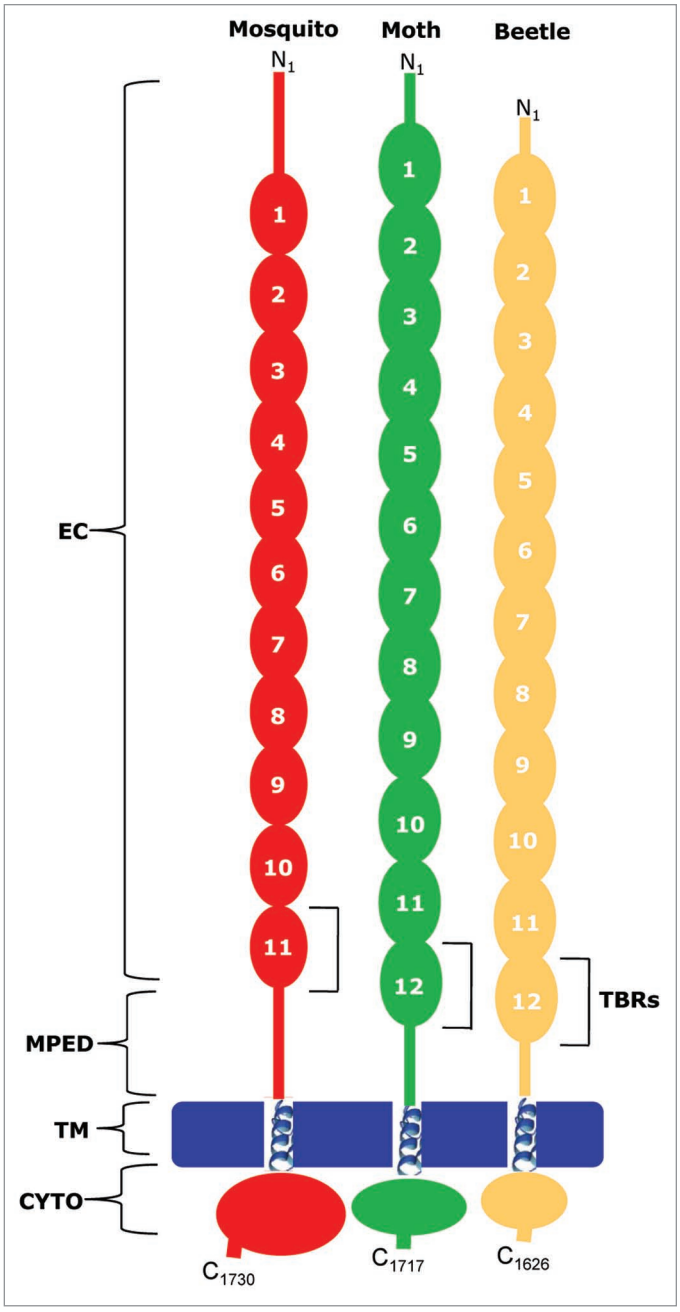

Figure 5. Domain structure and putative Cry toxin-binding regions in cadherin-like proteins from mosquito (red), moth (green) and beetle (golden). EC, ectodomain; MPED, membrane proximal extracellular domain; TM, transmembrane domain; CYTO, cytoplasmic domain, TBR, toxin binding region.

The toxin-binding motifs of BT-R_1, BT-R_3 and BT-R_4 are displayed in three-dimensional models (**Fig. 6B**) based on the known crystal structure of Domain 2 of the classical mouse E-cadherin (PDB code 113w), an N-cadherin (PDB code 1ncj) and a C-cadherin (PDB code 113w).[162-164] All three BT-R models are very similar topologically in that there are two essential sequences for toxin binding located at the N- and C-termini of

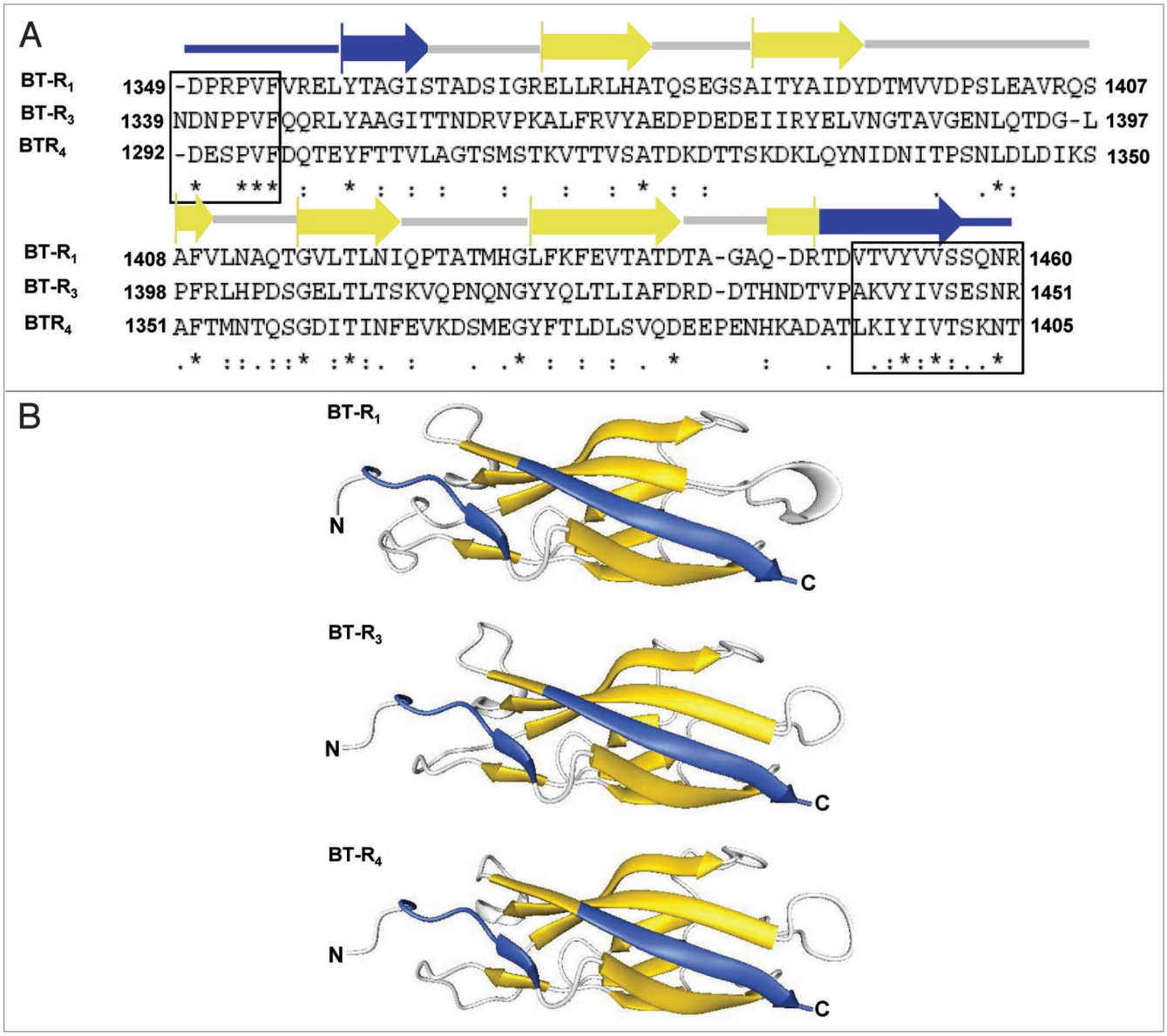

Figure 6. Predicted secondary structure and three-dimensional models of EC modules containing the TBRs for moth, mosquito and beetle. (A) Multiple sequence alignments and secondary structure prediction of the amino acid residues spanning the TBRs of BT-R_1 (residues 1349–1460), BT-R_3 (residues 1339–1451) and BT-R_4 (residues 1292–1405). The structures were generated using the Pfam[181] and SAS[182] databases. The two black boxes contain the conserved residues in the upstream and downstream regions of the Cry toxin-binding motifs. The designations beneath the alignments are as described in the legend to Fig. 4. (B) Three-dimensional models of the TBRs of BT-R_1, BT-R_3 and BT-R_4 generated from crystal structures of homologous cadherin domains from a mouse E-cadherin (PDB code 1edh), a mouse N-cadherin (PDB code 1ncj), and a frog C-cadherin (PDB code 1l3w). The three TBRs were templated onto the superimposed crystal structures by sequence alignment using the DeepView (Swiss-PdbViewer) computer program.[183] The alignment was guided by secondary structure prediction by the JPred 3 server[184] to match predicted β-strands in the receptor sequences to β-strands in the cadherin crystal structures. The templated models were then refined on the SWISS-MODEL protein modeling server.[173] Golden arrows and gray lines highlight predicted β-strands and loops, respectively. Blue arrows and blue lines represent sequences that flank the TBRs and are required for binding.

either EC11 or EC-12. The two signature sequences (**Fig. 6A**) form two adjacent β strands and a loop within the β-barrel fold of either EC11 or EC12 (**Fig. 6B**), providing an interface for toxin binding. The structural features of the TBRs on all three BT-Rs most likely are critical to the specificity, selectivity and affinity of their cognate toxins. And, apparently, the signaling events that lead to cell death depend on binding of Cry toxin to the conserved motifs in EC11 or EC12.

Mechanism of Cry Toxin Action

Just as the classification and taxonomy of Bt remains somewhat controversial, so does the explanation of how Cry toxins destroy insects. There are several models reviewed in the literature that seek to explain how Cry toxins exert their killing capacity.[165] For sake of brevity, we have chosen to describe only two mechanisms. The first one postulates that Cry toxin binds to midgut

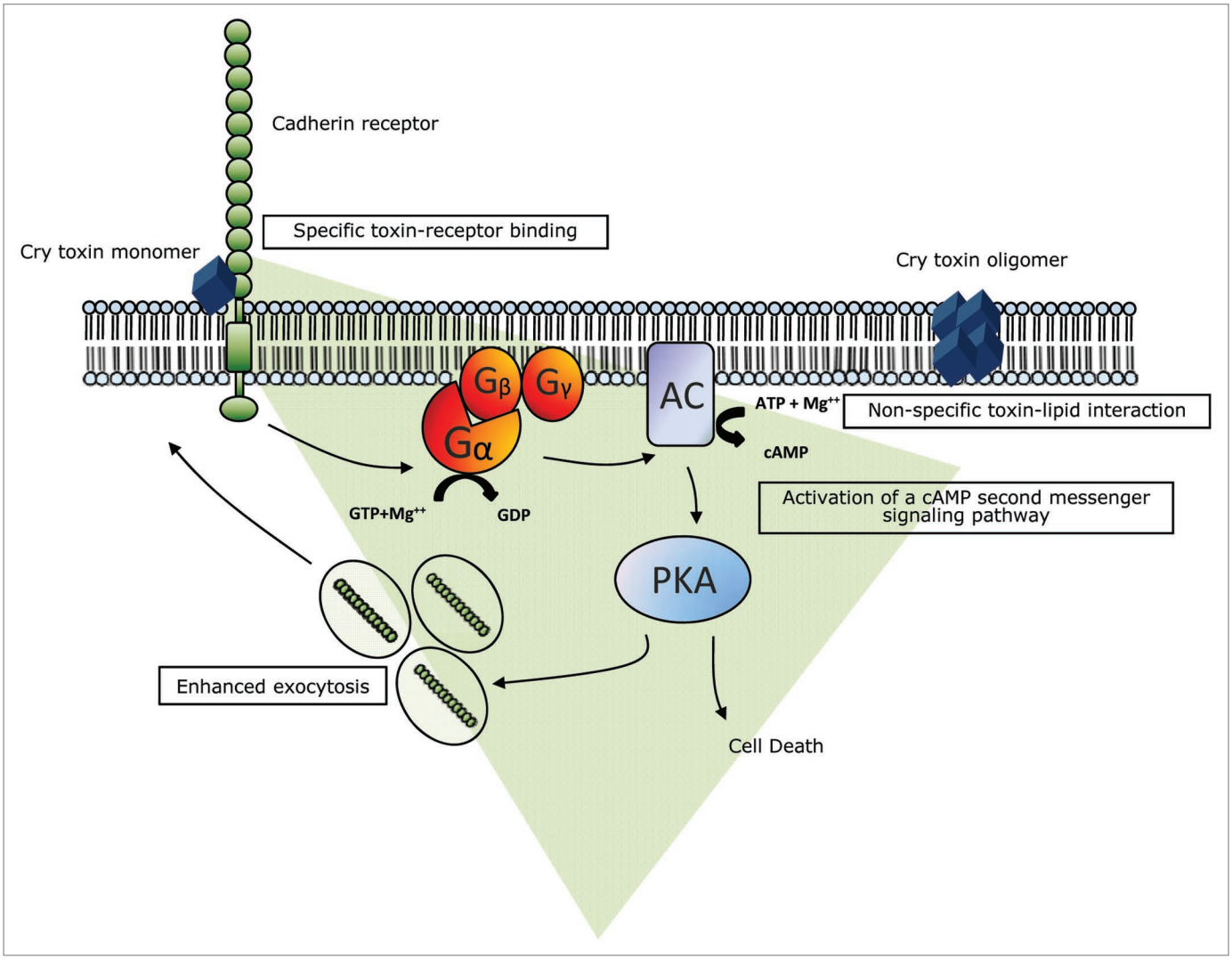

Figure 7. Proposed model for Cry toxin action. The univalent binding of Cry toxin monomer to BT-R initiates the progression of cell death by transmitting a death signal into the cell. A signal transduction pathway, involving G protein (G_α) adenylyl cyclase (AC) and protein kinase A (PKA), is activated. Activation of the signaling pathway mediates exocytosis of the BT-R receptor from intracellular vesicles to the cell membrane. The resulting enhanced display of BT-R on the cell surface facilitates recruitment of additional toxin molecules which, in turn, amplifies the original signal in a cascade-like fashion. The signaling kinase PKA modifies downstream molecules that promote the biochemical activities that destroy the cell. Toxin oligomers incorporated into the plasma membrane of living cells do not form lytic pores and are not toxic.

receptor(s), oligomerizes and inserts into the membrane to form lytic pores.[166] The notion that Cry toxins assemble lytic pores in the plasma membrane by forming oligomers is based on detection of ion fluxing in brush border membrane vesicles and synthetic lipid bilayers treated with Cry toxins.[90,166] However, no direct evidence has been provided for such a mechanism in either living cells or an insect. In fact, it has been shown that toxin oligomers incorporated into the plasma membrane of living cells do not form lytic pores and are not toxic.[143] Furthermore, studies of mutated Cry toxins demonstrate that neither toxin oligomers nor commensurate changes in membrane vesicle permeability correlate directly with toxicity.[167-169]

The second model (**Fig.** 7) advanced by Zhang et al.[143,144] challenges the notion that Cry toxin kills cells exclusively by osmotic lysis.[166] Instead, toxin monomer binds to the cadherin receptor $BT\text{-}R_1$ and activates a Mg^{2+}-dependent signal-transduction pathway leading to cell death. The model demonstrates that, in living cells, Cry1Ab oligomers, integrated into the cell membrane does not correlate with cytotoxicity (**Fig.** 7). Actually, toxin action is much more complicated than the proposed toxin-induced osmotic lysis. Cry toxin action is a complex, dynamic process that involves univalent binding of toxin to the highly conserved structural motif (described above) in the cadherin receptor $BT\text{-}R_1$.[132] In turn, a cascade of events is triggered that leads to a form of programmed cell death referred to as oncosis.[143] Binding of Cry1Ab toxin to the $BT\text{-}R_1$ receptor educes a molecular signal that stimulates heterotrimeric G protein and adenylyl cyclase with an accompanying dramatic increase in production of cAMP. The cAMP activates protein kinase A, bringing about an array of cellular alterations, which includes cytoskeletal rearrangement

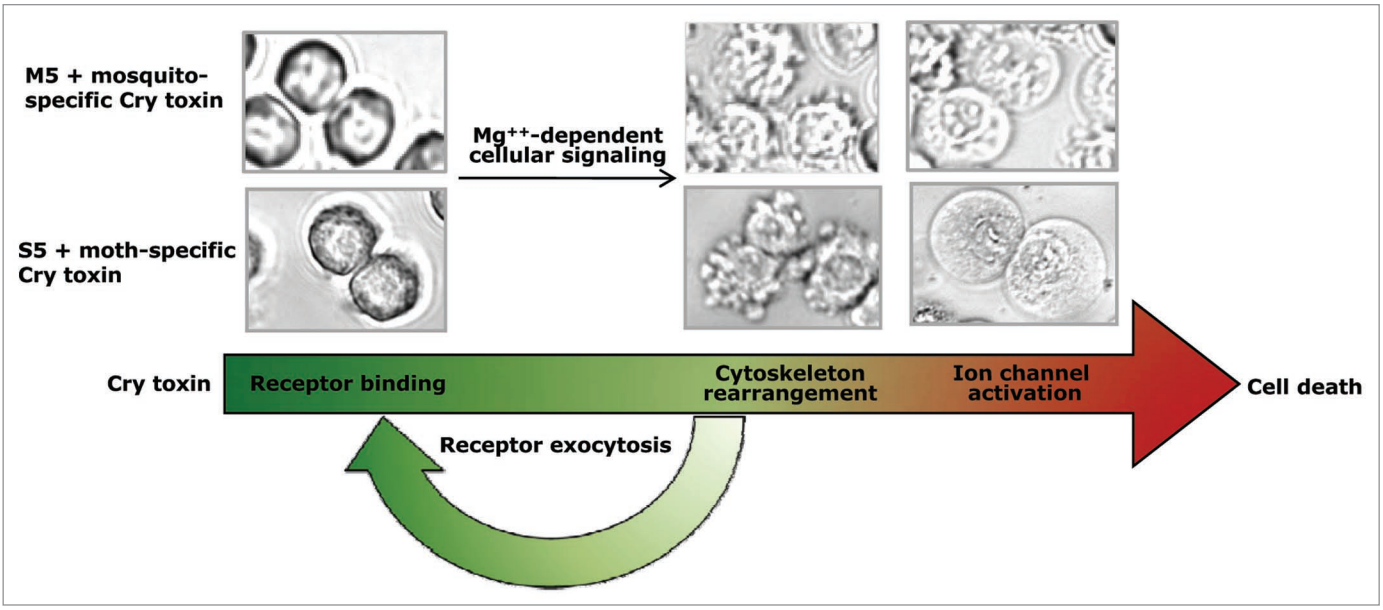

Figure 8. Morphological changes associated with BT-R-transfected High-Five cells treated with mosquito and moth toxins. High-Five cells transfected with BT-R_3 cDNA (M5 cells, upper photos) and BT-R_1 cDNA (S5 cells, lower photos) were treated with Cry4Ba and Cry1Ab, respectively. The sequence of cytological changes during the course of toxin-induced cell death was captured by phase contrast microscopy. The long arrow beneath the photographs indicates the stages of cell death: toxin binding, membrane blebbing and cellular swelling. Cytotoxicity is Mg^{2+}-dependent and involves exocytosis of the cadherin receptors (BT-R).

and ion fluxing. Acceleration of this second messenger pathway alters the chemistry of the cell and brings about cell death.[143,144] Furthermore, the killing mechanism involves promotion by the toxin of exocytotic translocation of BT-R_1 from intracellular membrane vesicles to the cell membrane.[170] Movement of the receptor is mediated by toxin-induced signal-transduction, and amplification of this signaling is correlated directly to the execution of cell death.

Phenotypic changes associated with the progression of Cry1Ab toxin-induced cell death are the hallmarks of the killing process. **Figure 8** summarizes the cytological changes in High Five cells transfected with BT-R_1 cDNA[143] and BT-R_3 cDNA. (Ibrahim M and Griko N, unpublished results). The BT-R_1-transfected cells (**Fig. 8**, lower) respond to the Cry1Ab toxin by undergoing a series of biochemical events, including activation of a signaling pathway, which leads to discrete morphological changes, modified cell membrane permeability and complete cellular lysis. Importantly, Cry4Ba toxin also induces similar biochemical and cytological changes in High Five cells transfected with BT-R_3 cDNA (**Fig. 8**, upper). This particular result corroborates the effects of Cry toxin on living cells and establishes a pattern of cell death events brought about by cellular machinery intrinsic to the cell (**Fig.** 7).

It is particularly noteworthy that toxin action can be inhibited by blocking the binding of the toxin to BT-R_1 either in an insect[126,159] or in cultured insect cells.[143,171] Recently, Liu et al.[172] reported that Cry1Ac cytotoxicity in the cotton bollworm can be reduced by a soluble toxin-binding cadherin fragment. All of these results demonstrate that the primary determinant of toxicity involves specific toxin-receptor interaction.

Determining the chemistry of the toxin-receptor structure-function relationship would provide a significant step toward better understanding Cry toxin action. In this regard, we have constructed and described a structural model for the toxin-binding cadherin domain of the BT-R_1 receptor generated from X-ray crystal structures of homologous cadherin domains.[132] The structure includes residues 1349–1460 that comprise EC12 (**Fig. 6**). A structural model for the Cry1Ab toxin also was generated by the SWISS-MODEL protein modeling server[173] using the crystal structure of the Cry1Aa toxin (protein data bank code 1CIY) as a template. The Cry1Ab and Cry1Aa amino acid sequences are 88.3% identical, ensuring a reliable model.

A cropped view of the modeled toxin-receptor complex (see ribbon structure in **Fig. 9A**) shows residues within the sequence 422–498 in Cry1Ab that most likely are required for BT-R_1 binding, suggesting that this sequence contains the receptor-binding site. In the model, this sequence spans the end of Domain II (blue) and the beginning of Domain III (gold) and contributes to two distinct regions (red) on the toxin surface. Also as seen in **Figure 9**, the two regions are oriented such that they can interact with the signature sequences of BT-R_1 depicted in **Figure 6**. Close inspection of the interface between the Cry1Ab toxin and the TBR of BT-R_1 reveals potential hydrophobic interactions involving surface-exposed Phe_{440} in Cry1Ab and Leu_{1358}, Tyr_{1453} and Val_{1455} in the TBR of BT-R_1. Furthermore, potential electrostatic interaction between the positively charged Arg_{437} in Cry1Ab and the negatively charged Glu_{1357} in the TBR is probable. Importantly, both Arg_{437} and Phe_{440} have been shown to be essential to receptor binding and insecticidal activity.[110] It is noteworthy that Glu_{1357}, Leu_{1358} Tyr_{1453} and Val_{1455} lie either within or immediately adjacent

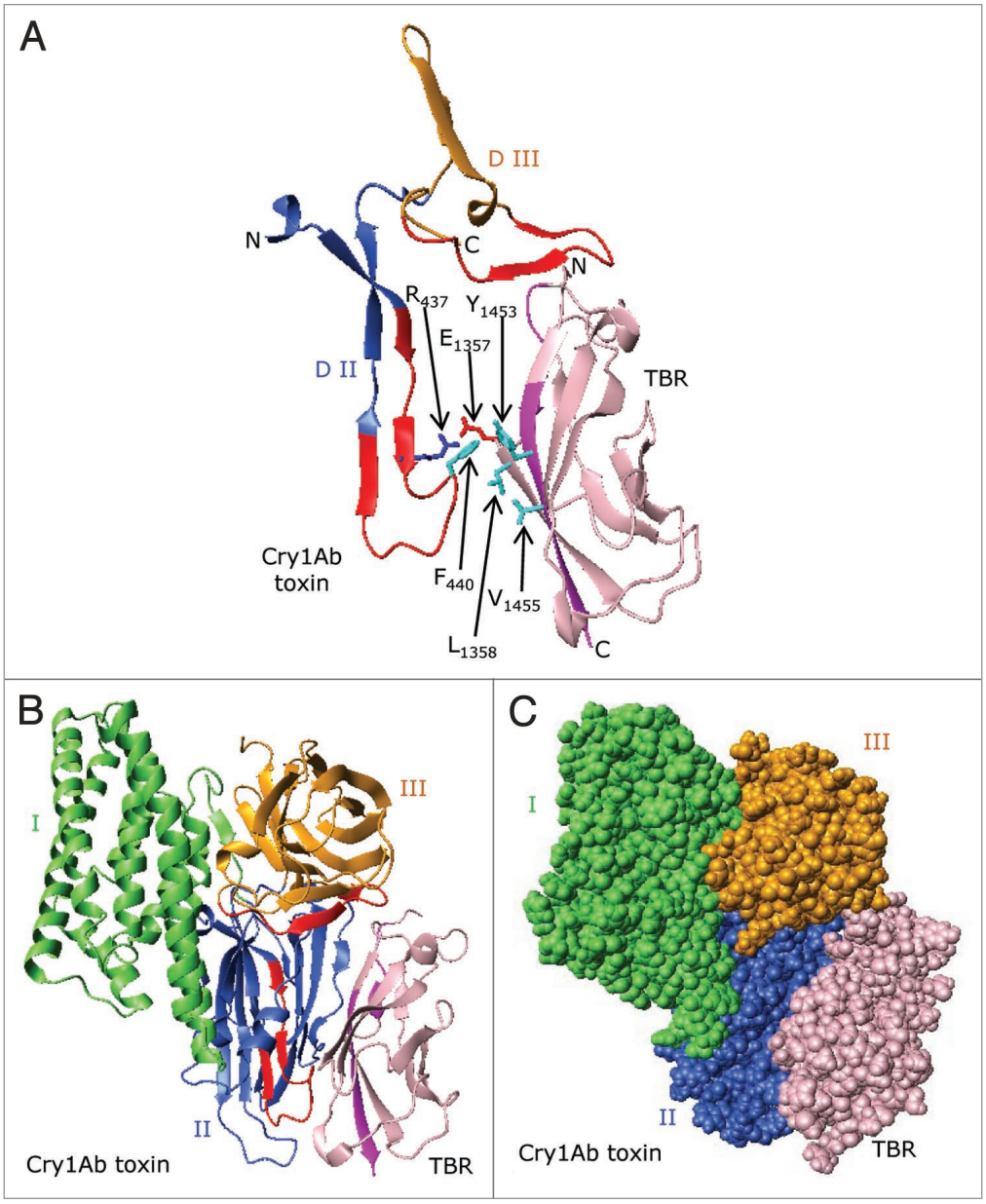

Figure 9. A proposed model showing docking of the Cry1Ab toxin to BT-R_1. (A) Ribbon structures showing docking of the putative receptor-binding sequence of the Cry1Ab toxin (residues 422–498) to the TBR (residues 1349–1460) in BT-R_1. The structure for the TBR was generated from the X-ray crystal structures of homologous cadherin domains as described in the legend to Figure 6. Note that in the interface between the Cry1Ab toxin and the TBR of BT-R_1 there are potential hydrophobic interactions involving surface-exposed Phe_{440} in Cry1Ab and Leu_{1358}, Tyr_{1453} and Val_{1455} in the TBR of BT-R_1. Also, there is probable electrostatic interaction between the positively charged Arg_{437} in Cry1Ab and the negatively charged Glu_{1357} in the TBR. Glu_{1357}, Leu_{1358}, Tyr_{1453} and Val_{1455} lie either within or immediately adjacent to the signature sequences in the TBR of BT-R_1 (Fig. 6 and blue arrows). Ribbon (B) and space-filling (C) representations showing docking of Cry1Ab toxin to TBR. Domains I, II and III of the toxin are colored green, blue and gold, respectively. The TBR is colored pink. Surface-exposed residues 432–449 and 480–493 in Cry1Ab are colored red, and residues 1349–1354 and 1451–1460 (signature sequences) in the TBR are colored purple.

to the signature sequences in the TBR of BT-R_1 (**Fig. 6** and blue arrows). **Figure 9B** shows a potential docking arrangement in a ribbon structure, with residues forming the two surface regions in the toxin highlighted in red (residues 432–449 and 480–493) and residues in the signature sequences of BT-R_1 highlighted in purple (residues 1349–1354 and 1451–1460). **Figure 9C** is a space-filling representation of the proposed docking. This docking arrangement maximizes the surface contact between the predicted binding residues on both proteins.

Insect Resistance to Cry Toxins

For the past 50–60 years, commercial formulations of Bt have been utilized to control economically important insect pests

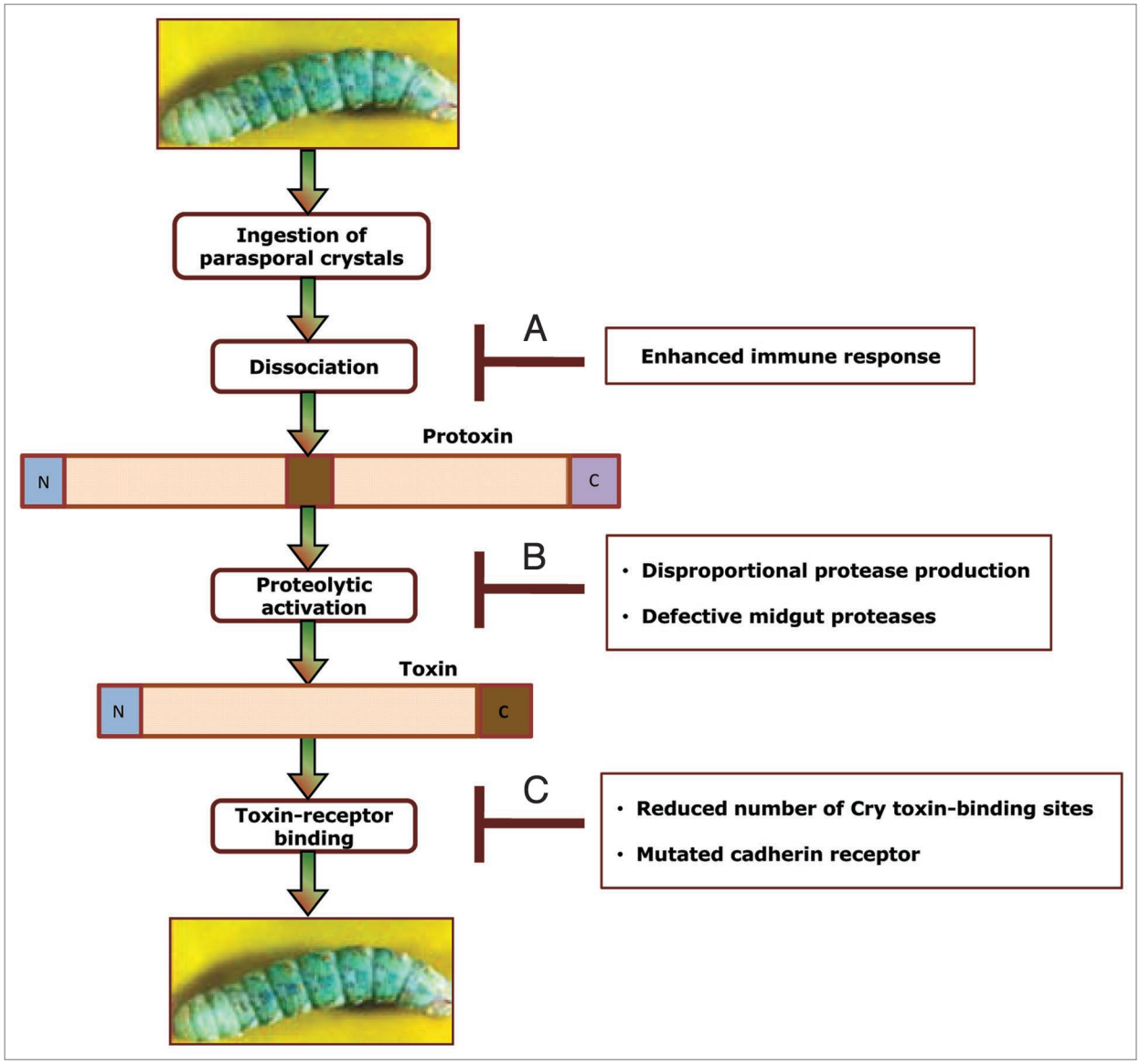

Figure 10. Possible mechanisms of insect resistance to Bt. Once Bt parasporal crystals are ingested by an insect larva, the crystals are dissociated, followed by proteolytic activation of protoxin and conversion to toxin. The activated toxin binds to the receptor, effecting a cascade of signal transduction events that lead to eventual larval death. There are three ways by which the insect larva could become resistant: (A) an enhanced immune response that inhibits dissociation and accessibility of protoxin; (B) erroneous protease production or defective protease activity that interferes with protoxin/toxin activation; (C) reduced number of binding sites or mutated receptors that retard or prevent appropriate toxin-receptor interaction.

worldwide. Today, a number of agricultural crops carry *cry* genes that render them resistant to insect infestation. Because the bacterium has co-existed and co-evolved with insects for millions of years, it was assumed that insects would not develop resistance to Bt or its insecticidal toxins. Furthermore, it has been taken for granted that Bt can adapt to an insect's defense system simply by altering the toxins produced to fit the situation or by generating several different toxins with varying specific activities. Although co-evolution and adaptability are relevant factors, interference with nature by questionable management practices involving widespread and intensive use of Bt and its *cry* genes has increased the likelihood of insects developing resistance to Bt-based bioinsecticides and transgenic plants. In other words, strong evolutionary pressure on insects by Bt will promote development of defense mechanisms that can circumvent bacterial and toxin attack. In fact, a number of different Bt-resistant insect species have been generated in the laboratory through special selection techniques or have been discovered naturally in the field.[174-176]

The insecticidal activity of Cry toxins involves a number of sequential events, including dissociation of the parasporal crystals into protoxins, activation of the protoxins to toxins by gut proteases, interaction of the toxins with midgut epithelium and binding to specific receptors. Toxin-receptor interactions trigger a cAMP-dependent signaling pathway, which leads to disruption of the structure and functionality of the midgut epithelium (**Fig.**

7).[143,144] When in continual touch with Bt, insects exhibit physiological changes and enhanced immune response. The upshot is resistance to the insecticidal activity of Bt. Three possible scenarios that describe the loss of sensitivity to Bt toxins are summarized in **Figure 10**. A heightened immune response primarily involves changes in the activity of the mucosal surface, causing increased secretion of proteases and pro-coagulants (**Fig. 10A**). For example, a 75-kDa pro-coagulant protein from the gut juice of the spruce budworm, which exhibits elastase-like activity, has been shown to bring about precipitation of the protoxin of Bt subsp. *sotto*.[177] Precipitation leads to sequestering of the toxin and limiting its accessibility to its target receptor.

The profile of gut juice proteases involved in protoxin activation changes with larval age. During larval growth and development, there is an observable increase in the concentration of proteases in the alimentary canal. This boost in the amount of gut proteases may account for the reduced sensitivity to Cry toxins (**Fig. 10B**). In the case of a resistant strain of the Colorado potato beetle (CPB), function-based activity profiling using zymographic gels containing gelatin as a substrate showed specific proteolytic bands present in midgut extracts and brush border membrane vesicles of the resistant strain not apparent in the sensitive strain. Furthermore, the aminopeptidase activity associated with the membrane vesicles was higher in the resistant strain than the sensitive one.[176] Increased levels of aminopeptidase also were seen in a resistant colony of Indian meal moth.[178] Unlike the CPB, proteolytic activation of Bt subsp. *kurstaki* protoxins was significantly reduced in a resistant strain of the Egyptian cotton leafworm compared to a sensitive strain (Ibrahim M, unpublished data), indicating that impaired proteolytic processing of toxins in insect guts is an essential mechanism for resistance. Similarly, in the Indian meal moth, a chymotrypsin-like enzyme was significantly reduced in the resistant moth.[178] Whether this proteolytic enzyme is essential for toxin activation is not known.

Decreased toxin binding due to reduction in the number of toxin-binding sites or to mutations that render the receptor incapable of binding to the Cry toxin has been implicated also in resistance (**Fig. 10C**).[147,176] Saturation-binding assays revealed that brush border membrane vesicles from the resistant CPB strain bound ~60% less Cry3A toxin than membrane vesicles from the sensitive strain. Homologous competition inhibition

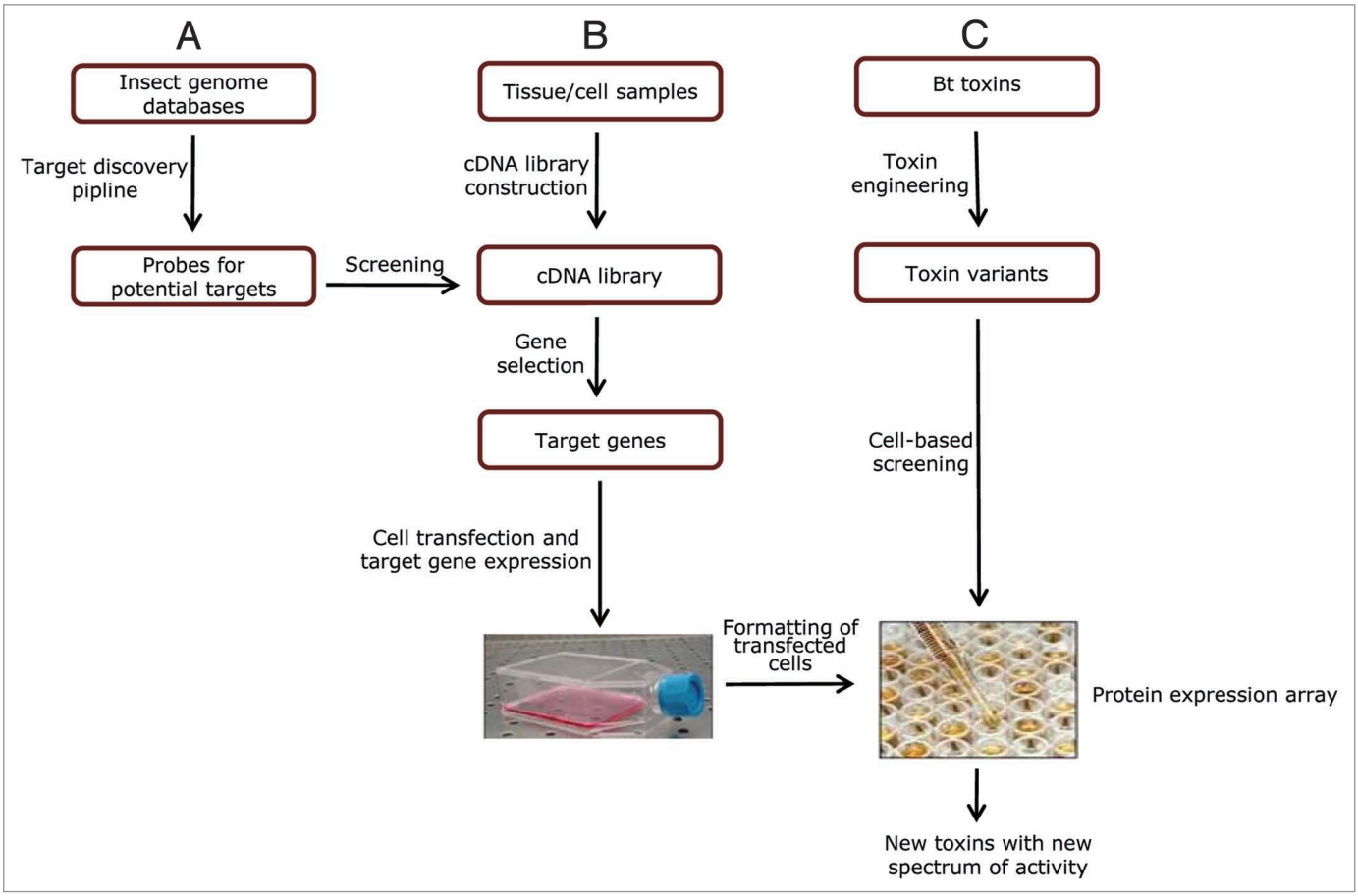

Figure 11. A genomics/proteomics platform for new insecticide discovery. There are three pathways to discovery, identification and validation of insecticide targets. (A) Bioinformatics pipeline to identify target proteins and to design specific primers or probes for cDNA screening. The process of target selection starts with data mining of a list of protein sequences from available genomic databases. The proteins are selected and categorized based on their potential to mediate insecticidal action. (B) Construction of cDNA libraries from tissue or cell samples, cloning and transfection of selected genes in insect cells and formatting of transfected cells for gene expression. (C) Construction of engineered toxins and target screening for evaluation and validation.

of ^{125}I-Cry3A binding to membrane vesicles also revealed differences in binding affinity between the sensitive and resistant strains.[176] Apparently, resistance in the CPB correlates with specific alterations in protease activity in the midgut as well as decreased toxin binding.

Future Considerations

What's next for Bt? Although scientific and technical knowledge about Bt has advanced during the last few decades, detailed studies involving global gene expression and regulation of physiological responses in addition to phenotypic differences and comparative analyses with related bacteria, particularly Ba and Bc, have been limited due to lack of functional genomics and proteomics information. Because Bt typically establishes a pathogenic relationship with its host, but also can exist symbiotically with some invertebrate animals, the bacterium provides an exceptional model to address questions related to microbe-host interactions and to those factors that influence pathogenic and symbiotic relationships. As already discussed, the pathogenicity of Bt involves targeting specific cadherin receptors within susceptible hosts, indicating that attacking cell adhesion molecules is evolutionarily significant for Bt and many other pathogens that disrupt and penetrate epithelial barriers in their hosts.[159,179] Therefore, deciphering the genome and proteome of Bt can help answer various questions related to bacterial disease processes as exemplified in insect systems. Likewise, understanding more about insect host immune response to Bt would help explain some of the features and characteristics of the immune system in invertebrates in general. Also, fathoming insect immunity a la Bt infections would enlighten us about vertebrate immunity as well because insects are similar in a number of ways to vertebrates in their ability to ward off disease.

A most promising area of investigation is the discovery, identification and validation of molecular targets for development of new insecticides. There are extensive databases for genome sequences of insects and other organisms which afford valuable information for identification of new targets. Recently, a web-based computational pipeline platform was developed for automated large-scale gene mining and insecticide target identification.[180] The platform utilizes bioinformatics, genomics and proteomics for high-throughput gene and protein target identification (**Fig. 11**). It brings together genome- and proteome-based target identification and target-directed screening for validating the action of engineered Bt toxins in cell-based assays. All targets selected for consideration can then be analyzed in silico by docking calculations, molecular dynamics simulations and other techniques to characterize appropriate target interactions with chemically or genetically altered Cry toxins. Such an approach should facilitate protein design for the creation of Cry protein and peptide mimics that might be more effective than the natural toxins themselves and less able to induce insect host resistance.

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