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Review Article

Biocidal Activity of the *Bacillus thuringiensis* 3D Cry Toxins, Molecular Crosstalk at the Insect Midgut with Implication in Insect Resistance Development

Gloria G Guerrero M*

Universidad Autónoma de Zacatecas, Campus II, Unidad Académica de Ciencias Biológicas, Zacatecas, Zac, México

***Corresponding Author:** Gloria G Guerrero M, Universidad Autónoma de Zacatecas, Campus II, Unidad Académica de Ciencias Biológicas, Zacatecas, Zac, México.

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Abstract

Bacillus thuringiensis (Bt) is a Gram-positive bacteria characterized by the production of parasporal crystalline proteins toxic to a wide range of insect orders. Cry toxins targeted pests of crops of economic importance. Nowadays, around 600 genes encode crystalline proteins with a range of molecular weight of 50 to 130 kDa. Cry proteins are comprised of three domains and their tridimensional structures have been elucidated by X-rays. Their mode of action remains to be defined and understood. However, most of these proteins follow a basic program for their biocidal activity. A critical step in the mode of action of the 3D Cry toxins is the specific binding to the receptors present in the midgut epithelial. These receptors are determinants of the specificity and susceptibility of targeted insects. Among them are the classical glycosyl phosphate inositol (GPI)-anchored membrane receptors, such as N-aminopeptidase, Alkaline Phosphatase, and classical epithelial cadherins DE-Cadherins. The second group of binding proteins includes ABC transporters, V-ATPase, and other lipid rafts-associated proteins. The hallmark of this molecular crosstalk at the insect midgut is that it is conserved between different Cry 3D toxins with diverse targets of insects. Moreover, the receptors in this tissue are also common resulting in a common mode of action that comprises the insect response to entomopathogens, which potentially can guide a design of safe and integrated management of crop pests.

Keywords: Crystalline Proteins (Cry); BTR1 Receptors; REPAT; G Proteins; PKA; ERK; MAPK p38

Introduction

Bacillus thuringiensis (*Bt*) is an entomopathogenic Gram-positive bacteria discovered and firstly isolated from a dead silkworm larvae *Bombyx mori* (Lepidoptera *Bombycidae)* in 1901. Ten year later was isolated from a flour moth larvae *Ephestia kuehniella* (Lepidoptera *Pyralidae)* in the province of Thüringen in 1911 [1]. However, it was until 1953 that *Bt* was referred by the parasporal crystals production as biopesticide [2]. Since then it has been the subject of intense research because a set of four phylogenetically non related proteins with different action. As pathogen, *Bt* comprise a vegetative phase and sporulation phase. In the first one produce a set of insecticidal proteins called Vegetative Insecticidal Proteins or VIP proteins (V1P1/VIP2), and VIP3) [3-5]. In the second produce parasporal crystalline delta-endotoxins or three domain Cry toxins (3D-Cry toxins) (4;5). These bacterial toxins belong to the family of bacterial toxins, able to form pores in the membranes, or pore-forming toxins (PFT) [6-9], such as Colicin A [8,9], Cholera toxin, Enterotoxins [10-12], and Aerolysin [13], The third group of toxins produced for some subspecies of *Bt svar israelensis (Bti)* are the Cyt toxins. and *Lysinibacillus sphaericus* or *Bt* var *sphaericus*, which contains one major the binary-like(Bin) and the mosquitocidal Cry toxins (Mtx) [1,3].

Featuring to the *Bt* 3D- Cry toxins

These proteins belong to a multigene family, around 500 genes [14], classified by their primary amino sequence onto sixty seven groups (Cry1-Cry67). These proteins are produced during the sporulation phase as protoxins or immature proteins with a range of molecular weight (MW) of 70 (i.e. Cry2Aa)-130 kDa (i.e. Cry1A) [15-17]. These proteins are highly specific towards different orders of insects [3,16-18], It can be distinguished two main families: Cyt (cytolitic) and Cry (Crystalline) protein. The protoxins or inmature proteins have a MW of 130 kDa. To be active, protoxins should be proteolitically processed to release the C-terminal region. The N-terminal or the fragment toxic of MW of 60-70 kDa. Based on sequence identity, it has been determined that most Cry toxins share

a common three domain structure and share five highly conserved blocks [16,17]. The three dimensional structure of the N-terminal of several Cry toxins elucidated by x-Rays of Cry1Aa [19]; Cry1Ac [20]; Cry2Aa [21,22], Cry3Aa [23]; Cry3Ba [24,25]; Cry4Ba [26] formed by three domains [21]. Domain I, a bundle of seven alpha helices participates in pore formation, specifically, helices alpha five and helix seven, which are highly conserved, implying their essential role in pore formation. Domain II with a topology of anti-parallel beta-sheet is the hypervariable or binding domain and determines the specificity of the receptor. Domain III, a sandwich of beta-folded sheets that plays a role in the protection of proteolytic cleavages. It also plays a key role in determining insect specificity [28]. Particularly in the case of the Cry1Aa and Cry1Ac where a loop extension in Cry1Ac creates a unique N-Acetylgalactosamine (GalNAc) binding pocket implicated in receptor binding [29]. The remarkable feature of the 3D-Cry toxins is their conservation between different Cry proteins with different insect specificity, at the same time a conserved and common mode of action relationships among them [1,2,15-17]. *Bt* 3D Cry toxins have adapted to the different insect host by a process named "i "domain II and III swapping" generating hybrid toxins, with a different specificity. But it also has been found that exchanging domain I and domain II, or domain I by domain III, resulted in hybrid toxins endowed with enhanced toxicity [29-31]. A second strategy during the long evolution is the horizontal genetic transference through the mobile genetic elements, the plasmids that in *Bt* encode the Cry protein. The presence of large plasmids in the strains of *Bt* have allowed also to subvert the harsh selection pressure and to transfer the bio insecticidal genes. In addition other cellular strategies a family of insect pathogen-related, (REPAT) and arylphorin proteins are differentially expressed leading to a protective and healing midgut resistant response [32-36] (Figure 1). The current knowledge of the complex interaction of the insect host and *Bt* remains to be understood and characterized. Hopefully th identification of novel targets utmost in the insect response might guide the design of safe and enhanced rational application of the bio pesticides based in 3D-Cry toxins, alone, in combination or as part of integrated management control programs.

Mechanism(s) of action of *Bt* Cry proteins to assure persistence and environmental survival

The mode of action of 3D Cry toxins remains to be understood and defined [37-43]. However, several lines of evidences supported a multistep mode of action based on a Basic Program [28,38-41] as outlined below. After insect ingestion of the mix spore crystal; solubilization of the protoxin, followed by proteolytic processing of the protoxin (half of the C-terminal end and 2 to 50 amino acids of the N-terminal end) by the action of the proteases trypsin and chymotrypsin, a step key to release the toxic fragment N-terminal comprised of three domains that have the information for specific reversible binding (loops of domain II) to the insect midgut Glycosylphosphatidylinositol (GPI)-anchored receptors, Alkaline phos-

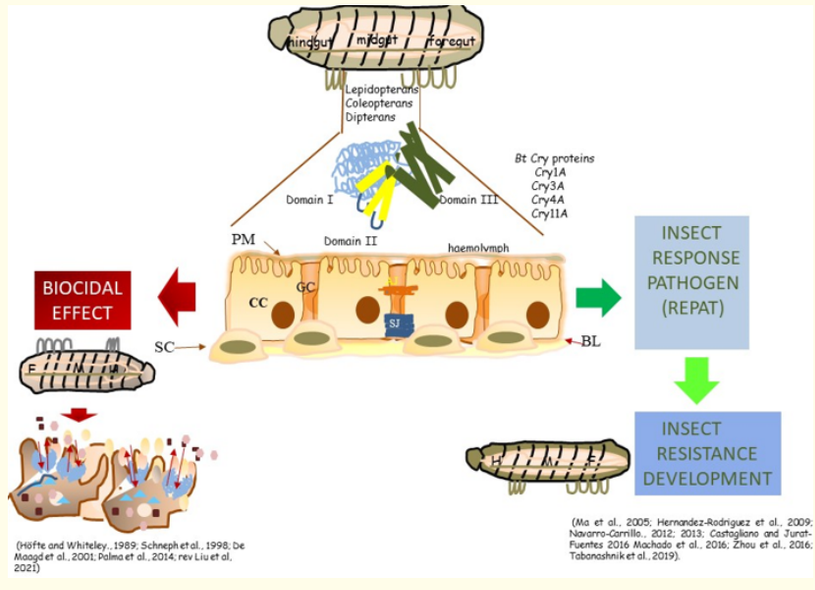
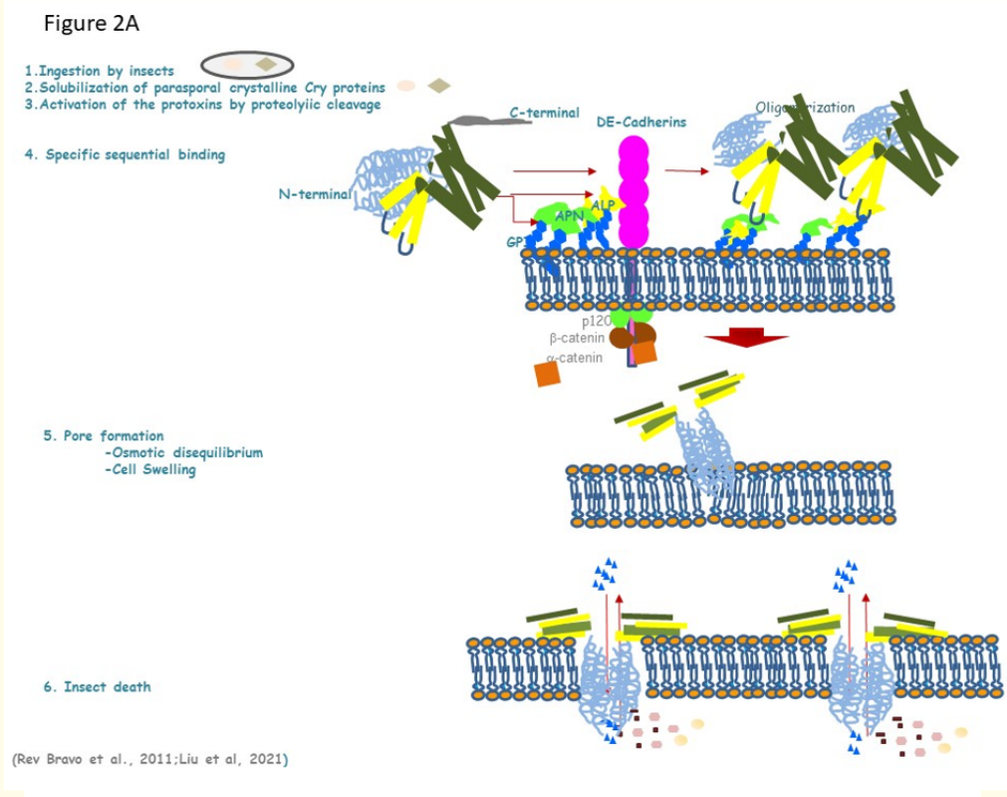


Figure 1: The interaction of host (insects) and *B. thuringiensis* (*Bt*), a molecular crosstalk at the insect midgut. Once ingested as a mix of spore+crystal, initiate a series of events that ultimately and of utmost will favor spore germination and cell death. While this complete the *Bt* cell cycle, the insects have evolved though evolution in the insect response (REPAT) as well as other cellular and physiological processes to cope with the action of Bt through their products. Nowadays, the identification of the molecular players involved in the insect resistance development represent a key to approach and guide a safe technological management of pest of agronomical crops.

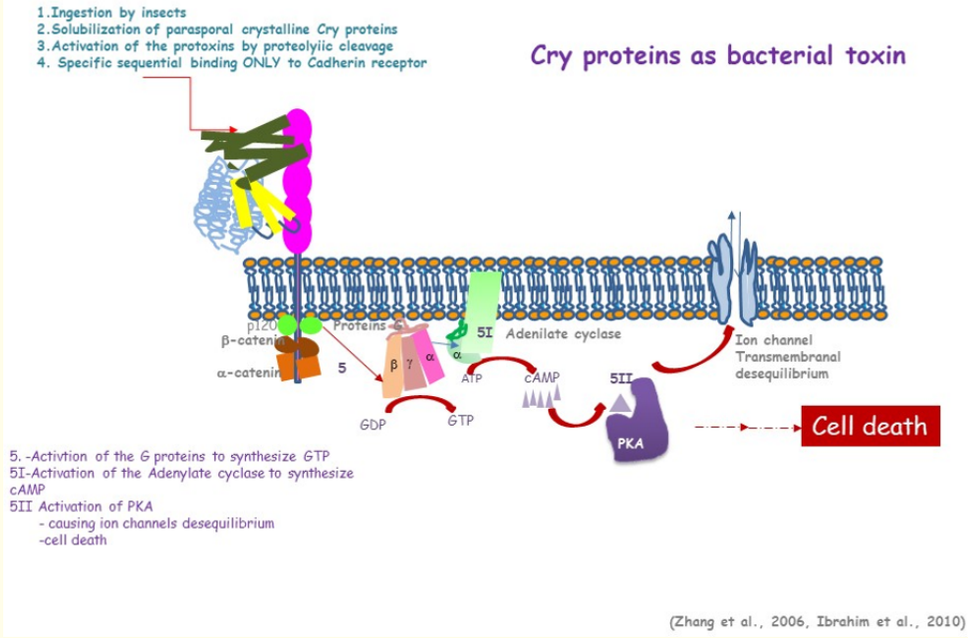
phatase (ALP) and N-Amino peptidase (APN) [17,38,39,41,42] and for the transmembrane ion channel (domain I) at the insect midgut, facilitating favoring a subsequent cleavage of alpha helix 1 from the N-terminal of domain I [43]. Then, mature Cry toxins forms firstly a pre-pore-oligomer [44] able to bind again to APN and ALP with greater affinity [16,42,44] and this complex drives their insertion into the cell membrane to form transmembrane cation ion channels [43,44], resulting in an efflux of ions, osmotic disequilibrium, cell swelling and cell death (Figure 2A). Ultimately intestinal contents leaks into the hemocoel causing septicemia and insect death [18,28,43,44].

Figure 2A



(Rev Bravo et al., 2011;Liu et al, 2021)

Figure 2B



(Zhang et al., 2006, Ibrahim et al., 2010)

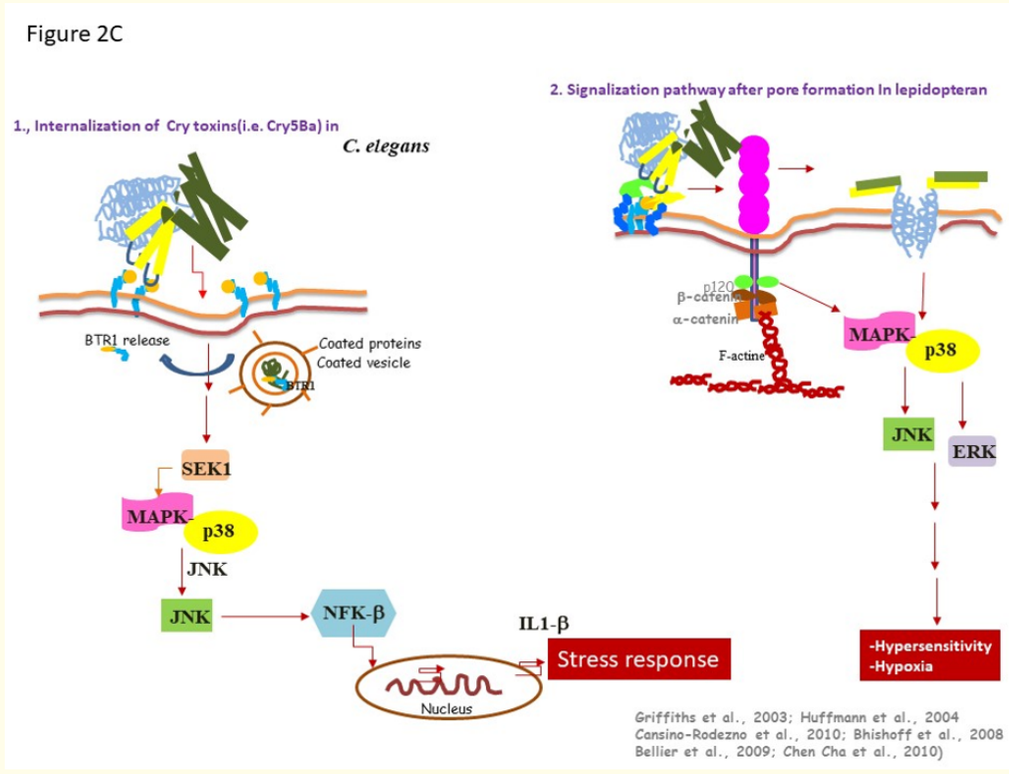


Figure 2: The mode of action of the 3D *B.thuringiensis* Cry toxins. A. A vast line of studies *in vivo* and *in vitro* accept a conserved mode of action of the *Bt* Cry toxins (A) that can be outlined as a program of: ingestion of the spore+crystalline protoxina, solubilization to release the protoxina, activation of the protoxina by proteolytic cleavage mainly of the C-terminal region, Sequential binding to receptor(s) and/or protein bindings present in the midgut of the insect(s) to toxin oligomerization and pore formation. *B. Cadherin* receptors (only to this) binding leads to signal transmition activating to the G proteins with production of the second messanger cyclic cAMP, and finally PKA activation that eventually can induce also cell death and/or ion transport disequilibrium. C. As a third mode of action that involves more the insect response to pathogens (REPAT), and the activation of other cellular signalization pathways such as stress responses, involving the activation of the MAPK-p38 SRK1 and the UPR response, resulting in mechanism of desintoxication and protection.

Basic conserved program of the mode of action of *Bt* Cry toxins

- Insect Ingestion of the parasporal crystalline proteins (ICPs)
- Solubilization of ICPs (pH alkaline)
- Activation of ICPs (by insect proteases, trypsin and chymotrypsin)
- Specific binding of Cry toxins to midgut receptors
- Pore formation and cell membrane disruption
- Cell lysis and insect death

Alternative mode of action of *Bt* Cry proteins

The alternative mode of action follow four basic steps of the program, ingestion, solubilization, activation by proteolytic cleavage to produce the toxic N-terminal fragment and binding to the brush border membrane receptors in the insect midgut epithelium. Secondly the 3D structure of the *Bt* pore-forming toxins have the information to act through other mechanism of action [1,2].

On referring to the 3D Cry toxins, it has been proposed that after cadherin binding, there is a process of internalization of the *Bt*R1 which further it can be released [2,43,44] (Figure 2B). The binding to cadherin's receptors, leads to Mg2+ dependent cell signaling cascades, such as the activation of G proteins, synthesis of Guanosine Triphosphate (GTP), and this are associated to the activation of the adenylate cyclase (AC) [2,43,44] and increase in the level of the intracellular second messenger molecule, cyclic adenosine monophosphate (cAMP). Thereafter activating protein kinases A (PKAs), triggering and affecting the flux and equilibrium in other ion channels and even cytoskeletons [2,45-48] (Figure 2B).

Alternative program No 1 for the mode of action of the Cry toxins [45-48]

- Ingestion by the target insects
- Solubilization of the ICPs

- Activation of the ICPs
- Specific binding ONLY to CADHERINS Receptor
- Downstream signalization cascades Mg 2+ dependent
- Activation of G-proteins, and Adenylate Cyclase
- Increase in the cAMP and therefore Activation of PKAs
- Disruption of ion channels and cytoskeleton
- Insect Death

Alternative program no 2 of the mode of Action of the 3D Cry toxins

Binding to BTR1 receptors (BTR1) can also aid in the internalization of the 3D Cry toxin leading to other fates. Using the nematode *Caenorabditis elegans* as model of study of the insect response to the action of *Bt* Cry toxins [49]. Interestingly, Cry5Ba and Cry21 [50] internalized and induced an upregulation of MAPK, p38 (PMK-1) SEK-1 and JNK kinases (Figure 2C) [50]. In particular, JNK and p38 are involved in the stress –associated stimuli (3D Cry toxins). The *C. elegans* mutants in PMK-1 and SRK pathway exposed to Cry5Ba toxins showed a hypersensitive response, suggesting that the activation of these kinases pathway are involved in the protective response in insects [50]. Two p38 dependent transcript, called tm-1 and tm2 [50], which role was determined by silencing animals using dsRNA. Once again, after exposure to Cry5Ba, the silenced animals showed a hypersensitivity. Of note is that one of the transcripts, tm-1 showed homology with the human zinc transporter ZnT3, Therefore, it is probable that activation of the above pathways are involved in the up regulation of the stress response and of the ion transporters, causing efflux of cytotoxic cations (desintoxication) [50]. Furthermore, other downstream targets of p38 SRK-1 kinases of the unfolded proteins of the reticulum endoplasmic (UPR) causing the hypersensitivity to the exposure of the Cry5Ba toxins [51]. Recent work using whole genome approach for the identification of the hypoxic response, and signal transduction ERK pathway of the nematode to the exposure of this toxin [52]. Indeed in mice, it has been reported that pCry1Ac is able to activate the ERK pathway, a similar effect of the exposure of the insects to the 3D Cry Toxins [50,52]. A similar effect was observed in *Manduca sexta* (Lepidoptera Sphingidae) or *Aedes aegypti (*Diptera Culicidae) a fast activation of p38 pathway by phosphorylation. When non toxic mutants of Cry1Ab or Cry11A were fed to the larvae of *M. sexta* or *Ae-aegypti* no phosphorylation of p38 was observed [52]. Through silencing p38 in each of these insects, and then exposure to these toxins caused a hypersensitivity to them, supporting once again the activation of the MAPK, p38 (PMK-1) and SRL-1 pathways leads to protective immune responses in the insects [52,53] (Figure 2C). In several orders of insects, specifically, in lepidopterans, it has been shown that after exposure to 3D Cry toxins, reduction of their digestive activity concomitant with an increase in immune related function (healing mechanism). Moreover, renewing midgut cells through stem cells proliferation, favoring the increased production of mitogen factors [27]. Furthermore, exposure to 3D Cry toxins activate a family of insect pathogen-related, (REPAT) and arylphorin proteins are differentially expressed [23,28-30] and play a role as mitogen factors for stem cells gut regeneration. Furthermore, the differential expression of REPAT genes and other proteins as arylphorin are concomitant with activation of stress responses pathways UPR response), as well as activation of desintoxication mechanism that as whole constitute an integrated protective response of the insects to the action of the 3D-Cry toxins [27-37].

The Receptors as gatekeepers of the insect midgut against the action of the *Bt* 3D-Cry toxins

Featuring the classical *B- thuringiensis* 3D Cry toxins Receptors

Bt Cry toxins function by binding specific receptors immersed on the brush-border membrane surface of the midgut epithelium of targeted insects [53-59]. One of the most common receptors are the Cadherin's, glycoproteins of MW around 220 kDa [53,56,57]-The case of the Aminopeptidase (APN) and Alkaline Phosphate receptors (ALP). These GPI-anchored proteins are widely distributed on the brush-border membrane surface of the midgut epithelium of several species of Lepidopterans [56,59]. These proteins bind to 3D Cry toxins binds in the *M. sexta* via the sugar N-acetyl-D-Galactosamine (GalNAc). At least five different subfamilies of APN proteins, and 2 two isoforms present in the midgut of leptidoptera have shown that bind with high or less affinity to Cry1A toxins [41]. The binding of the Cry1A toxins loops of domain II to APN and/or to ALP, it has been shown that these toxins bind with lower affinity (Kd 100 nM) than the to Cadherin receptor (Kd 1 nm). Indeed, ALP and APN despite their abundance onto the midgut are highly abundant low affinity binding sites for the toxin [57-62]. Therefore, the hallmark of the sequential binding resulted in that the initial binding with APN and ALP proteins concentrate the mononmeric activated toxin N. terminal fragment, favoring then; 2) A conformational change that allow to expose more the loops of domain II; 3) Binding with high affinity to the cadherin receptor, alpha 8-2 and particularly loop 3 in *M. sexta*, and the tobacco budworm *He-*

liothis virescens (Lepidoptera Noctuidae); 4) and *B. mori* [60-62]. This second interaction with a higher affinity facilitates the furhter cleavage of the N-terminal end that includes alpha 1-of domain I, leading to pre-pore-oligomer formation [43,60]. Several evidences support the role of the oligomerization process since it has been found that there is an increase by 200 fold the affinity of the toxin for the GP!-anchored receptors, ALP and APN [59,60]. Indeed, is this oligomeric GPI-binding structure that is favored for the insertion into the cell membrane for pore formation and eventually cell lysis [43,44,60,62]. -Furthermore, in Dipteran insects, the binding of the Cry11A and Cry4Ba toxins [109,110] to receptor(s) has been identified also as GPI-anchored, and Cadherins [65]. The ALP isoform binding to Cry11A and two more isoforms bind to Cry11A, specifically binding domain III and domain II loop alpha eight regions [66,67]. Indeed, recenlty this region has been identified as an important region involved in the binding of Cry11A toxin in *Ae. aegypti* BBMV with the cadherin receptor [66-68]. A similar binding process was shown between ALP and Cry11B and Cry4Ba [65-69]. The hallmark of these toxins, produced by *Bt svar israelensis* (*Bti*) is the synergistic effect of the Cyt1Aa on Cry4Aa, Cry4Ba and Cry11A toxin activity. Indeed, *Bti* toxins for the biological control of mosquitoes [69-72]. Interestingly, in the case of the insect midgut receptors of Coleopterans targeted by the Cry3A toxins [73], the binding is through GPI-anchored protein (ALP forms) identified in Coleopteran spp as the cotton pest, *Anthonomus grandis* (Coleoptera Curculionidae) targeted by Cry1B toxins as a putative receptor [1,16,17,73]. A second one, a cadherin protein in *Tenebrio molitor* (Coleoptera, Tenebrionidae), which also facilitates oligomer formation. This Cad receptor was also identified in another coleopteran *Diabrotica virgifera* (Coleoptera, Chrysomolidaeae), a mexican corn rootworm [3,73,74]. Furthermore, it was identified the binding site of the cadherin protein, the membrane proximal cadherin repeats 8-10 bound to Cry3A and Cry3Bb with high affinity (1.2 to 1.4 nM) and therefore, enhanced toxicity.

The case of the Cadherin's receptor(s) of the 3D-Cry toxins in Lepidopterans, Coleopterans and Dipterans, These glycoproteins plays an essential role in cell recognition, adhesion activities, and morphogenesis [74,75]. Adhesion properties of cadherin are due to the calcium-binding sites that play a critical role in keeping the extracellular domains and mediating binding between cadherins proteins on opposing cells. In invertebrates, cadherins are present in the intestinal midgut of lepidopterans and Dipteran insects to function as transmembrane glycoproteins like receptors of *Bt* Cry proteins, Cry1A, Cry3A [57,58]. The predicted structure of Cry-binding lepidopteran cadherins includes an amino-terminal signal peptide, 8-12 cadherin repeats (CRS), a membrane-proximal extracellular domain (MPED), a transmembrane domain, and a cytoplasmic domain [74-79].

In lepidopterans, most of Cry toxins binding sites are present at or near the membrane-proximal Cadherin Repeats [79]. Cadherin fragments containing the critical toxin binding region enhance the activities of Cry toxins in some lepidopteran [78]. The cadherin gene is essential for *Aede's* development [76]. Studies have shown that *Aae Cad* plays a role in the apical membrane and the maintenance of midgut integrity. In the most accepted model of the mechanism of action of Cry toxins, the binding to Cadherins receptors result in two utmost outcomes: 1) favored alpha-1 helix proteolytic cleavage for oligomer formation, and further oligomer insertion into the membrane for pore formation; 2) after sensing the external stimuli, transmit the signal to intracellular signalization pathways [45,46,72,73] (i.e. cell death pathway [2,39,45,56].

Moreover, a diverse proteins present in the insect midgut that can function as receptor of *Bt* Cry toxins such as: Chlorophyllide-binding proteins P252 [80], the BTR-270 glycoconjugate [81,82], the V- -ATPAse subunit A and actin (proteomic analysis) [82], lipids rafts associated proteins, flotilin and prohibitin; which represent the other componentsor additional proteins ins as well as intracellular proteins may have an active role in the mode of action of Cry toxins in mosquitoes [82]. Glycolipids, and alpha amylase has been proposed as Cry toxins receptors in lepidopteran insects [58,59,82,83]. In addition another protein binding to Cry3Aa was identified the ADAM-3 metalloprotease in the mdigut of the beetle *Leptinotarsa decemlineata* (Lepidoptera Chrysomeliadae) which bind through loop 1, and therefore an enhance pore formation activity, implying again that this binding play a key role in the toxicity of the Cry3Aa toxin [84].

Furthermore, referring to the ABCC transporters that consist of two transmembrane domain (TNMD12) and two nucleotide-binding domain (NBD12), play a key role in all living beings, as transporter of specific molecules, playing roles in absorption, distribution, and excretion of different types of molecules [85]. They are expressed in different tissues, kidney, intestine, liver and brain, playing a role in diverse physiological process such as to keep the

homeostasis of the osmotic equilibrium, immunity, lipid and cholesterol trafficking, immunity. In humans have described at least 40 ABC transporters. 11 of these that include other glycoproteins and multidrug resistant proteins are involved in multidrug resistance (MDR). Despite this direct evidence of the ABCC2 as a receptor for Cry1A toxins is lacking. The ABCC transporters in Lepidopteran was identified when mapping the locus that caused the resistance to Cry1A toxins identified as s an ATP-binding cassette (ABC) transporter family C2 [86]. Thereafter, using binding *in vitro* studies, Surface Plasmon Resonance (SPR) and deletion mutants of Cry1Aa in loop 2 or loop 3, it was shown that can function as receptor binding proteins for Cry1A toxins. This ABCC transporters was also found in another diamondback moth, *Plutella xylostella (*Lepidoptera Plutellidae) [87,88]. Indeed, the resistance of several strains of the silkworm larvae, *B. mori* to Cry1Ab was found due to insertion of one amino acid residue in ABCC2 [89]. Interestingly, through these experiments and the mutants in the loops 2 and 3 of Cry1A toxins, it was also shown that the binding sites of the ABCC2 transporters to the Cry1A toxins reside in the loops 2 and 3 [90]. Furthermore, ABCC2 transporters showed high binding affinity to Cry1A toxins, promoting cell swelling and cation pore formation [86,90].

The Molecular Cross talk at the insect midgut epithelium with implication in the (a) biocidal action of the 3D Cry Toxins

The hallmark of the biocidal action of the *Bt* 3D-Cry toxins starts right after the ingestion, solubilization and activation of the parasporal crystalline proteins (Figure 2A). at the insect midgut. One of the functions of the insect midgut epithelium is digestive enzymes production, and vectorial transport of small organic nutrients, ions, and water. Another important midgut function is the ability to produce signaling molecules that regulate its own physiology and the activity of other organs [27-29]. The two main mature cell types present in the midgut of all insects, i.e., columnar and endocrine cells, are responsible for these functions [27-29]. In addition, stem cells, located at the base of the midgut epithelium, ensure the growth and renewal of the midgut during development and after injury. In insects belonging to specific orders, midgut physiology is deeply conditioned by the presence of unique cell types, i.e., goblet and copper cells, which confer peculiar features to this organ [27-31].

Importantly, the insect midgut is a key player in insect development and homeostasis. At molecular level, the cadherins, catenin, integrins are known to play a fundamental role in morphogenesis and in pathways that function in morphogenesis and cell development. Besides, they participate dynamically in intercellular interactions, cell-cell adhesion adherents junction, pathways that contribute to the physiology and development of whole organism [78-82].

Cadherins is that along with these function (adhesive linkage of neighboring cells, architecture in morphogenesis) in vertebrates and invertebrates, have a role as sensors and transmitters of extracellular signals to the nucleus. A wealth of studies have highlighted the cadherin role of connection between cadherin-catenin protein complexes and importantly as major transmitters of intracellular signalization pathways. This role can be well accomplished in the insect midgut, through binding to the 3D Cry toxins and serving as intracellular sensor for intracellular signaling (Figure 2B) or by gathering monomeric toxins for pore formation (Figure 2A). One of the most studied are the neural cadherins of *Drosophla melanogaste*r (Diptera Drosophillidae) which differ in function and in the number of ectodomains of the rest of insect, at least of the Lepidopterans. Classical cadherin's have a large contribution to the construction of the animal body. There is a fundamental difference in the mode of classic cadherin-mediated cell-cell adhesion between chordate and non-chordate metazoans. These cadherins have a unique extracellular domain that is absent from vertebrate and ascidian classic cadherins. Different lines of experimental evidence have recently indicated that the site responsible for mediating adhesive interactions is localized to the first extracellular domain of cadherin. Invertebrate cadherin's may utilize multiple EC domains to form intercellular adhesive bonds. Remarkably, by sequence analysis it has been shown that similar Ca2+ linkers are widely distributed in the ectodomains of both invertebrate and vertebrate domains [80]. An interesting molecular conversation between Cry proteins and the different isoforms of cadherins, aminopeptidase, phosphatase alkaline and potentially of the ABC transporters (Figure 2A-B; Figure 3).The receptor-ligand interaction is the first step in the signaling of a variety and diverse biological and physiological processes. A hallmark of the molecular crosstalk of the 3D-Cry toxins with receptors present in insect midgut is their highly degree of conservation between different Cry proteins with different insect specificity, would implying a conserved and common mode of action relationships among them, giving the opportunity to broad their biocidal action.

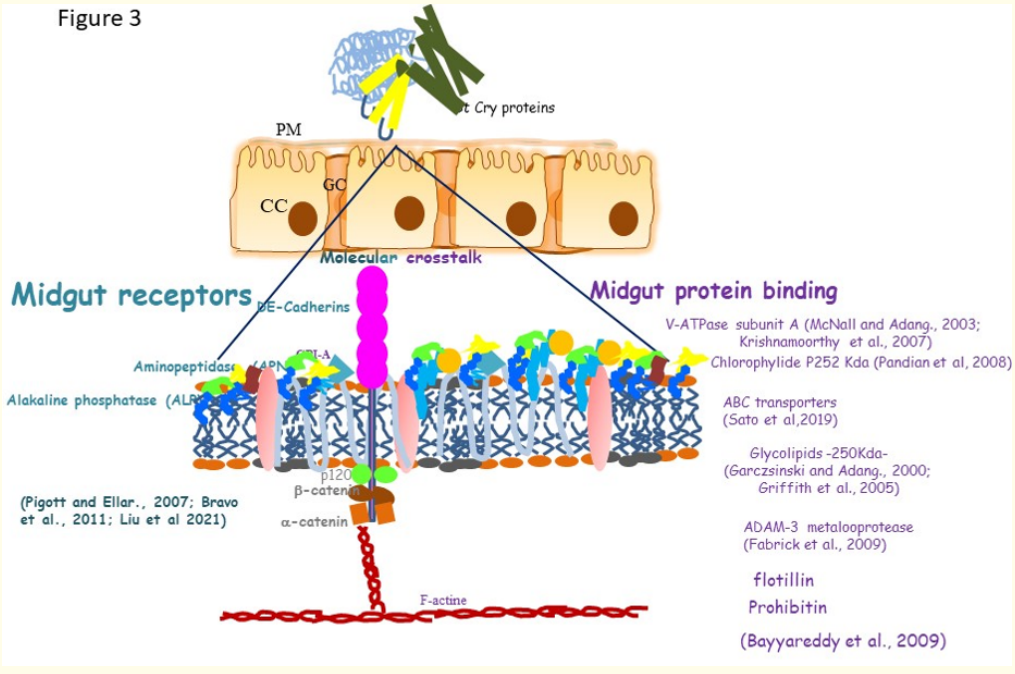


Figure 3: The entomopathogenic bacteria *B. thuringiensis* once ingested is able to reach their targets in the insect midgut. The receptors immersed in the brush border membrane, and/or different types of binding proteins, which will lead to a series of cellular and molecular events ultimately leading to a cell death or an insect defense responses (resistance). Indeed, is through the pattern of expression of the different isoforms of the receptors that the insect can cope with the action of the 3D Cry toxins. So, it seems that insects have evolved long through to evolution to activate all their genomic information and epigenetics to cope with the action of the 3D Cry toxins. *Bt* as a pathogen has also its strategies to target successfully the insect midgut.

Molecular basis of the insect resistant to Bt 3-D Cry toxins for agriculture.

Since decades it is known that *Bt* have colonized the insect world through mechanism of adaptation, homologous recombination and under the selection pressure [88,91-96]. Nowadays, the climatic changes have exerting selection pressures on organisms, allowing the expression on genotypes that allow to circumvent abiotic and biotic stresses. Under the absence of any selection pressure the expressed genotypes are naturally eliminated [88,96-101]. This could explain the symbiosis of the insects and *Bt.* In addition, depending of the form of the gene (dominant, recessive, and co-dominant) encoding some molecular component involved in the resistant to insect to chemical and/or to *Bt* 3D-Cry toxins. Meanwhile, at cellular level, insects are the reservoir and the host for *Bt* spore germination [101-104]. Moreover, the insect response (REPAT) [30-34] as well as other physiological (gut epithelial tissue regeneration, detoxifying enzymes), and molecular responses to weaken and overcome the action of *Bt* [26,27,92,94,98].

Furthermore insects have unique feeding and digestion characteristics that allowed to be resistant to the action of the *Bt* 3D Cry toxins. Thus, for example, when larvae are fed with high concentrations of Cry1C and Cry32A, the architecture of the BBMV, the development and the four intestinal digestive enzymes remains unaffected. Interestingly when aphids ingest Cry1Ac no observable damage because these insects are able to expel it along with a large amount of liquid food quickly [105]. In addition, detoxification enzymes involved in the insect resistance mechanism such as glutathione-S-transferase in the subalpine *Aedes rusticus* (Diptera *Culicidae*) [106]. In addition, the expression levels of esterase and dynein involved in the processes of detoxification and mid-intestinal repair to increase resistant in *Diabrotica virgifera (*Coleptera, *Chrysomelidae*) [107]. At molecular level, receptors and the diversity of protein bindings, play a crucial role in the protection of the insects to the action of the Cry toxins. The regulation of the expression of the diverse receptors present in the BBMV (ABBC transporter, alpha-amylase, ALP, APN, DE-Cad, glucolipids) that are the target of the 3D-Cry toxins. Cadherins are transmembrane proteins,

calcium-dependent adhesive proteins, present in the insect midgut of lepidopterans with a molecular weight of 220 kDa [56,58] (Figure 3). Aspartate amino acid residues coordinate calcium ions at the base for the cadherin extracellular domains. In Lepidopterans, most of Cry toxins binding sites are present at or near the membrane-proximal Cadherin Repeats [79]. Cadherin fragments containing the critical toxin binding region enhance the activities of Cry toxins in some lepidopteran [56,79]. Indeed, engineering of the amino acids of the domain II loops that can have more affinity with a K_d in several orders of magnitude) for the phospholipid bilayer membrane of the insect midgut tissue and therefore for the recognition and binding to the receptors like molecules. Furthermore, the cellular communication and the transmission of the extracellular signal downstream to the nucleus and therefore activation of the insect response are the anchor glycosylphosphatidylinositol (GPI) attached to the proteins (ALP, APN) like binding receptors, at outer leaflet of the cell membrane midgut which putative role is to function in lipid raft portioning, signal transduction, cellular communication, apical membrane targeting [103,104,108].

The insect host has adapted to *Bt* in relatively short evolutionary time scale highly possible through regulation of the epigenetic mechanism transformed into transgenerational inherited variation [103,109,110], or the transmission to the first (F1) and second filial generations (F2), also known as the paternal trans-generational immune priming [111,112]. This regulatory mechanism include to the molecular mechanism of DNA methylation [113], histone, acetylation modification [114] and the levels changes in microRNA (miRNA). All together comprise the evolutive insect defense (immunity) to the action of the bio pesticides [115]. Furthermore, other molecular components that play a role in the crosstalk at the insect midgut are some trans-regulatory mechanisms that has been found are involved in the downregulation in the expression levels of several of the Cry binding receptors. One of them is the role of the mitogen-activated protein kinase (MAPK) signaling cascade can trans-regulate the expression of ALP and ABCC genes, which expression is downregulated inducing insect resistance development in four strains of *P. xylostella* [116-118]. Other molecular components triggered by the action of the 3D-Cry toxins are the antimicrobial encoding genes of apidaecin, and hymenoptaecin *Apis mellifera* (Hymenoptera *Apidae*) [119,120]. Which improve the host immune response and promote its resistance [121,122]. Besides, in the midgut of the Lepidoptera, *Spodoptera littoralis* (Lepidoptera *Noctuidae*) it produces an antimicrobial peptide Mundticin KS, which can also have a role in the inhibition of some potential pathogens [123].

Highlights and perspectives

A plethora of studies regarding the mode of action of the 3D Cry toxins, still remains to be understood the interaction of the insect and *Bt*. While most of the studies have focused in the mode of action of these toxins *in vitro* and *in vivo*, The identification of the biomarkers in the insect-*Bt* interaction at transcriptional, transduction, and epigenetics levels (DNA methylation), as well as many other insect physiology process and even cellular (intestinal microbiota) could give an input for the design of safe biological control strategies of insects. A hot spot nowadays is toward harnessing to the multigene family above described as most possible in combination for the improvement of the biocidal activity of the 3D Cry toxins in front of the insects' evolution.

Disclosure Statement

The authors declare not conflict of interest.

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