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Review

Signaling versus punching hole: How do Bacillus thuringiensis toxins kill insect midgut cells?

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Abstract. Cry proteins, produced by *Bacillus thuringiensis* (Bt), are widely used for the control of insect pests in agriculture as spray products or expressed in transgenic crops, such as maize and cotton. Little was known regarding the mechanism of action of these toxins when the first commercial Bt product was introduced fifty years ago. However, research on the mechanism of action over the last two decades has enhanced our knowledge of toxin interaction with membrane receptors and their effects in insect midgut cells. All this information allowed for the rational design of improved toxins with higher toxicity or toxins that overcome insect resistance, which could compromise Bt use and effectiveness in the field. In this review we discuss and evaluate the different models of the mode of action of Cry toxins, including a discussion about the role of various receptors in toxin action.

Keywords. Cry toxins, *Bacillus thuringiensis*, pore formation, signal transduction, toxin receptors, oligomerization, Cry toxin resistance.

Use of Bt toxins as bioinsecticides to control insect pests

Bacillus thuringiensis (Bt) is a member of the *Bacillus cereus* group of bacteria [1]. One important characteristic of Bt strains is that they are entomophatogenic because they produce insecticidal δ-endotoxin proteins (named Cry and Cyt toxins) during the sporulation phase. These proteins are highly specific to their target insects, are innocuous to humans, other vertebrates and plants, and are completely biodegradable. Therefore, they represent a viable alternative for the control of insect pests in agriculture and disease vectors of importance in human public health [2].

Cry and Cyt δ-endotoxins are defined as any parasporal inclusion protein produced by Bt bacteria that is toxic to a target organism, or any protein that has sequence similarity to known Cry or Cyt proteins. Presently these proteins have been classified as Cry1 to Cry55; and Cyt1 and Cyt2 based on their primary sequence identity [3]. The Cry toxins are organized in three main groups that are not related phylogenetically (the three domain, the mosquitocidal-like and the binary-like Cry toxins) and it is proposed that each of these groups of Cry toxins may have a different mechanism of action [4]. The mosquitocidal-like Cry toxins (Mtx-like) and the binary-like Cry toxins (Bin-like) have some similarity with the Mtx or Bin toxins produced by *B. sphaericus,* although in the case of *B. sphaericus* these toxins are toxic against mosquitoes and in Bt they are toxic against coleopteran larvae [4].

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The three domain Cry toxins (3d-Cry) represent the biggest group of Cry proteins. It is classified into more than 40 different groups (Cry1, Cry2…) and many subgroups (Cry1Aa, Cry1Ab, Cry2Aa, Cry1Ba, Cry1Ca, Cry2Aa…) [3]. They are globular proteins composed of three distinct domains connected by single linkers. Domain I is a seven α-helix bundle implicated in membrane channel formation. Domain II consists of a β-prism of three anti-parallel β-sheets packed around a hydrophobic core [2] and domain III is a β-sandwich of two antiparallel β-sheets. Domains II and III are implicated in insect specificity [2]. Figure 1 shows a representative structure of one member of the 3d-Cry group, the Cry3Aa, which was the first solved three-dimensional structure [5]. The structures of other 3d-Cry members that have also been resolved (Cry1Aa, Cry3B, Cry2Aa, Cry4Aa and Cry4Ba) are highly similar to Cry3Aa and it was proposed that all members in this group of Cry toxins might function by a similar mode of action [6–10].

Cyt toxins comprise two highly related groups (Cyt1 and Cyt2) [3]. Cyt toxins have a single α-β domain consisting of two outer layers of α-helix hairpins wrapped around a β-sheet [11]. Cyt proteins are almost exclusively found in dipteran-active Bt strains, although a few exceptions have been found [12]. The Cyt toxins synergize the toxic effect of some Cry proteins active against mosquitoes and also that of the Bin toxin produced by *B. sphaericus* [13, 14].

The number of different δ-endotoxin proteins that have been isolated and cloned is quite big: more than 168 different *cry* and nine *cyt* holotype sequences [3]. However, their application in agriculture and in human health is still limited, since only a few Cry and Cyt toxin variants have been developed as commercial spray products or have been incorporated into the plant genome to produce insect-resistant transgenic crops [15].

The commercialized spray products formulated as liquid concentrates, wettable powders, and ready-to-use dusts and granules have been widely adopted by growers in large-scale crop production. Most of these products (Biobit, Condor, Cutlass, Dipel, Full-Bac, Javelin, and M-Peril,) are composed of spore-crystal preparations derived from wild-type strains such as *B. thuringiensis* var. *kurstaki* (Btk) HD1 and HD73 strains that express some Cry1A and Cry2A proteins. These products are highly effective in controlling many common leaf-feeding caterpillars, including pests of vegetables, cereals and cotton [15]. Btk-based products have been successfully used for the control of lepidopteran defoliator pests of conifers, such as bag worms, tent caterpillars and other forest caterpillars [16]. Other products, including Certan, Agree and Xentari are based on other Bt strains such as *B. thuringiensis* var. *aizawai* HD137 which produces slightly different Cry toxins, Cry1A, Cry1B, Cry1C and Cry1D. These products kill other lepidopterans, including the Indian meal moth larvae in stored grain. In addition, Bt formulations that are effective against beetles seem to offer great promise. Products such as M-Trak, Foil and Novodor are based on *B. thuringiensis* var. *san diego* and *B. thuringiensis* var. *tenebrionis*, which produce Cry3 toxins active against some coleopteran insects, including the Colorado potato beetle, *Leptinotarsa decemlineata* [17]. Finally, *B. thuringiensis* var. *israelensis* (Bti), containing Cry4, Cry10, Cry11 and Cyt toxins, is the base of several commercial products (Vectobac, Teknar, Bactimos, Skeetal, and Mosquito Attack) that have been developed for the control of disease vector mosquitoes such as *Aedes aegypti* (vector of dengue fever), *Simulium damnosum* (vector of onchocerciasis) and certain *Anopheles* species (vectors of malaria) [18, 19]. The high insecticidal activity, the lack of mosquito resistance to this Bti subspecies due to the presence of Cyt and Cry toxins, the lack of activity against other organisms and the reported mosquito resistance to chemical insecticides has made Bti an effective alternative control method of mosquitoes and black flies.

Bt products are highly selective. For example, those that kill lepidopteran insects are not effective against other types of pests such as coleopteran or dipteran pests and vice versa. Because of this narrow selectivity of Cry toxin action, Bt products usually kill only certain lepidopteran pests while others are not controlled at all, either because they show low susceptibility or due to their feeding habits; those insects that bore into plant tissues do not consume a significant amount of the Bt applied to plant surfaces. Insects that live in the soil or sucking insects are also poorly controlled by spraying Bt formulations [20]. However, the creation of transgenic plants in which the Cry toxin is produced continuously in the plant tissues and is in this way protected from degradation facilitates the control of insects that bore into plant tissues and those that feed on plant roots. These transgenic crops have caused a revolution in agriculture by substituting an environmental friendly alternative for chemical insecticides. Consequently, Bt-crops are now grown on over 162 million hectares worldwide and have proven to be effective in controlling some insect pests and to help reduce the use of chemical insecticides [20].

Proposed models of the mode of action of Cry toxins

Two models have been reported to describe the mode of action of Cry toxins. For more than 20 years, the 3d-

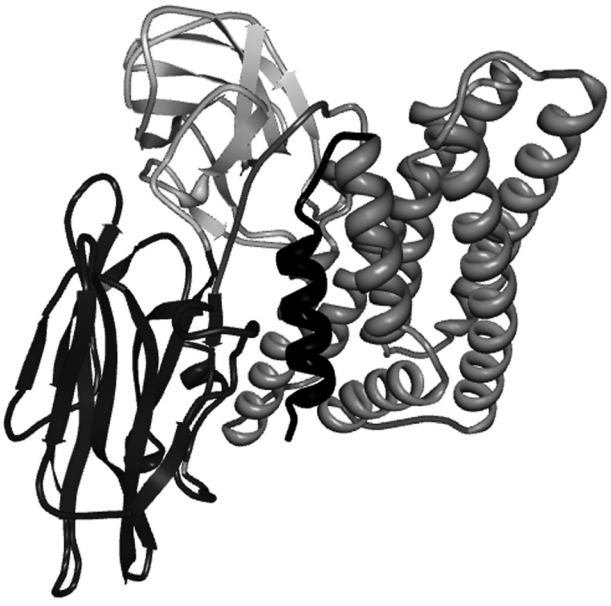

Figure 1. Ribbon representation of Cry3Aa toxin structure. Cry3Aa is a member of the 3d-Cry family group. Domain I is composed of seven α-helices, domain II and domain III are composed of β-sheets. The helix alpha-1 is shown in black. This figure was constructed using Swiss-PDB Viewer and coordinates of Cry3Aa (PDB number 1DLC).

Cry toxins have been described as pore–forming toxins that induce cell death by forming ionic pores following insertion into the membrane, causing osmotic lysis of midgut epithelial cells in their target insect [2, 6, 21–23]. Since then, multiple publications have provided evidence that supports this model. However, an alternative model recently proposed that 3d-Cry toxins are not pore-forming toxins, but toxins that activate a cascade signal pathway mediated only by its interaction with a specific receptor named cadherin [24, 25]. The initial steps in the mode of action in both models are similar, from ingestion of the toxin to the interaction with the primary cadherin receptor (Fig. 2).

Pore formation model

The pore formation model of 3d-Cry toxin action comprises several steps [23] (Fig. 2). Most of the 3d-Cry proteins are produced as crystalline inclusions of 130 kDa protoxins while some others are 70 kDa protoxins. The larvae ingest the crystalline inclusions which are solubilized in the gut lumen due to its high pH and reducing conditions. After solubilization the protoxins are cleaved by midgut proteases at the N- and C-terminal ends to yield activated monomeric 60 kDa toxins with a three domain structure [2] (Fig. 1). The activated toxins bind to primary receptors that are present in the larval midgut cells. In the case of the Cry1A toxins that are active against several lepidopteran pests, the cadherin-like proteins function as the primary receptors [26, 27, 28, 29]. Recently cadherin was also recognized as a functional receptor in the dipteran insect *An. gambiae* [30]. Binding with cadherin facilitates additional protease cleavages of the N-terminal end of the toxins, eliminating helix α-1 of domain I [31, 32]. This cleavage induces assembly of oligomeric forms of the toxin. The oligomers have increased binding affinity to the secondary receptors, which are glycosylphosphatidyl-inositol (GPI)-anchored proteins, such as aminopeptidase N (APN) in *Manduca sexta* and alkaline phosphatase (ALP) in the lepidopteran *Heliothis virescens* or in the dipteran *Ae. aegypti* [23, 33, 34]. After the oligomers bind to secondary receptors, they insert into membrane microdomains where GPI-anchored receptors are localized, and create pores in the apical membrane of midgut cells causing osmotic shock, bursting of the midgut cells and finally ending in the insect's death [2, 4, 23] (Fig. 2).

Signal transduction model

Recently, an alternative model proposed that the toxicity of Cry proteins is due to the activation of a Mg^{+2}-dependent signal cascade pathway that is triggered by the interaction of the monomeric 3d-Cry toxin with the primary receptor, the cadherin protein. This interaction activates a guanine nucleotide-binding protein (G protein), which in turn activates an adenylyl cyclase promoting the production of intracellular cAMP. The increased cAMP levels activate protein kinase A which activates an intracellular pathway resulting in cell death [24]. This model states that insect cell death occurs without the participation of oligomeric structures that form lytic pores of Cry toxin or the participation of other receptors as GPI-anchored proteins [24, 25] (Fig. 2).

Role of receptors in mode of action of Cry toxins

As mentioned previously, Cry toxins are highly selective and kill only a limited number of insect species. This selectivity is mainly due to the interaction of Cry toxins with larval proteins located in the midgut epithelium cells. A major research effort has taken place in the identification of insect proteins that bind Cry toxins and mediate toxicity. Among these, two major types of receptors have been identified: transmembrane proteins, such as cadherins, and proteins anchored to the membrane such as the GPI-anchored proteins that have been proposed to be involved in the action of Cry toxins.

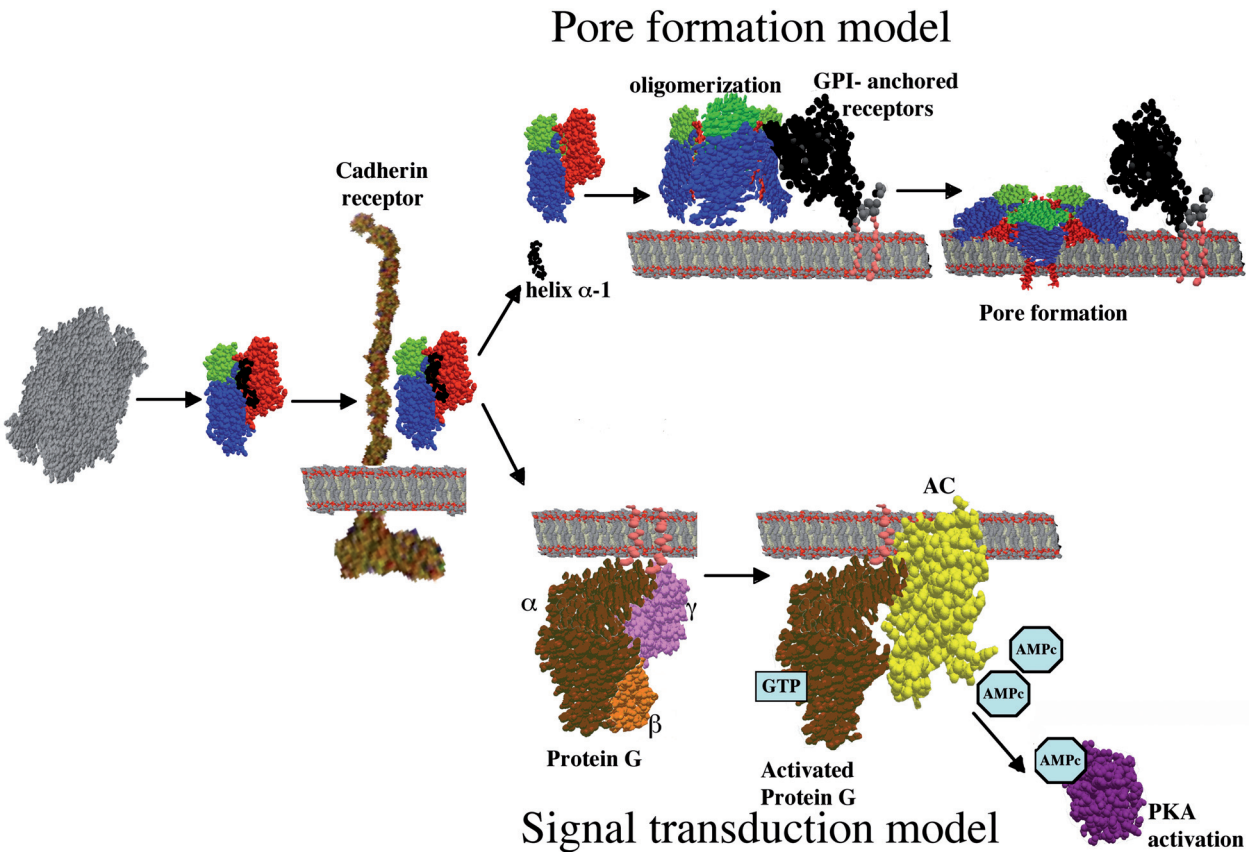

Figure 2. Mechanism of action of the 3d-Cry toxins. The pore formation model and the signal transduction model. Both models include identical initial steps. In these steps, the protoxin is solubilized in the insect gut lumen, then the soluble protoxin is activated by midgut proteases resulting in the 3-d Cry monomeric toxin and finally the toxin binds to the cadherin receptor. In the pore forming model, the interaction with cadherin initiates the cleavage of helix α-1, leading to toxin oligomerization. The oligomeric Cry toxin binds to GPI-anchored receptors which help in toxin insertion into the membrane. The formed pore is important for cell death. In the signal transduction model, toxin interaction with cadherin activates a G protein that increases activity of adenylyl cyclase (AC), resulting in increased cAMP levels which in turn activate protein kinase A (PKA) that is responsible for the activation of an intracellular pathway resulting in cell death. Only the structure of the monomeric Cry toxin was modelled using Swiss-PDB Viewer and coordinates of Cry1Aa (PDB number 1CIY). The rest of the proteins depicted in the figure are hypothetical structures.

Cadherin receptors

Cadherin proteins were first identified as Cry1A toxin-binding proteins in the lepidopteran insect *M. sexta* [26, 35] and later shown to be involved in Cry toxin binding and toxicity in several other lepidopteran species such as *Bombyx mori* [36], *H. virescens* [37], *Helicoverpa armigera* [38], *Pectinophora gossypiella* [39], *Ostrinia nubilalis* [40] and more recently in the dipteran *An. gambiae* [30]. Insect cadherins are modular proteins composed of three domains, the ectodomain formed by 11 to 12 cadherin repeats (CR), the transmembrane domain and the intracellular domain [35]. In contrast to vertebrate cadherins that are involved in inter-cellular contacts and are located in the basolateral membrane, in *M. sexta* and *An. gambiae* larvae the cadherin proteins were shown to be located in the microvilli of midgut epithelium cells, the site of action of Cry toxins [30, 41].

Different experimental evidence indicates that cadherin proteins are involved in the toxic effect of Cry toxins in different insect species.

Expression of cadherin receptors in cell lines

The *M. sexta* cadherin (Bt-R_1) and the *B. mori* cadherin protein (Bt-R_{175}) have been expressed in different cell lines and shown to mediate toxicity to Cry1A toxins in these cell lines, although the Cry toxin-induced toxicity levels were variable. In the case of Bt-R_1, its transient expression in mammalian cell lines COS7 or HEK193 induced susceptibility of these cell lines to Cry1Ab toxin [42]. Bt-R_1 has also been expressed in insect cell lines such as the *Drosophila melanogaster* Schneider 2 (S2) cell line [43] and the *Trichoplusia ni* High Five cell line and was found to render cells susceptible to Cry1Ab toxin in both cases [25]. Also, the cadherin protein from *H. virescens* (HevCaLP) was expressed in S2 cells conferring

susceptibility to Cry1A toxin [44]. Interestingly, a fragment of a truncated cadherin Bt-R_1 protein containing the cadherin repeat 12 (CR12) expressed in the surface of S2 cells also induced susceptibility to Cry1Ab toxin, suggesting that this protein fragment is important in mediating Cry1Ab-induced cytotoxicity [45]. In *H. virescens* cadherin CR11, that corresponds to the *M. sexta* CR12, was shown to be a Cry1Ac binding site [29]. In the case of Bt-R_{175}, this protein was expressed in the SF9 insect cell line [36] and also in the mammalian HEK193 cell line [46] and in both cases it induced susceptibility to Cry1Aa toxin.

Blocking toxin binding to the cadherin receptor

Another approach to determine the role of receptor molecules in the toxicity of Cry toxins has been the use of binding molecules such as antibodies that bind specific epitopes in the toxin or in the protein receptors and block the toxin-receptor interaction, hence showing an effect on Cry toxicity when fed together with the toxin in bioassays. In the case of cadherin receptors, single chain variable Fragments (scFv's) antibodies that bind to loop 2 or loop 3 of domain II of Cry1Ab toxin and inhibit binding of Cry1Ab to Bt-R_1 but not to the APN receptor were shown to lower the toxicity of Cry1Ab toxin to *M. sexta* larvae [27, 47]. Also, certain cadherin protein fragments that contain Cry1A binding regions inhibited the toxicity of Cry1Ab protein to *M. sexta* larvae or to the COS7 cell line expressing Bt-R_1 [28, 42]. In the case of *H. virescens*, an anti-cadherin antibody and a cadherin fragment containing a Cry1A binding site lowered Cry1Ab and Cry1Ac toxicity [29]. Finally, an anti-Bt-R_{175} antibody protected detached *B. mori* midgut cells from Cry1Aa toxin, as judged by the analysis of the release of lactate dehydrogenase activity after exposure of the cells to Cry1Aa toxin [48, 49]. Nevertheless, a Cry1Aa mutant (F328A) that showed 23-fold reduced binding to Bt-R_{175} was shown to have only a 4-fold reduction in the toxicity to *B. mori* larvae, suggesting that an additional protein in the larval gut could in part fulfill the role of Bt-R_{175} in this insect species [50].

Synergism of Cry toxin action by fragments of the cadherin protein

As mentioned previously, when a cadherin fragment of Bt-R_1 named CR12- MPED (membrane proximal ectodomain) which contains a Cry1A toxin-binding site was expressed in S2 cells, the susceptibility to Cry1Ab toxin was induced [45]. Interestingly, this CR12-MPED fragment produced in *E. coli* cells enhanced the activity of Cry1Ab toxin in different lepidopteran insect larvae when fed in bioassays [51].

It was suggested that the CR12-MPED fragment increases the toxin concentration in the microvilli membrane of the larvae, since this fragment is localized in the microvilli of the CR12-MPED fed larvae. The Cry toxin would then be able to interact with GPI-anchored Cry1A secondary-receptors [51]. A similar effect was observed with the corresponding fragment CR12 of the *An. gambiae* cadherin, suggesting that a cadherin receptor also mediates toxicity of Cry4Ba toxin in dipteran insects [30].

Mutations and silencing of the cadherin receptor

Toxin receptor interaction is a limiting step in the mode of action of Cry toxins, but theoretically the resistance to Cry toxins can occur by different mechanisms affecting any step of the mode of action of Cry toxins (Fig 2). However, the most common mechanism of resistance observed so far involves mutations that affect the assembly of cadherin receptor molecules [52]. The resistance to Cry1A toxins in three resistant colonies of different lepidopteran insect species, pests of cotton, was shown to be genetically linked to mutations in the cadherin gene. The first example was the resistance of the laboratory selected YHD2 *H. virescens* population that contained a retrotransposon insertion in the cadherin gene [37]. In the case of *P. gossypiella*, a resistant population (AZP-R) to Cry1Ac toxin was obtained after crossing individuals collected from ten different locations in Arizona cotton fields. It was shown that resistance in the AZP-R population was linked to three different cadherin deletion alleles [39]. In *H. armigera*, both a laboratory-selected population and a population obtained after screening of field individuals by crossing and selection of offspring in toxin, resistance was linked to three cadherin alleles, two retrotransposon insertions and one stop codon mutation [38, 53]. Finally, in the case of *M. sexta*, silencing of the Bt-R_1 transcript by dsRNA resulted in high tolerance to Cry1Ab toxin [54]. These data show that presence of the cadherin protein in the insect midgut is necessary for Cry1A toxicity.

Role of the cadherin receptor in inducing toxin oligomerization

As mentioned above, binding of Cry1A toxins to cadherin protein is an important step in mediating toxicity. Binding of Cry1Ab protoxin to cadherin in the presence of *M. sexta* midgut proteases induces the cleavage of helix α-1 of domain I of the toxin and oligomerization of the toxin, forming a 250 kDa structure [31] (Fig. 2). Figure 1B shows the localization of helix α-1 in the crystal structure of 3d-Cry toxins. An important observation was that scFv73 antibody binds to loop 2 of domain II of Cry1Ab toxin

and blocks binding to Bt-R_1. The scFv73 antibody also facilitates the formation of a Cry1Ab 250 kDa oligomeric structure [31]. Formation of the oligomeric structure was also observed when Cry1Ab protoxin was proteolytically activated in the presence of cadherin fragments containing Cry1A binding sites, including CR12 [28, 54] or in the presence of brush border membrane vesicles (BBMV) that contain the complete cadherin protein [31]. Further characterization of the oligomeric structure of Cry1Ab toxins showed that the oligomer structure is competent in membrane insertion and also induces stable pores with high open probability in contrast to monomers, indicating that the oligomer is the membrane insertion-competent structure [55] (Fig. 2).

Role of the cadherin receptor in mediating signal transduction in an H5 cell line

The role of Bt-R_1 cadherin mediating cell death by Cry1Ab was studied in a stable *T. ni* H5 insect cell line expressing Bt-R_1 (named S5 cells) [25]. The S5 cell line was susceptible to activated Cry1Ab toxin in a dose-dependent manner and toxicity was inhibited in the presence of a cadherin fragment (TBR1) that contains a Cry1Ab toxin-binding site. Cry1Ab toxicity mediated by Bt-R_1 was dependent on the presence of Mg^{+2} ions in the medium and it was speculated that a Mg^{+2}-dependent cellular signal was responsible for triggering cell death [24]. Further characterization of this signal transduction pathway by using some inhibitors revealed the participation of a G protein which activated an adenylyl cyclase, causing increased levels of cAMP that activated a protein kinase A (PKA). Activation of this PKA correlated with cell death [24] (Fig. 2).

GPI-anchored receptors

The first Cry1A toxin-binding protein that was described was an APN protein in the lepidopteran *M. sexta* [56]. This protein was glycosilated and anchored to the membrane by a GPI anchor. Since then, other GPI-anchored APNs have been recognized as Cry toxin receptors in different lepidopteran species such as *H. virescens* [57–60], *Spodoptera litura* [61], *H. armigera* [62], *B. mori* [63], *Lymantria dispar* [64], *Plutella xylostella* [65], and in the dipteran *An. quadrimaculatus* [66]. Phylogenetic analyses suggest that in lepidopteran insects there are at least five different APN families and at least three of them have been shown to bind Cry1 toxins in different insect species [67, 68]. A GPI-anchored ALP that binds Cry toxins has also been described in the lepidopterans *M. sexta* [69] and *H. virescens* [33] and in the dipteran *Ae. aegypti* [34]. In the coleopteran *Leptinotarsa decemlineata*, a GPI-anchored ADAM metalloprotease was shown to bind Cry3A toxin [70]. GPI-anchored proteins are preferentially partitioned to membrane microdomains or lipid rafts, and lipid raft integrity has been shown to be important for Cry1A toxin insertion and pore formation in *M. sexta* and *H. virescens* [71]. In the case of *M. sexta*, the APN and ALP proteins that bind Cry1A toxins were shown to be located in the microvilli of epithelial cells that is the site of action of Cry1A toxins [41].

Different types of evidence suggest that GPI-anchored proteins are involved in Cry toxicity in different insect orders:

Blocking toxin binding to GPI- anchored receptors

As with cadherin proteins, blocking the interaction of Cry toxins with GPI-anchored receptors has been useful in some cases to show the role of these proteins in Cry insecticidal activity. In the case of *M. sexta*, a scFv-phage that bound Cry1Ab toxin through β16-β22 of domain III blocked binding of Cry1Ab with APN but not with Bt-R_1 and inhibited the toxicity of Cry1Ab in bioassays [47]. Nevertheless, in *B. mori* detached midgut cells, an anti-APN antibody did not affect toxicity of Cry1Aa in contrast to an anti-cadherin antibody that inhibited toxicity, suggesting either that this APN may not be involved in toxicity or that other additional GPI-anchored proteins or lipids could substitute APN function [48, 49]. In *Ae. aegypti,* a peptide-phage that bound the 65 kDa ALP competed binding of the Cry11Aa to BBMV of mosquito and inhibited Cry11Aa toxicity in bioassays, suggesting that GPI-anchored ALP is a functional receptor of Cry11Aa [34]. Finally, in the coleopteran *L. decemlineata* a synthetic peptide corresponding to domain II loop 1 of Cry3Aa could bind the GPI-anchored ADAM metalloprotease and inhibit Cry3Aa-induced pore formation in BBMV isolated from *L. decemlineata* larvae, suggesting that the GPI-anchored metalloprotease is a functional receptor of Cry3Aa [70].

In the case of Cry1Ac toxin it has been shown that the toxin binds APN through a domain III binding pocket that recognizes an N-acetyl galactosamine (GalNAc) moiety in the receptor [72, 73]. Mutagenesis studies of Cry1Ac domain III identified the residues ^{509}QNR^{511}, N^{506} and Y^{513} as the sugar-binding epitope [74, 75]. Interestingly, these mutants which had altered *M. sexta* APN-binding were barely affected in toxicity [74, 75], suggesting that either APN is not an important receptor determining toxicity or that additional receptor molecules can fulfill the role of APN. As mentioned previously, Cry1Ac toxin binds to both APN and ALP molecules in *M. sexta* [56, 69] and ALP

also contains the GalNAc moiety [33]. Thus, the role of ALP in toxin action cannot be discarded.

Mutations and silencing of GPI-anchored receptors

As mentioned previously, an *H. virescens* laboratory selected population, YHD2, contained a retrotransposon insertion in the cadherin gene [37]. However, the mutation in the cadherin gene only accounted for 40-80 % of the resistance phenotype. Additional mutations were responsible for the rest of the resistant phenotype in YHD2. These additional mutations were shown to affect GPI-ALP production, indicating that ALP is likely a functional receptor of Cry1Ac toxin in *H. virescens* [33].

A resistant *S. exigua* population that is resistant to Cry1Ca toxin was shown to lack the RNA transcript of APN-1, suggesting that this APN is involved in Cry1C toxicity to this insect species [67]. Finally, in the case of *S. litura*, silencing an APN with dsRNA resulted in a lower susceptibility to Cry1Ca toxin, also indicating a role of APN in Cry1C toxicity in this insect species [76].

Role of GPI-anchored APN in facilitating membrane insertion and pore formation

The APN has been implicated in toxin insertion, since cleavage of APN by phosphatidyl-inositol specific phospholipase C treatment which cleaves out the GPI anchored proteins substantially decreased the levels of Cry1Ab incorporation into insoluble lipid raft membranes [23, 71] and drastically reduced the pore-formation activity of the toxin assayed in BBMV from *Trichoplusia ni* [77]. In addition, the incorporation of APN into the lipid bilayer enhanced Cry1Aa pore-formation activity [78].

The sugar GalNAc in the APN receptor is an important epitope in the interaction with Cry1Ac toxin [72, 73]. In the case of the lepidopteran *Lymantria dispar*, it was proposed that the monomeric Cry1Ac toxin interacts with APN following a sequential binding model [79]. In this model, APN is first recognized by domain III of Cry1Ac through the GalNAc moiety, followed by a protein-protein contact of the domain II loop region of Cry1Ac. The first contact is fast and reversible, and mutations close to a domain III cavity affect this initial binding, while mutations in domain II affect the rate constants of the second interaction step which is slower and tighter [79]. Li et al. [80] reported that the binding of GalNAc to monomeric Cry1Ac correlates with an increase of temperature factors in the pore-forming domain I. However, there was no indication of a clear conformational change in the monomeric-Cry1Ac toxin [80]. In contrast, the fluorescence spectroscopy studies of Cry1Ac in its oligomeric state showed that GalNAc induces a conformational change in domain III of the oligomeric structure of Cry1Ac in the vicinity of the sugar pocket [81]. The interaction of Cry1A-oligomer with GalNAc enhanced membrane insertion of the soluble pre-pore oligomeric structure [81], supporting the model that interaction of the Cry1A pre-pore with GPI-anchored receptors facilitates membrane insertion and pore-formation. The APN-oligomer interaction may be especially critical when low toxin protein concentrations reach the midgut epithelium, conditions that may occur *in vivo* in the larvae gut where the Cry toxins are exposed to high concentrations of proteases.

Participation of oligomer structures in larvae intoxication

Other Cry toxins besides Cry1A toxins are also able to form oligomeric structures when they are activated in the presence of their natural receptor. This is the case for several Cry1 toxins such as Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da, Cry1Ea and Cry1Fa that are active against *M. sexta* and result in the formation of an oligomeric structure after activation in the presence of *M. sexta* BBMV midgut membranes [31, 55, 82–85]. Oligomeric structures of Cry1C were also found when this toxin was activated in the presence of BBMV from a target insect, *S. exigua* [85], and oligomeric structures of Cry1Aa were also observed when this toxin was activated in the presence of BBMV from *Bombyx mori* larvae [86]. The presence of oligomeric structures in these toxin preparations correlated with higher K^+ permeability than in samples containing only monomeric toxins [31, 55, 82], supporting the hypothesis that the oligomeric structure of Cry toxins is the intermediate responsible for its insertion into the membrane. The Cry3Aa, Cry3Ba and Cry3Ca toxins, which are specific against coleopteran insects, also formed oligomeric structures after activation in the presence of BBMV membranes from the susceptible insect *L. decemlineata* and correlated with higher pore formation [87].

Regarding dipteran-specific toxins, it was reported that Cry11Aa was able to form oligomeric 250 kDa structures after activation in the presence of BBMV of the mosquito *Ae. aegypti* [88]. The Cry4Ba also formed oligomeric structures after activation [89]. In fact, the membrane-associated structure of the Cry4Ba toxin was further analyzed by atomic force microscopy (AFM) [90] and by electron crystallography [91]. The AFM studies indicated that the toxin preferentially inserts into the membrane in a self-assembled structure, showing a pore-like structure with a four-fold symmetry, suggesting that tetramers

are the preferred oligomerization state of this toxin [90]. However the calculated projection structures from 2D crystal patches analyzed by electron crystallography at 17 Å resolution showed a trimeric organization [91]. The AFM was also used to analyze the structure of Cry1Aa toxin inserted into monolayer membranes. These studies suggested that the pores are composed of four subunits surrounding a 1.5 nm diameter central depression [92].

Finally it was reported that Cyt1Aa synergizes the toxic activity of Cry11Aa by functioning as a membrane-bound receptor of Cry11Aa [93]. The proposed mechanism is that Cyt1Aa inserts into the midgut epithelium membrane and exposes protein regions that are recognized by Cry11Aa. It was demonstrated that this interaction facilitates the oligomerization of Cry11Aa and its pore formation activity [88, 93]. Cry11Aa binds Cyt1Aa using the loop α-8 that is also involved in interaction with its ALP receptor [34]. Mutations in the binding regions of Cry11Aa or Cyt1Aa affected the specific interaction between these proteins, reduced their synergism [93] and reduced the formation of a Cry11Aa oligomeric structure [88].

Modified Cry toxins that kill insects resistant to Cry1A toxins

According to the pore formation model, the cadherin receptor binds the monomeric toxin and induces toxin oligomerization after further cleavage of helix α-1. Recently, modified toxins lacking helix α-1 (named CryMod toxins) were constructed. These Cry1AMod toxins form oligomers of 250 kDa when treated with trypsin in the absence of the cadherin receptor [54]. The oligomeric structures formed by CryMod toxins are active in pore formation and are toxic against *M. sexta* and *P. gossypiella* larvae. The most important characteristic of Cry1AMod toxins is that they are able to kill insects that are resistant to native Bt toxins due to mutations in the cadherin receptor or that have reduced susceptibility due to silencing of cadherin protein expression by using RNAi [54] (Fig. 3). The Cry1AMod toxins were tested against Cry1Ac-susceptible and -resistant *P. gossypiella* larvae. The Cry1Ac-resistant *P. gossypiella* AZP-R strain has deletion mutations in the cadherin gene [39] and was able to survive on Bt cotton producing Cry1Ac [39, 94] but was highly sensitive to Cry1AMod toxins [54]. The Cry1AMod toxins were also toxic to the susceptible *P. gossypiella* APHIS-S strain, but the modified toxins were slightly less potent than the wild type toxins.

Moreover, silencing of cadherin expression in *M. sexta* larvae using RNAi resulted in inhibition of the expression of the cadherin protein and a decreased larval susceptibility to Cry1Ab. However the *M. sexta* larvae with cadherin silenced by RNAi were still highly susceptible to Cry1AbMod toxins [54].

Cry1A mutants in oligomerization

Oligomeric structures in some other proteins are stabilized by α-helical coiled-coil structures which constitute an important protein-folding motif formed in the interaction between two to five α-helices [95, 96]. The primary structure of coiled-coil structures is characterized by heptads of residues, $(abcdefg)_n$, with a unique pattern of internal *a* and *d* positions occupied mostly by apolar residues forming a hydrophobic core and positions *g* and *e* occupied by charged residues. The four positions *a, d, e* and *g* are important to maintain the coiled-coil interaction. Prediction of coiled-coil formation in Cry toxins showed that within the 60 kDa Cry toxin, helix α-3 of domain I has the highest provability score [32]. The role of helix α-3 in Cry toxin oligomerization was supported by the fact that a synthetic peptide corresponding to Cry1Ab helix α-3 inhibited formation of the Cry1Ab 250 kDa oligomer. In contrast, synthetic peptides corresponding to other helices did not affect oligomer formation [32]. Some residues of helix α-3, located in the important positions of coiled-coils, were mutagenized in Cry1Aa and Cry1Ab toxins, resulting in proteins affected in the rate of pore-formation but not in the pore characteristics and they showed extremely low toxicity to *M. sexta* larvae [32, 97]. The phenotype in these mutants could be explained if they were affected in oligomer formation, since this is an important step for channel formation. The substitutions R99E and Y107E of Cry1Ab (positions *g* and *d* of the putative coiled coil, respectively) were further analyzed. Both were non-toxic to *M. sexta* and unable to form ionic channels when analyzed in black lipid bilayers [32]. The low response in pore formation of these mutants correlated with their inability to form the 250 kDa oligomeric structure [32]. One mutant, L100E, showed increased pore formation and higher toxicity, suggesting that helix α-3 is important for the rate of pore formation *in vivo.* One important characteristic of these mutants (L100E, Y107E and R99E) was that their binding characteristics were similar to the wild type toxin [32]. Firstly, the binding competition of biotinylated-Cry1Ab toxin with unlabelled mutant proteins was identical to that of the unlabelled wild type toxin [32]. Secondly, binding affinity to a Bt-R_1 protein fragment that contains all the Cry1A binding sites was also similar to the wild type Cry1Ab toxin [32], suggesting that the nontoxic phenotype observed

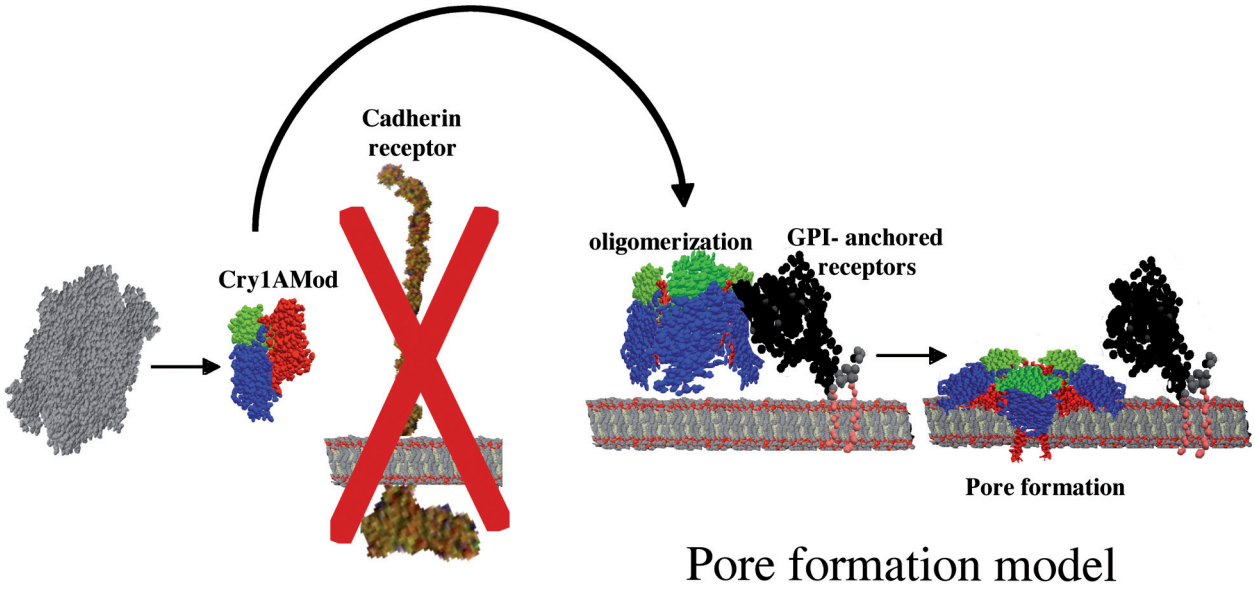

Figure 3. Model for the mechanism of action of the CryMod toxins. CryMod toxins that lack helix α-1 do not require interaction with cadherin to induce oligomerization, binding to GPI-anchored receptors and pore formation. Thus, CryMod toxins are able to kill insects which lack the cadherin receptor and are resistant to wild type Cry toxins.
Only the structure of the monomeric Cry toxin was modelled using Swiss-PDB Viewer and coordinates of Cry1Aa (PDB number 1CIY). The rest of the proteins depicted in the figure are hypothetical structures.

in the mutants affected in oligomerization, as well as the higher toxicity observed in the mutant that was able to oligomerize, were not linked to cadherin binding. Also, binding to cadherin was not enough to kill the larvae.

Other mutations performed in helix α-3 of Cry1Ac toxin are those introduced in residues R93 and A92. Most of the mutated proteins were severely affected in toxicity, and only the conservative change R93K was fully active [98, 99]. In mutants A92E and A92D, the loss of toxicity correlated with the loss of pore formation [98]. Residue R93 is located in position *a* of the predicted coiled-coil structure of helix α-3. In other coiled-coil structures the presence of polar residues in the apolar interface correlates with proper alignment, orientation and selectivity of the coiled-coil interaction and contributes considerably to their stability [95, 96]. Thus, it is possible that residue R93 plays an important role in coiled-coil conformation and stability.

It was also suggested that helix α-5, located in the central position of domain I, could be involved in Cry1Ac toxin oligomerization, since several point mutations in this helix disrupted oligomerization and the mutated toxin became severely affected in toxicity against *M. sexta* [83]. The only exception was mutant H168R which showed high insecticidal activity but could not form the 200 kDa oligomeric structure observed with wild type Cry1Ac in SDS-PAGE. However, these authors also showed that mutant H168R as well as the wild type Cry1Ac form oligomers of >200 kDa [83].

Finally, there are examples of mutants outside the domain I that affect oligomerization. The Cry1C mutants Q374A and T440A, located in loops 2 and 3 of domain II respectively, showed a major decrease in toxicity against *S. exigua* larvae and were also severely affected in oligomer formation when activated in the presence of *S. exigua* BBMV. Apparently the affinity of this mutant to the BBMVs was two-fold lower when compared with the wild type, suggesting that changes in domain II loops affect the interaction with membrane receptors which is necessary for oligomer formation of Cry1Ca in *S. exigua* [85]

Signal transduction induced by pore formation?

Other pore-forming toxins produced by other bacteria cells, such as the α-toxin from *Staphylococcus aureus* and aerolysin produced by *Aeromonas hydrophila* kill their target cells by forming pores, but they also induce cell death by triggering an apoptotic signal cascade pathway [100, 101]. The mechanism of cell death, pore-formation or apoptosis depends on the cell type and on the dose of toxin. Higher doses induce death by pore formation and subnanomolar doses trigger the apoptotic pathway. Over-expression of anti-apoptotic protein could block aerolysin-induced apoptosis, although this effect was overcome if higher toxin

concentrations were used, in which case cells died quickly due to pore formation. The apoptotic pathway was not observed [101]. In the case of aerolysin it was demonstrated that apoptosis was neither directly triggered by binding of the toxin to its receptor nor by oligomerization of the toxin, but rather that it was caused by the production of a small number of channels in the membrane [101], indicating that the intracellular downstream effects were triggered by membrane depolarization induced by pore formation. In the case of Cry toxins, intracellular signaling related to cell death has only been determined in one insect cell line. So far there are no published data showing that signal transduction is involved in cell death in whole larvae. This remains to be determined.

Concluding remarks

In the case of Cry toxins, we cannot exclude the possibility that intracellular responses also play a role in insect death or in synergizing the effect of the toxin. The fact that CryMod toxins are able to kill larvae that lack the cadherin receptor clearly indicates that this receptor is not the only responsible for triggering insect death. The CryMod toxin supported the pore formation model as the mechanism of action of these toxins. In this case, if any type of intracellular response is involved in Cry toxin intoxication *in vivo*, this may be triggered by other events that occur after toxin binding to cadherin, such as oligomerization, binding to GPI-anchored receptors or pore formation. The oligomerization process could result in simultaneous clustering of receptors on the cell surface, and this could lead to an intracellular signal. Alternatively, toxin binding to GPI-anchored receptors could also be important since they are located in lipid rafts which are directly involved in signal transduction, sorting and trafficking of plasma membrane proteins in mammalian cells [102] and functions such as pathogen portals for different viruses, bacteria and toxins [102, 103]. Finally, the formation of a small number of channels may be essential for triggering a putative intracellular response as shown for aerolysin and α-toxin in mammalian cells. It is still necessary to determine if other events besides pore formation are involved in insect death. Nonetheless, we can speculate that toxin concentration may play an important role, in which case the intracellular response is triggered by low concentration of Cry toxins and cell death by pore formation occurs only when the larvae ingest high concentrations of the toxin. Thus it is attractive to propose that intracellular responses will most often be triggered *in vivo* where low toxin concentrations are most probably encountered.

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1 Helgason, E., Okstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. and Kolsto, A. B. (2000). *Bacillus anthrancis, Bacillus cereus*, and *Bacillus thuringiensis* one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66, 2627–2630.
2 Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., R., Feitelson, J., Zeigler, D. and Dean, D. H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 705–806.
3 Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D. H. (1998) Revision of the Nomenclature for the *Bacillus thuringiensis* Pesticidal Crystal Proteins. Microbiol. Mol. Biol. Rev. 62, 807–813. http,//www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html
4 de Maagd, R. A., Bravo, A., Berry, C., Crickmore, N. and Schnepf, H. E. (2003) Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. Ann. Rev. Genet. 37, 409–433.
5 Li, J., Carroll, J. and Ellar, D. J. (1991) Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. Nature. 353, 815–821.
6 Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J. L., Brousseau, R. and Cygler, M. (1995) *Bacillus thuringiensis* Cry1A(a) insecticidal toxin, crystal structure and channel formation J. Mol. Biol. 254, 447–464.
7 Morse, R. J., Yamamoto, T. and Stroud. R. M. (2001) Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure. 9, 409–417.
8 Galitsky, N., Cody, V., Wojtczak, A., Debashis, G., Luft, J. R., Pangborn, W. and English, L. (2001) Structure of insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis.* Acta. Cryst. D57, 1101–1109.
9 Boonserm, P., Davis, P., Ellar, D. J. and Li, J. (2005) Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. J. Mol. Biol. 348, 363–382.
10 Boonserm, P., Mo, M., Angsuthanasombat, Ch. and Lescar, J. (2006) Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8 Angstrom resolution J. Bacteriol. 188, 3391–3401
11 Li, J., Pandelakis, A. K. and Ellar, D. J. (1996) Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J. Mol. Biol. 257, 129–152.
12 Guerchicoff, A., Delécluse, A. and Rubinstein, C. P. (2001). The *Bacillus thuringiensis cyt* genes for hemolytic endotoxin constitute a gene family. Appl. Environ. Microbiol. 67, 1090–1096.
13 Wu, D., Johnson, J. J. and Fredereci, B. A. (1994). Synergism of mosquitocidal toxicity between CytA and CryIVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis.* Mol. Microbiol. 13, 965–972.
14 Wirth, M. C., Delécuse, A. and Walton, W. E. (2001) CytAb1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *Bacillus thuringiensis* subsp. *israelensis* synergize *Bacillus sphaericus* against *Aedes aegypti* and resistant *Culex quinquefasciatus* (Diptera, Culicidae). Appl. Environ. Microbiol. 67, 3280–3284.
15 Roh, J. Y., Choi, J. Y., Li, M. S., Jin, B. R. and Je, Y. H. (2007) *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J. Microbiol. Biotechnol. 17, 547–59.
16 van Frankenhuyzen, K. (2000) Application of *Bacillus thuringiensis* in forestry. In: Entomopathogenic bacteria, from laboratory to field application. pp. 371–376. Charles, J. F., Delécluse, A. and Nielsen-LeRoux, C. (ed.) Kluwer Academic Publishers, Netehrlands.

17 MacIntosh, S.C., Stone, T. B., Sims, S. R., Hunst, P. L., Greenplata, J. T., Marrone, P. G., Perlak, F. J., Fischhoff, D. A. and Fuchs, R. L. (1990) Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. J. Invertebr. Pathol. 56, 258–66.

18 Guillet, P., Kurstak, D. C., Philippon, B. and Meyer, R. (1990) Use of *Bacillus thuringiensis israelensis* for Onchocerciasis control in West Africa. In: Bacterial control of mosquitoes and blackfies. pp. 187 de Barjac, H. and Sutherland, D. J. (ed.) Rutgers University Press, New Jersey.

19 Becker, N. (2000) Bacterial control of vector-mosquitoes and black flies. In: Entomopathogenic bacteria, from laboratory to field application pp. 383–396. Charles, J. F., Delécluse, A. and Nielsen-LeRoux, C. (ed.) Kluwer Academic Publishers, Netehrlands.

20 James, C. (2006) Global status of commercialized biotech/GM crops, 2006. ISAAA Briefs 35, 1–9.

21 Haider, M. Z. and Ellar, D. J. (1989) Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin, interaction with phospholipid vesicles. Biochim. Biophys. Acta. 978, 216–22.

22 Knowles, B. H. and Ellar, D. J. (1987) Colloid-osmotic lysis is a general feature of the mechanism of action *of Bacillus thuringiensis* δ-endotoxins with different insect specificity. Biochim. Biophys. Acta. 924, 507–518.

23 Bravo, A., Gómez, I., Conde, J., Muñoz-Garay, C., Sánchez, J., Miranda, R., Zhuang, M., Gill, S. S. and Soberón, M. (2004) Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochim. Biophys. Acta. 1667, 38–46.

24 Zhang, X., Candas, M., Griko, N. B., Taissing, R. and Bulla, L. A. Jr. (2006) A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis.* Proc. Natl. Acad. Sci. USA. 103, 9897–9902.

25 Zhang, X., Candas, M., Griko, N. B., Rose-Young, L. and Bulla, L. A. Jr. (2005) Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R1 expressed in insect cells. Cell Death Differ. 12, 1407–16.

26 Vadlamudi, R. K., Ji, T. H. and Bulla, L. A. Jr. (1993) A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner.* J. Biol. Chem. 268,12334–12340.

27 Gómez, I., Oltean, D. I., Sánchez, J., Gill, S. S., Bravo, A. and Soberón, M. (2001) Mapping the epitope in Cadherin-like receptors involved in *Bacillus thuringiensis* Cry1A toxin interaction using phage display. J. Biol. Chem. 276, 28906–28912.

28 Gómez, I., Dean, D. H., Bravo, A. and Soberón, M. (2003) Molecular basis for *Bacillus thuringiensis* Cry1Ab toxin specificity, two structural determinants in the *Manduca sexta* Bt-R_1 receptor interact with loops α-8 and 2 in domain II of Cry1Ab toxin. Biochemistry. 42, 10482–10489.

29 Xie, R., Zhuang, M., Ross, L. S., Gómez, I., Oltean, D. I., Bravo, A., Soberón, M. and Gill, S. S. (2005) Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. J. Biol. Chem. 280, 8416–8425.

30 Hua, G., Zhang, R., Abdullah, M. A. and Adang, M. J. (2008) *Anopheles gambiae* cadherin AgCad1 binds the Cry4Ba toxin of *Bacillus thuringiensis israelensis* and a fragment of AgCad1 synergizes toxicity. Biochemistry. 47, 5101–5110.

31 Gómez, I., Sánchez. J,, Miranda, R., Bravo, A. and Soberón, M. (2002) Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. FEBS Lett. 513, 242–246.

32 Jiménez-Juárez, N., Muñoz-Garay, C., Gómez, I., Saab-Rincon, G., Damian-Alamazo, J. Y., Gill, S. S., Soberón, M. and Bravo, A. (2007) *Bacillus thuringiensis* Cry1Ab mutants affecting oligomer formation are non toxic to *Manduca sexta* larvae. J Biol. Chem. 282, 21222–21229.

33 Jurat-Fuentes, J. L. and Adang, M. J. (2004) Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur. J. Biochem. 271, 3127–3135.

34 Fernandez, L. E., Aimanova, K. G., Gill, S. S., Bravo, A. and Soberón, M. (2006) A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. Biochem. J. 394, 77–84.

35 Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H. and Bulla, L. A. Jr. (1995) Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis.* J. Biol. Chem. 270, 5490–5494.

36 Nagamatsu, Y., Toda, S., Koike, T., Miyoshi, Y., Shigematsu, S. and Kogure, M. (1998) Cloning, sequencing and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* insecticidal Cry1Aa toxin. Biosci. Biotechnol. Biochem. 62, 727–734.

37 Gahan, L. J., Gould, F. and Heckel, D. G. (2001) Identification of a gene associated with *Bacillus thuringiensis* resistance in *Heliothis virescens.* Science. 293, 857–860.

38 Xu, X., Yu, L. and Wu, Y. (2005) Disruption of a cadherin gene associated with resistance to Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera.* Appl. Environ. Microbiol. 71, 948–954.

39 Morin, S., Biggs, R. W., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, Heckel, D. G., Carriere, Y., Dennehy, T. J., Brown, J. K. and Tabashnik, B. E. (2003) Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Nat. Acad. Sci. 100, 5004–5009.

40 Flannagan, R. D., Yu, C. G., Mathis, J. P., Meyer, T. E., Shi, X., Siqueira, H. A. and Siegfried, B. D. (2005) Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera, Crambidae). Insect Biochem. Mol. Biol. 35, 33–40.

41 Chen, J., Brown, M. R., Hua, G. and Adang, M. J. (2005) Comparison of the localization of *Bacillus thuringiensis* Cry1A delta endotoxins and their binding proteins in larval midgut of tobacco hornworm, *Manduca sexta.* Cell Tissue Res. 321, 123–129.

42 Dorsch, J. A., Candas, M., Griko, N. B., Maaty, W. S., Midboe, E. G., Vadlamudi, R. K. and Bulla, L. A. Jr. (2002) Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R(1) in *Manduca sexta*, involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis.* Insect Biochem. Mol. Biol. 32, 1025–1036.

43 Hua, G., Jurat-Fuentes, J. L. and Adang, M. J. (2004) Fluorescent-based assays establish *Manduca sexta* Bt-R(1a) cadherin as a receptor for multiple *Bacillus thuringiensis* Cry1A toxins in Drosophila S2 cells. Insect Biochem. Mol. Biol. 34, 193–202.

44 Jurat Fuentes J.L., and Adang M.J. (2006) The *Heliothis virescens* cadherin protein expressed in *Drosophila* S2 cells functions as a receptor for *Bacillus thuringiensis* Cry1A but not Cry1Fa toxins. Biochemistry 45, 9688–9695

45 Hua, G., Jurat-Fuentes, J. L. and Adang, M. J. (2004) Bt-R1a extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and cytotoxicity. J. Biol. Chem. 279, 28051–28056.

46 Tsuda, Y., Nakatani, F., Hashimoto, K., Ikawa, S., Matsuura, C., Fukada, T., Sugimoto, K. and Himeno, M. (2003) Cytotoxic activity of *Bacillus thuringiensis* Cry proteins on mammalian cells transfected with cadherin-like Cry receptor gene of *Bombyx mori* (silkworm). Biochem. J. 369, 697–703.

47 Gómez, I., Arenas, I., Benitez, I., Miranda-Ríos, J., Becerril, B., Grande, R., Almagro, J.C., Bravo, A. and Soberón, M. (2006) Specific epitopes of Domains II and III of *Bacillus thuringiensis* Cry1Ab toxin involved in the sequential interaction with cadherin and aminopeptidase-N receptors in *Manduca sexta.* J. Biol. Chem. 281, 34032–34039.

48 Hara, H., Atsumi, S., Yaoi, K., Nakanishi, K., Higurashi, S., Miura, N., Tabunoki, H. and Sato, R. (2003) A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins on midgut epithelial cells of *Bombyx mori* larvae. FEBS Lett. 538, 29–34.
49 Ibiza-Palacios, M. S., Ferré, J., Higurashi, S., Miyamoto, K., Sato, R., and Escriche, B. (2008) Selective inhibition of binding of *Bacillus thuringiensis* Cry1Ab toxin to cadherin-like and aminopeptidase proteins in brush-border membranes and dissociated epithelial cells from *Bombyx mori.* Biochem. J. 409, 215–221
50 You, T. H., Lee, M. K., Jenkins, J. L., Alzate, O. and Dean, D. H. (2008) Blocking binding of *Bacillus thuringiensis* Cry1Aa to *Bombyx mori* cadherin receptor results in only a minor reduction of toxicity. BMC Biochemistry 9, 3.
51 Chen, J. Hua, G., Jurat-Fuentes, J. L. Abdullah, M. A., and Adang, M. J. (2007) Synergism of *Bacillus thuringiensis* toxins by a fragment of a toxin-binding cadherin. Proc. Natl. Acad. Sci. USA. 104, 13901–13906.
52 Ferré, J. and Van Rie, J. (2002) Biochemistry and genetics of insect resistance to *Bacillus thuringiensis.* Annu. Rev. Entomol. 47, 501–533.
53 Yang, Y., Chen, H., Wu, Y,, Yang, Y. and Wu, S. (2007) Mutated Cadherin alleles from a field population of *Helicoverpa armigera* confer resistance to *Bacillus thuringiensis* toxin Cry1Ac. Appl. Environ. Microbiol. 73, 6939–6944.
54 Soberón, M., Pardo-López, L., López, I., Gómez, I., Tabashnik, B. and Bravo, A. (2007) Engineering Modified Bt Toxins to Counter Insect Resistance. Science. 318, 1640–1642
55 Rausell, C., Muñoz-Garay, C., Miranda-CassoLuengo, R., Gómez, I., Rudiño-Piñera, E., Soberón, M. and Bravo, A. (2004) Tryptophan spectroscopy studies and black lipid bilayer analysis indicate that the oligomeric structure of Cry1Ab toxin from *Bacillus thuringiensis* is the membrane-insertion intermediate. Biochemistry. 43, 166–174
56 Knight, P. J., Knowles, B.H. and Ellar, D. J. (1995) Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. J. Biol. Chem. 270, 17765–17770.
57 Gill, S. S., E. A. Cowles, and Francis, V. (1995) Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens.* J. Biol. Chem. 270, 27277–27282.
58 Oltean, D. I., Pullikuth, A. K., Lee, H. K. and Gill, S. S. (1999) Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. Appl. Environ. Microbiol. 11, 4760–4766.
59 Luo, K., Sangandala, S., Masoon, L., Mazza, A., Brousseau, R. and Adang, M. J. (1997) The *Heliothis virescens* 170 kDa aminopeptidase functions as receptor A by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation. Insect Biochem. Mol. Biol. 27, 735–743.
60 Banks, D. J., Hua, G. and Adang, M.J. (2003) Cloning of a *Heliothis virescens* 110 kDa aminopeptidase N and expression in *Drosophila* S2 cells. Insect Biochem. Mol. Biol. 33, 499–508.
61 Agrawal, N., Malhotra, P. and Bhatnagar, R. K. (2002) Interaction of gene-cloned and insect cell-expressed aminopeptidase N of *Spodoptera litura* with insecticidal crystal protein Cry1C. Appl. Environ. Microbiol. 68, 4583–4592.
62 Rajangopal, R., Agrawai, N., Seivapandiyan, A., Sivakumar, S., Ahmad, S. and Bhatnagar, R. K. (2003) Recombinantly expressed isozymic aminopeptidases from *Helicoverpa armigera* midgut display differential interaction with closely related Cry proteins. Biochem. J. 370, 971–978.
63 Yaoi, K., Naknishi, K., Kadotani, T., Imamura, M., Koizumi, N., Iwahana, H. and Sato, R. (1999) cDNA cloning and expression of *Bacillus thuringiensis* Cry1Aa toxin binding 120 kDa aminopeptidase N from *Bombyx mori.* Biochim. Biophys. Acta 1444, 131–137.
64 Garner, K. J., Hiremath, S., Lehtoma, K. and Valaitis, A. P. (1999) Cloning and complete sequence characterization of two gypsy moth aminopeptidase N cDNAs including the receptor for *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem. Mol. Biol. 29, 527–535.
65 Nakanishi, K., Yaoi, K., Nagino, Y., Hara, H., Kitami, M., Atsumi, S., Miura, N. and Sato, R. (2002) Aminopeptidase N isoforms from the midgut of *Bombyx mori* and *Plutella xylostella;* their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry1A toxin. FEBS Lett. 519, 215–220.
66 Abdullah, M. A., Valaitis, A. P. and Dean, D. H. (2006) Identification of a *Bacillus thuringiensis* Cry11Ba toxin binding aminopeptidase from the mosquito *Anopheles quadrimaculatus.* BMC Biochem. 7, 16.
67 Herrero, S., Gechev, T., Bakker, P. L., Moar, W. J. and de Maagd, R. A. (2005) *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four Aminopeptidase N genes. BMC Genomics. 6, 96.
68 Pigott, C. R. and Ellar, D. J. (2007) Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol. Mol. Biol. Rev. 71, 255–281.
69 McNail, R. J. and Adang, M. J. (2003) Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. Insect Biochem. Mol. Biol. 33, 999–1010.
70 Ochoa-Campuzano, C., Real, M. D., Martínez-Ramírez, A. C., Bravo, A. and Rausell, C. (2007) An ADAM metalloprotease is a Cry3Aa *Bacillus thuringiensis* toxin receptor. Biochem. Biophys. Res. Com. 362, 437–442.
71 Zhuang, M., Oltean, D. I., Gómez, I., Pullikuth, A. K., Soberón, M., Bravo, A. and Gill, S. S. (2002) *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. J. Biol. Chem. 277, 13863–13872.
72 Masson, L., Lu, Y.-J., Mazza, A., Brosseau, R. and Adang, M. J. (1995*)* The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. J. Biol. Chem. 270, 20309–20315.
73 Cooper, M. A., Carroll, J., Travis, E., Williams, D. H. and Ellar, D. J. (1998) *Bacillus thuringiensis* Cry1Ac toxin interaction with *Manduca sexta* aminopeptidase N in a model membrane environment. Biochem. J. 333, 677–683.
74 Burton, S. L., Ellar, D. J., Li, J. and Derbyshire, D. J. (1999) N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin, J. Mol. Biol. 287, 1011–22.
75 Lee, M. K., You, T. H., Gould, F. L. and Dean, D. H. (1999) Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. Appl. Environ. Microbiol. 65, 4513–4520.
76 Rajagopal, R., Sivakumar, S., Agrawai, N., Malhotra, P. and Bhatnagar, R. K. (2002) Silencing of midgut aminopeptidase N of *Spodoptera litura* by double stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. J. Biol. Chem. 277, 46849–46851.
77 Lorence, A., Darszon, A. and Bravo, A. (1997) The pore formation activity of *Bacillus thuringiensis* Cry1Ac toxin on *Trichoplusia ni* membranes depends on the presence of aminopeptidase N. FEBS Lett. 414, 303–307.
78 Schwartz, J.L., Lu, Y.J., Sohnlein, P., Brousseau, R., Laprade, R., Masson, L. and Adang, M. J. (1997) Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors. FEBS Lett. 412, 270–276.
79 Jenkins, J. L., Lee, M. K., Valaitis, A. P., Curtiss, A. and Dean, D. H. (2000) Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase *N* receptor. J. Biol. Chem. 275, 14423–14431.
80 Li, J., Derbyshire, D. J., Promdonkoy, B. and Ellar, D. J. (2001) Structural implications for the transformation of the *Bacillus*

thuringiensis δ-endotoxins from water-soluble to membrane-inserted forms. Biochem. Soc. 29, 571–577.
81 Pardo-López, L., Gómez, I., Rausell, C., Sánchez, J., Soberón, M. and Bravo, A. (2006) Structural changes of the Cry1Ac oligomeric pre-pore from *Bacillus thuringiensis* induced by *N*-acetylgalactosamine facilitates toxin membrane insertion. Biochemistry 45, 10329–10336.
82 Muñoz-Garay, C., Sánchez, J., Darszon, A., de Maagd, R. A., Bakker, P. L, Soberón, M. and Bravo, A. (2006) Permeability changes of *Manduca sexta* midgut brush border membranes induced by oligomeric structures of different Cry toxins. J. Membr. Biol. 212, 61–68.
83 Aronson, A.I., Chaoxianm G. and Wu, L. (1999) Aggregation of *Bacillus thuringiensis* Cry1A toxin upon binding to target insect larval midgut vesicles. Appl. Environ. Microbiol. 65, 2503–2507.
84 Tigue, N. J., Jacoby, J. and Ellar, D. J. (2001) The alpha-helix 4 residue, Asn135, is involved in the oligomerization of Cry1Ac1 and Cry1Ab5 *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 67, 5715–20.
85 Herrero, S., González-Cabrera, J., Ferré, J. Bakker, P. L. and de Maagd, R. (2004) Mutations in the *Bacillus thuringiensis* Cry1Ca toxin demonstrate the role of domains II and III in specificity towards *Spodoptera exigua* larvae. Biochem. J. 384, 507–513.
86 Ihara H., and Himeno M. (2008) Study of irreversible binding of *Bacillus thuringiensis* Cry1Aa to brush border membrane vesicles from *Bombyx mori* midgut. J. Invertebr Pathol 98, 177–183
87 Rausell, C., García-Robles I., Sánchez, J., Muñoz-Garay, C., Martínez-Ramírez, A. C., Real, M. D. and Bravo, A. (2004) Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* [Say]. Biochem. Biophys. Acta 1660, 99–105.
88 Pérez, C., Muñoz-Garay, C., Portugal, L. C., Sánchez, J., Gill, S. S., Soberón, M. and Bravo A. (2007) *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa enhances activity of Cry11Aa toxin by facilitating the formation of a pre-pore oligomeric structure. Cellular Microbiol. 9, 2931–2937.
89 Likitvivatanavong, S., Katzenmeier, G. and Angsuthanasombat, Ch. (2006) Asn183 in helix α5 is essential for oligomerization and toxicity of the *Bacillus thuringiensis* Cry4Ba toxin. Arch. Biochem. Biophys. 445, 46–55.
90 Puntheeranurak, T., Stroh, C., Zhu, R., Angsuthanasombat, C. and Hinterdorfer, P. (2005) Structure and distribution of the *Bacillus thuringiensis* Cry4Ba toxin in lipid membranes. Ultramicroscopy. 105, 115–124
91 Ounjai, P., Unger, V. M., Sigworth, F. J. and Angsuthanasombat, C. (2007) Two conformational states of the membrane-associated *Bacillus thuringiensis* Cry4Ba delta-endotoxin complex revealed by electron crystallography: implications for toxin-pore formation. Biochem. Biophys. Res. Commun. 36, 890–895.
92 Vié, V., Van Mau, N., Pomarède, P., Dance, C., Schwartz, J. L., Laprade, R., Frutos, R., Rang, C., Masson, L., Heitz, F. and Le Grimellec, C. (2001) Lipid-induced pore formation of the *Bacillus thuringiensis* Cry1Aa insecticidal toxin. J. Membr, Biol. 180, 195–203.
93 Pérez, C., Fernandez, L. E., Sun, J., Folch, J. L., Gill, S. S., Soberón, M. and Bravo A. (2005). *Bti* Cry11Aa and Cyt1Aa toxins interactions support the synergism-model that Cyt1Aa functions as membrane-bound receptor. Proc. Natl. Acad. Sci. U.S.A. 102, 18303–18308.
94 Tabashnik, B. E., Patin, A. L., Dennehy, T. J., Liu, Y. B., Carriere, Y., Sims, M. A. and Antilla, L. (2000) Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc. Natl. Acad. Sci. U.S.A. 97, 12980–12984.
95 Burkhard, P., Stetefeld, J. and Strelkov, S. V.(2001) Coiled coils: a highly versatile protein folding motif. Trends Cell Biol. 11, 82–88.
96 Chothia, C., Levitt, M., and Richardson, D. (1981) Helix to helix packing in proteins. J. Mol. Biol. 145, 215–25.0
97 Vachon, V., Prefontaine, G., Coux, F., Rang, C., Marceau, L., Masson, L., Brousseau, R., Frutos, R., Schwartz, J. L. and Laprade, R. (2002) Role of helix 3 in pore formation by *Bacillus thuringiensis* insecticidal toxin Cry1Aa. Biochemistry 41, 6178–6184.
98 Chen, X. J., Curtiss, A., Alcantara, E. and Dean, D. H. (1995) Mutations in domain I of *Bacillus thuringiensis* δ-endotoxin Cry1Ab reduced the irreversible binding of toxin to *Manduca sexta* brush border membrane vesicles. J. Biol. Chem. 270, 6412–6419.
99 Wu, D. and Aronson, A. I. (1992) Localized mutagenesis defines regions of the *Bacillus thuringiensis* δ-endotoxin involved in toxicity and specificity. J. Biol. Chem. 267, 2311–2317.
100 Menzies, B. E. and Kourteva, I. (2000) *Staphylococcus aureus* alpha-toxin induces apoptosis in endothelial cells. FEMS Immunol. Med. Microbiol. 29, 39–45.
101 Nelson, K. L., Brodsky, R. A. and Buckley, J. T. (1999) Channels formed by subnanomolar concentrations of the toxin aerolysin trigger apoptosis of T lymphomas. Cell. Microbiol. 1, 69–74.
102 Rosenberger, C. M., Brumell, J. H. and Finlay, B. B. (2000)Microbial pathogenesis: lipid rafts as pathogen portals. Current. Biol. 10, 823–825.
103 Cabiaux. V., Wolff, Ch. and Ruysschaert, J. M. (1997) Interaction with a lipid membrane: a key step in bacterial toxins virulence. Int. J. Biol. Macromol. 21, 285–298.