

Cytotoxicity Analysis of Three *Bacillus thuringiensis* Subsp. *israelensis* δ -Endotoxins towards Insect and Mammalian Cells

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Abstract

Three members of the δ -endotoxin group of toxins expressed by *Bacillus thuringiensis* subsp. *israelensis*, Cyt2Ba, Cry4Aa and Cry11A, were individually expressed in recombinant acrySTALLIFEROUS *B. thuringiensis* strains for *in vitro* evaluation of their toxic activities against insect and mammalian cell lines. Both Cry4Aa and Cry11A toxins, activated with either trypsin or *Spodoptera frugiperda* gastric juice (GJ), resulted in different cleavage patterns for the activated toxins as seen by SDS-PAGE. The GJ-processed proteins were not cytotoxic to insect cell cultures. On the other hand, the combination of the trypsin-activated Cry4Aa and Cry11A toxins yielded the highest levels of cytotoxicity to all insect cells tested. The combination of activated Cyt2Ba and Cry11A also showed higher toxic activity than that of toxins activated individually. When activated Cry4Aa, Cry11A and Cyt2Ba were used simultaneously in the same assay a decrease in toxic activity was observed in all insect cells tested. No toxic effect was observed for the trypsin-activated Cry toxins in mammalian cells, but activated Cyt2Ba was toxic to human breast cancer cells (MCF-7) when tested at 20 μ g/mL.

Citation: Teixeira Corrêa RF, Ardisson-Araújo DMP, Monnerat RG, Ribeiro BM (2012) Cytotoxicity Analysis of Three *Bacillus thuringiensis* Subsp. *israelensis* δ -Endotoxins towards Insect and Mammalian Cells. PLoS ONE 7(9): e46121. doi:10.1371/journal.pone.0046121

Editor: Alejandra Bravo, Universidad Nacional Autonoma de Mexico, Instituto de Biotecnologia, Mexico

Received: December 23, 2011; **Accepted:** August 29, 2012; **Published:** September 21, 2012

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Funding: This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (www.cnpq.br) and Fundação de Apoio à Pesquisa do Distrito Federal-FAPDF (www.fap.df.gov.br). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Crop-damaging insects are responsible for a significant decrease in world crop yields every year [1]. Insects are also important vectors of human disease and thus are the focus of considerable control efforts [2]. The use of biological control of insects has been shown to be a safe, successful, cost-effective and promising alternative to chemical control [3]. The gram-positive, spore-forming bacterium *Bacillus thuringiensis* (Bt), which occurs naturally in several environments [4], is largely employed in biological control of various insect pests. Different Bt subspecies are toxic to specific insect groups, producing, during the sporulation phase, crystalline inclusion bodies consisting of host-specific insecticidal Cry and/or Cyt proteins known as δ -endotoxins [5]. Due to the high specificity to target insects and little evidence of toxicity to vertebrates and plants, the use of Bt as an alternative to chemical insect control has increased greatly [6]. Cry protoxins are solubilized under alkaline pH in the insect midgut and activated by endogenous gut proteases; these protoxins then interact with specific receptors present in the epithelial cells of the insect midgut, causing pore formation and consequent osmotic cell lysis [7,5]. Cyt protoxins also need solubilization and proteolytic cleavage in the insect's midgut to become active, but in contrast to Cry toxins, Cyt proteins bind nonspecifically to unsaturated phospholipids present in the host's epithelial cell, forming pores [8] or disrupting

the membrane through a detergent-like mode of action [9]. Relevant differences in the midgut physiology and consequent variation in proteolytic activity among different insect orders may determine the specificity of each toxin. For example, the main digestive lepidopteran and dipteran proteases are serine-proteases, whereas those found in coleoptera are cysteine and aspartic-proteases [10]. Cyt proteins are toxic to mosquito larvae, although their toxicities are usually lower than those from Cry proteins toxic to dipteran larvae [11]. It has also been reported that native toxins belonging to the Cyt family are toxic *in vitro* to erythrocytes and other mammalian cells, and lethal to mice after intravenous injection [12–15]. Cyt toxins have been shown to act synergistically with mosquitocidal Cry toxins [16]; Cyt1Aa can, for example, function as a receptor for the binding of Cry11Aa to *Aedes aegypti* gut cell membranes [17], thus playing a crucial role in the general activity of the parasporal inclusion. *Bacillus thuringiensis* subsp. *israelensis* (Bti) is highly toxic to dipteran insects and is utilized in mosquito control in many countries [18] e.g., to combat malaria and dengue fever. The entomopathogenic activity of Bti for *Culex spp.*, *Aedes spp.* and *Anopheles spp.* is due to the presence of a megaplasmid, pBtoxis, which encodes at least four Cry and two Cyt toxins: Cry4Aa, Cry4Ba, Cry10, Cry11Aa, Cyt1Aa and Cyt2Ba [19]. This plasmid also contains coding sequences for two other proteins (P19 and P20) believed to act as chaperones, helping toxin crystal formation [19].

In our study acrySTALLIFEROUS Bt strains were engineered to individually express Cry4Aa, Cry11A and Cyt2Ba proteins; the strains could then be tested *in vitro* against different cultured cell lines to determine their individual toxic activity and possible synergistic interactions with other toxins under controlled experimental conditions.

Results and Discussion

Bti Toxin Genes

Blast analysis of the sequences from amplified Bti genes showed that the *cyt2Ba* gene was identical to the sequence described elsewhere [20] (Genbank accession number = U52043), except for a 6xHis tag sequence in the N-terminal end, introduced by a PCR primer. The *cry11A* gene showed 100% nucleotide sequence identity with the *cry11A* gene described earlier by Berry *et al.* [19] (Genbank accession number = AL731825). The *cry4Aa* gene showed 98% nucleotide sequence identity with the first described *cry4Aa* gene [21] (Genbank accession number = Y00423) with eight nucleotide base differences, resulting in eight amino acid changes at the following positions: S16T, E32G, T301P, F569L, H572R, D980N, F1168V, I1170K.

Expression, Purification and Solubilization of Cyt and Cry Toxins

The amplified DNA fragments containing the *cyt2BaHis*, *cry11A* and *cry4Aa* coding sequences were first cloned in a PCR cloning vector (not shown), then transferred to the binary *B. thuringiensis* expression vector pSVP27A under control of the sporulation phase promoter *P_{cyt}* (Figures 1A, B, and C). The recombinant plasmids were purified and inserted into an acrySTALLIFEROUS Bt by electroporation, giving rise to the recombinant BtCyt2BaHis, BtCry11A and BtCry4Aa strains. The recombinant colonies were grown in selective media for 72 h and the spore/crystal of the mixture was subjected to centrifugation in discontinuous sucrose gradients.

After purification, Cry11A and Cry4Aa crystals were solubilized and analyzed by SDS-PAGE. Protein bands with the expected molecular masses were detected (66 kDa and 130 kDa, respectively) (Figure 1D). Despite the absence of the accessory proteins P20 and P19, usually associated with the folding of Cry11A, the recombinant Cry11A protein was largely expressed with the expected molecular mass. Xu *et al.* [22] have shown that the Cry11A protein was successfully expressed in recombinant *E. coli*, in the presence or absence of P20. On the other hand, in another recombinant Gram-negative bacterium, *Pseudomonas putida*, the Cry11A protein was produced in satisfactory amounts only when P20 was co-expressed [22]. Furthermore, high levels of Cry11A expression and toxic activity against *A. aegypti* larvae were shown in a recombinant acrySTALLIFEROUS Bt strain, 4Q2-81 [18]. In our work the Cyt2BaHis protein was obtained by centrifugation of the recombinant Bt extracts in a discontinuous sucrose gradient, followed by solubilization with Na₂CO₃ and affinity chromatography due to the large number of contaminants present after centrifugation (data not shown). After this additional purification step, SDS-PAGE analysis was carried out, showing a single 27 kDa band as expected (Figure 1E).

Toxicity of Dipteran-specific Cry4Aa and Cry11A Proteins to Dipteran and Lepidopteran Cell Lines

Cry4Aa and Cry11A proteins have been shown to be toxic to mosquitoes *in vivo* [4,23,24,25]. Our study used these two δ -endotoxins in cytotoxicity assays against both lepidopteran and dipteran cell cultures. Solubilized and trypsin-activated Cry4Aa

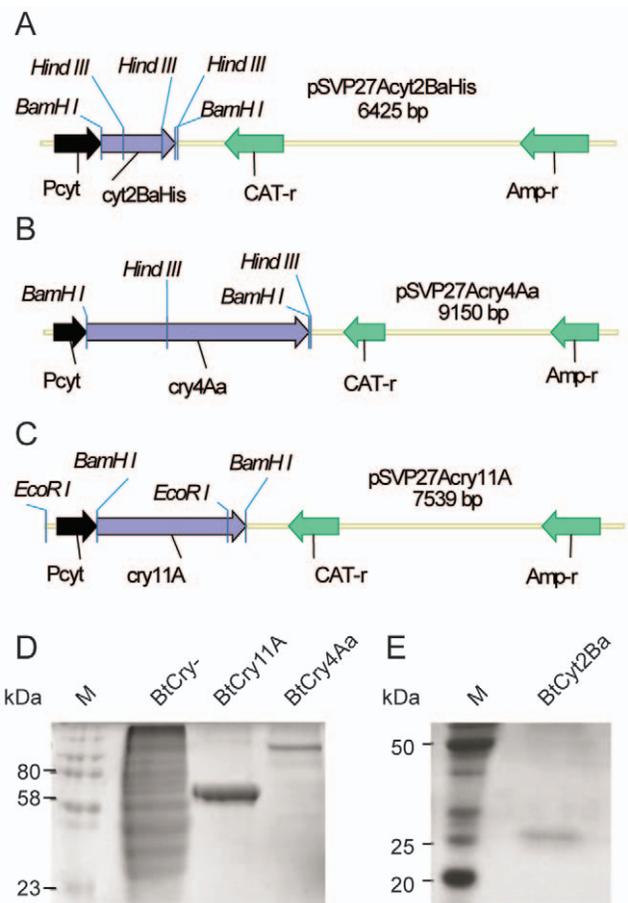


Figure 1. Schematic linear representation of the expression plasmids and SDS-PAGE analysis of Cry11A, Cry4Aa and Cyt2Ba expression. Panels A, B and C: *cyt2BaHis*, *cry4Aa* and *cry11A* genes inserted, respectively, into the *Bam*HI cloning site of the pSVP27A plasmid. The recombinant genes are under the control of a sporulation phase promoter, *P_{cyt}* (Figures 1A, B, and C). The genes for positive clone selection in *E. coli* (*Amp-r*) and in *B. thuringiensis* (*CAT-r*) are shown. Selected restriction enzyme sites used to confirm cloning are also shown. Panels D and E: SDS-PAGE of purified protoxins (Cry11A, Cry4Aa and Cyt2Ba) from recombinant *B. thuringiensis* strains. As a control, a sporulated acrySTALLIFEROUS *B. thuringiensis* strain (4Q7) extract was used. Selected molecular masses of the protein markers are shown (ColorPlus™ Prestained Marker-NEB, panel D and BenchMark™ Protein Ladder-Invitrogen, Panel E) on the left side of both panels. doi:10.1371/journal.pone.0046121.g001

and Cry11A toxins were incubated, separately or in combination, with dipteran and lepidopteran cells. Use of equal amounts of Cry4Aa and Cry11A toxin (20 μ g/mL) resulted in similar toxic effects in all cells tested. A slightly significant difference in toxicity was observed only when the toxins were tested separately against lepidopteran IPLB-SF-21AE and IPLB-LD-652Y cells. Interestingly, the mixture of the two toxins showed higher toxic activity to all insect cell lines (Figure 2A). In contrast, Chilcott and Ellar [26] observed no cytotoxicity when incubating trypsin-activated 130 kDa and 65 kDa Bti proteins with a cell line derived from *A. albopictus*. In our assays, the Cry4Aa and Cry11A activated toxins showed 10 to 15% of the toxicity of wild type Bti-activated toxins (see below) in *A. albopictus* C6/36 cells. Furthermore, the mixture of the two recombinant proteins increased toxicity to approximately 36% of the wild type Bti-activated toxins. As reported earlier [27], different cell lines from *Choristoneura fumiferana* (spruce budworm)

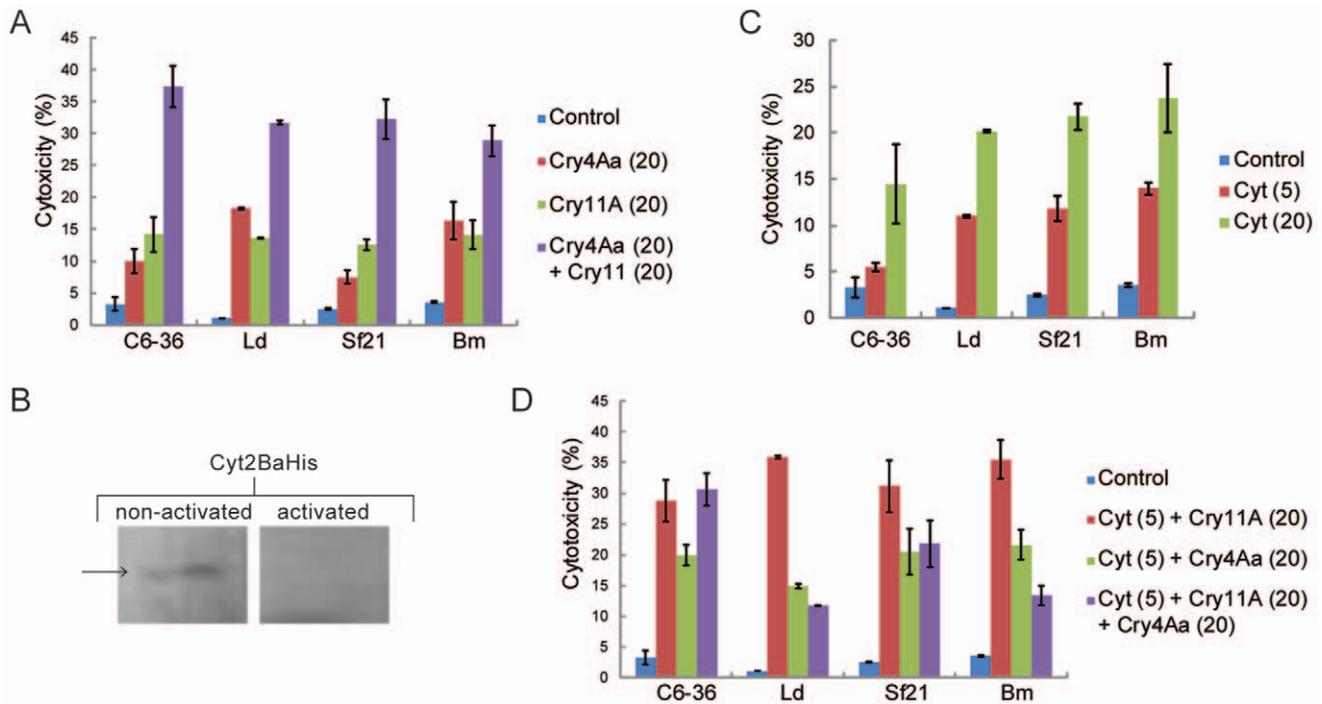


Figure 2. Cytotoxicity assays in insect cell cultures and western blot analysis of Cyt2Ba activation. Panel A: 20 $\mu\text{g}/\text{mL}$ of each activated toxin, Cry4Aa and Cry11A, were incubated with different insect cell lines for 30 min. Untreated cells (control) were not incubated with any of the toxins. The percentage of cytotoxicity (cell mortality) was determined by luminometric readings based on luciferase activity. Panel B: Trypsin activation of Cyt2BaHis was proceeded and both protoxin and the activated toxin samples were analyzed by western blot with an anti-His antibody. The arrow shows a band of 27 kDa expected for the non-activated protein. The proteolytic cleavage of Cyt2BaHis resulted in the 6xHis tag loss, making the activated toxin undetectable in western blot analysis using anti-His antibody. Panel C: The activated Cyt2Ba toxin was incubated with insect cells at two different concentrations (5 and 20 $\mu\text{g}/\text{mL}$). Cytotoxicity was determined as for panel A. Panel D: Combinations of Cyt2Ba (5 $\mu\text{g}/\text{mL}$) and the two Cry toxins (20 $\mu\text{g}/\text{mL}$ for each toxin) were incubated with insect cell lines. Cytotoxicity percentages were measured according to the same method employed in panel A and C. The toxins concentrations are shown in brackets. One type of dipteran cells (C6/36) and three types of lepidopteran cells (Ld, Sf-21 and Bm) are shown on the cytotoxicity assays graphics. doi:10.1371/journal.pone.0046121.g002

showed distinct relative susceptibility when incubated with Bt activated δ -endotoxins. The presence, absence or availability of suitable receptors on the cell surface could explain the diverse levels of susceptibility among different cell lines deriving from one insect species. The increased toxicity of combined Cry4Aa and Cry11A to *A. albopictus* cells was also observed in previous research [18]. The earlier study showed lower individual toxicity for Cry4Ba, Cry4Aa and Cry11Aa proteins to fourth-instar *A. aegypti* than native crystals produced by Bti. Sauka *et al.* [28] demonstrated that a mixture of Cry1Ba and Cry1Da toxins yielded a synergism factor higher than 1 [29] when tested against neonate larvae of *Epinotia aporema* (bean shoot borer), suggesting a possible synergism between these Cry toxins. However, no synergistic interaction between 130kDa and 65 kDa Cry proteins was detected *in vivo* against *A. aegypti* larvae [26,29]. Although Cry4Aa and Cry11A protoxins used in our work may share the same molecular mass as the respective proteins reported previously by other research groups, they are not the same polypeptides; there may be differences in the specificity of the molecular interactions. Using a model for synergism proposed by Tabashnik [29], we calculated the synergism factor (SF) for the Cry4Aa and Cry11A mixture in all cell lines tested. For this purpose, the median lethal concentration (LC_{50}) was estimated (Table 1). For the dipteran C6/36 and the lepidopteran SF-21 cell lines, the SF was higher than 1, suggesting possible synergistic effects (Table 2). Poncet *et al.* [30] detected higher SF values, and thus, stronger synergism when using a mixture consisting of Cry4A and Cry11A against three

different species of mosquito larvae. The dipteran cell line used in our assays, *in vitro*, is not necessarily derived from the midgut, but from a minced *A. albopictus* larvae. In contrast, the SF was lower than 1 for the mixture with the other two lepidopteran cell lines tested (Ld and Bm5) and some antagonism was detected (Table 2). Purified crystals from wild type Bti, containing all the δ -endotoxins expressed by this bacterium, were solubilized and added separately to the cell cultures at a concentration of 5 $\mu\text{g}/\text{mL}$. This amount of solubilized protein caused 100% cell lysis (data not shown) and was used as a positive reference in the cytotoxicity studies.

Cyt2Ba Cytotoxicity to Insect Cells

Although Cyt toxins are produced by some dipteran-specific Bt strains [31,32], these toxins have been reported to be cytolytic to a broad range of cells [12]. To determine any possible relationship between dose and toxicity to different cell lines, we activated purified Cyt2Ba toxin (Figure 2B), which has been proposed to bind cell membranes in a non-specific manner [8], and incubated it with four types of insect cells using two different concentrations (5 or 20 $\mu\text{g}/\text{mL}$). As demonstrated in our assays, Cyt2Ba showed toxicity to all insect cells tested (Figure 2C). There was also a significant increase in the toxic activity when Cyt2Ba was used at a concentration of 20 $\mu\text{g}/\text{mL}$.

Incubation of Cell Lines with Both Cry and Cyt Toxins

To verify that Cyt2Ba and Cry toxins (Cry4A and Cry11A) interact cytotoxically, assays were performed using combinations

Table 1. Cytotoxicity of mixtures of Cry and Cyt toxins of *Bacillus thuringiensis* subsp. *israelensis* to insect cell lines.

	Cry4Aa/Cry11A			Cyt2Ba/Cry11A			Cyt2Ba/Cry4Aa		
	LC ₅₀ ^a (95% limits)	S. ^b	χ ^{2c}	LC ₅₀ ^a (95% limits)	S. ^b	χ ^{2c}	LC ₅₀ ^a (95% limits)	S. ^b	χ ^{2c}
C6/36	59.46 (49.48–80.261)	2.07	0.77	61.44 (53.27–74.65)	2.44	2.30	78.96 (66.60–105.51)	2.52	2.17
Bm5	111.65 (99.23–132.47)	3.29	1.34	147.03 (93.77–618.44)	1.45	0.68	59.93 (46.96–92.23)	2.47	3.31
Ld	108.97 (78.45–274.18)	2.07	4.40	54.79 (43.11–78.29)	2.50	3.62	106.47 (79.75–200.03)	1.81	1.30
Sf	75.85 (50.66–119.51)	2.20	4.30	55.47 (44.41–76.275)	2.63	3.31	97.45 (73.02–175.76)	1.68	1.76

^a-mean lethal concentration. Numbers in parentheses are 95% fiducial limits.

^b-slope.

^c-chi-square.

doi:10.1371/journal.pone.0046121.t001

of these toxins. The Cry toxins were used at a concentration of 20 μg/mL, whereas the Cyt2Ba toxin was used at 5 μg/mL, since any increase in the obtained cytotoxicity could be misinterpreted as activity of only Cyt2Ba at its highest concentration (Figure 2C). The data showed a higher level of toxic activity when Cry11A and Cyt2Ba were used in combination (Figure 2D). In all cells tested the toxicity demonstrated by this combination was significantly higher than the Cry4Aa and Cyt2Ba combination (Figure 2D). Nevertheless, in both cases different types of toxic interactions were observed when determining the LC₅₀ of the mixtures and calculation of SF values (Tables 1 and 2). SF >1 was achieved with the mixture Cyt2Ba/Cry11A in dipteran C6/36 and lepidopteran Sf-21 cells as well as with the mixture Cyt2Ba/Cry4Aa in lepidopteran Bm5 cells. These data indicate synergism between the Cyt and Cry toxins tested, which agrees with other works showing this kind of interaction [33,16]. Weak additive effects (SF ~ 1) were obtained for the Cyt2Ba/Cry11A mixture when it was tested against lepidopteran Ld cells. Similar SF values were detected after incubation of Cyt2Ba/Cry4Aa with C6/36 and Sf-21 cells. It was also demonstrated that some antagonism (SF lower than 1) may occur between Cyt and Cry toxins depending on the cell line tested. SF <1 was observed for the Cyt2Ba/Cry11A mixture in Bm5 cells and for Cyt2Ba/Cry4Aa in Ld cells. Hughes *et al.* [34] also showed slight antagonism *in vivo* when Cry11A and Cry4B were assayed in combination with Cyt1A against *Chironomus tepperi* (Diptera: Chironomidae). Aside from this report, it has been demonstrated that binding of Cry11Aa to *A. aegypti* membranes is greatly enhanced by preincubation of “brush border membrane vesicles” (BBMV) with Cyt1A [17]. Except for the C6/36 cells, our combination of the three toxins (Cyt2Ba, Cry4Aa and Cry11A) led to a decrease of the toxic activity in relation to the Cry11A and Cyt2Ba mixture

(Figure 2D). These results indicate that Cyt2Ba acts as a receptor for both Cry toxins, as shown by Pérez *et al.* [17], and Cry4Aa competes with Cry11A for the binding to Cyt2Ba, leading to a weaker interaction than that of Cyt2Ba and Cry11A. There is also evidence that Cry toxins may interact, forming hetero-oligomers *in vivo* [35]. In our work, such molecular interactions between Cry toxins could be more or less effective depending on the cell line tested, after the putative binding of Cry toxins to Cyt2Ba. This may explain why the cytotoxicity of the three toxins together did not decrease in C6/36 in contrast to the other insect cell lines.

Cry4Aa and Cry11A Toxic Activity When Processed by Lepidopteran Gastric Juice

The Cry4Aa and Cry11A protoxins were also processed by proteases found in the gastric juice extracted from *S. frugiperda* larvae. As previously described, besides the three-domain based structure and the intrinsic amino acid sequence, proteolytic activation is a relevant factor for the toxicity of Cry toxins [36,37]. The same incubation conditions as well as the protein ratio used for trypsin activation were maintained for gastric juice (GJ) protein processing. The cleavage pattern obtained for Cry4Aa and Cry11A after incubation with *S. frugiperda* midgut proteases resulted in a different SDS-PAGE banding profile than with trypsin activation (Figure 3). According to Christeller *et al.* [38], the GJ from different lepidopteran larvae contain predominantly alkaline proteases with different patterns of trypsin- or chymotrypsin-like activities. Although both types of proteases are members of the serine protease group, Cry proteins may undergo different proteolytic processing upon trypsin or chymotrypsin treatment. For instance, Cry3Aa toxin produced different polypeptides after treatment with each protease, but retained its ability

Table 2. Observed and expected cytotoxicity of Cry and Cyt toxin mixtures to insect cell lines and the calculated synergism factor.

	Cry4Aa/Cry11A			Cyt2Ba/Cry11A			Cyt2Ba/Cry4Aa		
	LC ₅₀ ^a	Exp. LC ₅₀ ^b	SF ^c	LC ₅₀ ^a	Exp. LC ₅₀ ^b	SF ^c	LC ₅₀ ^a	Exp. LC ₅₀ ^b	SF ^c
C6/36	59.46	77.70	1.30	61.44	74.92	1.21	78.96	81.54	1.03
Bm5	111.65	100.7	0.89	147.03	95.67	0.65	59.93	91.94	1.53
Ld	108.97	65.47	0.60	54.79	55.45	1.01	106.47	89.64	0.84
Sf	75.85	75.85	1.10	55.47	76.45	1.37	97.45	97.48	1.01

^a-observed mean lethal concentration (μg/mL).

^b-expected mean lethal concentration (μg/mL).

^c-synergism factor.

doi:10.1371/journal.pone.0046121.t002

to bind Colorado potato beetle's (*Leptinotarsa decemlineata*) BBMV's. In contrast, the Cry3Ba toxin was unable to bind *L. decemlineata*'s BBMV's using identical proteolytic treatments [39]. In our work, the processed toxins were dialyzed and incubated with insect cell cultures at a concentration of 20 $\mu\text{g}/\text{mL}$. To ensure that neither trypsin nor GJ was responsible for any toxic activity to the cells, cytotoxicity was measured after incubation of both proteases with the cell lines. No cytotoxicity was detected for the processed Cry toxins or for the protease samples (data not shown). Furthermore, Cry11A and Cry4Aa crystals and GJ-activated proteins (20 $\mu\text{g}/\text{mL}$) were also tested *in vivo* against second-instar larvae of *A. aegypti*. Cry11A and Cry4Aa were toxic to *A. aegypti* larvae with LC_{50} values of 53.72 ng/mL and 92.24 ng/mL, respectively (Table 3). In contrast, no toxic activity was observed for the one-dose bioassay using GJ-activated proteins (data not shown). It is possible that the new cleavage pattern yielded by the lepidopteran gastric juice does not provide a functional structure of an active toxin [25]. However, we cannot rule out the possibility that Cry proteins were overdigested by insect gastric juice and consequently inactivated the toxins.

Cytotoxic Activity of Cry4Aa, Cry11A and Cyt2Ba to a Mammalian Cell Line

The specificity of Cry proteins is also due to the fact that, when ingested by susceptible insect larvae, each toxin binds to specific receptors on the midgut epithelial cell brush border membrane [40]. Thomas and Ellar showed that the pool of purified and solubilized crystal proteins of Bt was toxic to mouse fibroblasts, primary pig lymphocytes and mouse epithelial carcinoma cell lines [12]. To determine whether the dipteran-specific toxins, Cry4Aa and Cry11A, are toxic to mammalian cells via trypsin activation, we performed cytotoxicity assays with human breast cancer cells,

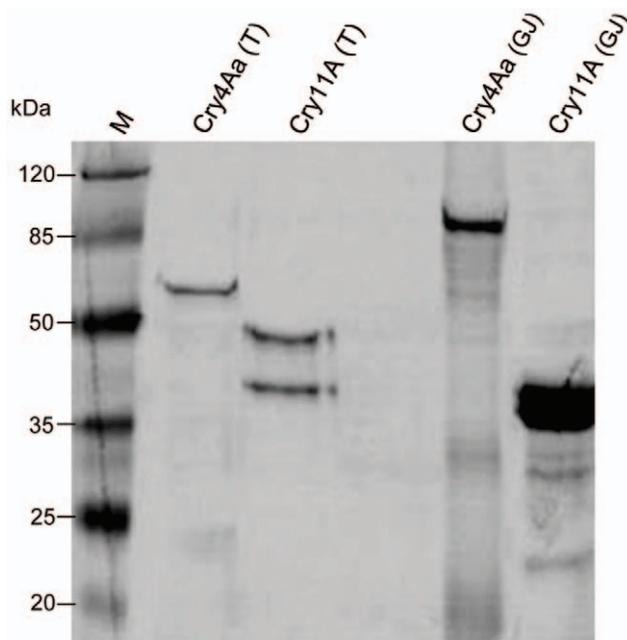


Figure 3. SDS-PAGE analysis of Cry4Aa and Cry11A proteolytic activation. The solubilized Cry4Aa and Cry11A protoxins were activated either by standard incubation using trypsin (T) or by using *S. frugiperda*' gastric juice (GJ). The processed proteins were stained with Coomassie blue. Molecular masses in kDa are shown on the left (Prestained Protein Molecular Weight Marker, Fermentas). doi:10.1371/journal.pone.0046121.g003

Table 3. Toxicity of individual Cry proteins to second-instar larvae of *A. aegypti*.

Toxin	n*	LC_{50} (95% limits)**	σ ***
Cry11A	75	53.72 (25.13–86.54)	3.814
Cry4Aa	75	92.24 (55.64–123.58)	2.673

*: number of tested larvae;

** : mean lethal concentration for 50% of larvae exposed ($\mu\text{g}/\text{mL}$).

*** σ : standard deviation.

doi:10.1371/journal.pone.0046121.t003

MCF-7 (Figure 4). Some Bt strains have been reported to produce parasporal proteins that are capable of discriminately killing cancer cells [41,42]. However, this anticancer activity is achieved by a different category of parasporal proteins, the so-called parasporins, which exhibit very low homologies (<25%) to Cry and Cyt proteins [42,43]. Since the wild type Bt δ -endotoxins used here as our positive reference control did not yield 100% mortality when observed by light microscopy, a lysis buffer was added to the cell culture so that all cells were lysed and a reliable reference value was obtained by luminometric reading. No cytotoxicity was detected when any of the Cry4Aa and Cry11A-activated toxins were incubated individually or in combination with the human cells. Mammalian cells do not display receptors for the binding of Cry toxins on their surfaces, which may explain why Bt is not toxic to mammals. In addition, the Cyt2Ba toxin was incubated at two different concentrations with MCF-7 cells (Figure 4). This experiment demonstrated that Cyt2Ba was cytotoxic only at the highest concentration used in our assays (20 $\mu\text{g}/\text{mL}$). This toxicity is probably due to the ability of Cyt2Ba to bind cell membranes without specific receptors. The activity value shown for the activated toxins expressed by Bt, approximately 70% (Figure 4), may be explained by the synergistic effects among additional toxins in the Bt crystals [11]. (For instance, Cyt1Aa has been shown to synergize Cry4 and Cry11 toxins in *A. aegypti* gut cells [17].) To investigate this hypothesis, another cytotoxic assay was carried out using 20 $\mu\text{g}/\text{m}$ concentrations of the three toxins (Figure 4) in combination. The mixture of Cry and Cyt toxins showed higher toxicity to MCF-7 cells than Cyt2Ba alone, but non-parametric variance analysis of the cytotoxicity values demonstrated that there was no significant difference in toxicity between Cyt2Ba alone and in combination with the two Cry toxins. It is not clear whether the significantly higher cytotoxic value obtained for a 60 min incubation period was due to the combination of the three toxins. Despite the variance, the cytotoxic effect measurements plotted in the graph (Figure 4) suggest an interaction between the Cry toxins with Cyt2Ba. If this is true, Cyt2Ba may be functioning as a receptor for Cry4Aa and Cry11A, which could lead to pore formation in human cell membranes. Further work is needed to characterize the toxicity interactions between Cry and Cyt proteins in mammalian cells.

Conclusion

During evolution insects from different orders and species developed differences in the enzymatic composition of gastric juices in order to adapt to a wide variety of diets. Microorganisms with entomopathogenic capabilities, such as Bt, may have co-evolved to take advantage of digestive enzymes evolved in insect midguts. The toxicity of Bt to insects is chiefly based on δ -endotoxins that must be processed correctly in the intestinal lumen of susceptible insects. The specificity of Cry toxins is, in part, due to the type of proteolytic cleavage that takes place in the insect

provided by Dr. David Ellar, University of Cambridge, UK [50,51], and transformed into *E. coli* DH5- $\alpha^{\text{®}}$. The positive clones were selected by ampicillin resistance in culture medium (100 μ g/mL). The recombinant plasmids pSVP27Acyt2BaHis, pSVP27Acry4Aa and pSVP27Acry11A were isolated by alkaline lysis as described in [49]. The cloning of each gene was confirmed by restriction enzyme digestion followed by electrophoresis in agarose gels as described above.

Expression, Purification and Solubilization of Cry and Cyt Proteins

An acrySTALLIFEROUS Bti strain, 4Q7, kindly provided by Dr. Colin Berry, Cardiff University, UK, was used as a host for the constructed plasmids. The Bti cells were transformed with the recombinant plasmids pSVP27Acyt2BaHis, pSVP27Acry4Aa and pSVP27Acry11A by electroporation as previously described [52] with the following modifications. The electroporation parameters were adjusted on the BTX Electro Cell ManipulatorTM 600 apparatus, using the following setup: 15 kV cm⁻¹, 200 Ω e 25 μ F. After electroporation, the cell suspensions were diluted in 1 mL LB broth and incubated with agitation (200 rpm) for 2 h at 28°C. After cell recovery by centrifugation, the pellets were resuspended and incubated on L-agar with 10 μ g mL⁻¹ chloramphenicol [48]. The positive clones were grown in selective NYSM media [53] with 10 μ g/mL chloramphenicol at 30°C for 72 h. After this period, crystals and spores were separated in a discontinuous sucrose gradient according to protocol described below and as described elsewhere [12] with modifications. The bacterial cultures were collected and centrifuged at 16,000 \times g for 20 min and the supernatant was discarded. The pellets were resuspended and washed in 60 mL of a solution containing 0.3 M NaCl, 0.01 M EDTA pH 8.0 and centrifuged at 16,000 \times g for 20 min. This procedure was repeated twice; at the end of the third centrifugation the supernatant was discarded and the pellets were resuspended in 30 mL 1.0 mM PMSF. Fresh 1.0 mM PMSF solution was used to wash the samples by centrifugation at 16,000 \times g for 20 min. The sediments were then resuspended in TTN buffer (20 mM Tris HCl, 0.1% Triton X-100, 300 mM NaCl, pH 7.2). The samples were centrifuged at 25,000 \times g for 1 h in a sucrose gradient (84%, 79%, 72%, 67% and 54%). The protein bands corresponding to Cry11A, Cry4Aa and Cyt2BaHis, formed in the gradient, were collected and added to a 0.1% Triton X-100 solution. A new centrifugation at 25,000 \times g for 45 min was performed and the sediments were resuspended in 1,0 mM PMSF. The crystals of Cyt2BaHis and Cry11A were solubilized in 0.1 M Na₂CO₃, pH 10.5 at 37°C for 1 h. The Cry4Aa crystals were solubilized in the same solution supplemented with the reducing agent 0.2% β -mercapto-ethanol. Any insoluble material was removed by centrifugation. The samples were neutralized with 0.1 M Tris-HCl pH 7.5 [54]. The 6xHis tag Cyt2Ba C-terminal fused protein was further purified by affinity chromatography using an agarose Ni-NTA column (The QIA expressionistTM, QIAGEN), according to the manufacturers instruction, to remove any contaminants. The Bti S-1989 strain was also grown in the sporulation medium NYSM and the protein crystals were purified in a sucrose gradient, as described above for the individual Cry and Cyt proteins. The purified crystals were then solubilized with 0.2% β -mercapto-ethanol in 0.1 M Na₂CO₃, pH 10.5 solution at 37°C for 1 h and then neutralized with 0.1 M Tris-HCl, pH 7.5. The samples were analysed by electrophoresis in a 12% SDS-PAGE (Mini-protean II – Biorad), following the manufacturer's instruction.

Toxin Activation

The samples containing the solubilized protoxins were quantified by fluorospectrophotometry using the NanoDrop 3300 apparatus together with the Quant-ItTM Assay Kit (Invitrogen). The individually purified Cry toxins were activated by two methods. In the first method, the protoxins were incubated with pancreatic bovine trypsin (Sigma) at a 10:1 ratio (w/w) for 2 h at 37°C. In the second activation method, the protease-containing gastric juice was obtained from ten *S. frugiperda* midguts following the procedure described in [55]. The Cry4Aa and Cry11A protoxins were incubated with the gastric juice at a 10:1 ratio (w/w) for 2 h at 37°C. The samples obtained from both activation methods were dialyzed against distilled water using 0.025 μ m membrane discs (MilliporeTM) and analyzed in a 12% SDS-PAGE. Purified Cyt2BaHis was also trypsin activated and dialyzed in the same conditions described for the Cry proteins. Furthermore, western blot analysis to check the Cyt2BaHis protein cleavage was carried out using a monoclonal anti-His antibody (GE Healthcare), following the manufacturer's instruction. The solubilized wild type Bti protoxins were incubated with pancreatic bovine trypsin (Sigma) only at a 10:1 (w/w) ratio for 2 h at 37°C, for protein activation, and dialyzed as above.

Cytotoxicity Assays

The insect (IPLB-SF-21AE, IPLB-LD-652Y, BM-5, C6/36) and mammalian (MCF-7) cell lines, grown in 25 cm² TPP flasks (Techno Plastic Products, Switzerland), were collected and added to 96-well plates (10⁴ cells/well). The culture medium of MCF-7 cells was removed and the cells washed with 1 mL PBS. A trypsin/EDTA (0.05%) solution was added to the cells, incubated at 37°C and 5% CO₂ for 4 min and 1 mL of DMEM medium was added to stop trypsinization. The loose cells were centrifuged at 1,000 \times g for 3 min, resuspended in DMEM medium and added to 96-well plates (10⁴ cells/well). The activated Cyt2Ba, Cry4Aa and Cry11A toxins were added to the wells at predetermined concentrations. Cyt2Ba toxin was tested at two concentrations (5 or 20 μ g/mL) separately or in combination with the other toxins. Cry4Aa and Cry11A toxins were used at the concentration of 20 μ g/mL, individually or in the presence or absence of Cyt2Ba. The pool of wild type Bti activated toxins were incubated with all cell lines at the concentration of 5 μ g/mL. All toxins were also incubated, at the mentioned concentrations, with different culture media in the absence of cells. Trypsin and gastric juice were individually incubated with C6/36 cells. All assays were carried out in triplicate. The time of all incubations with the three toxins as well as with the proteases, was 30 min. Furthermore, the culture media were incubated with CellTiter-Glo[®] Reagent from the CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega) for 10 min to quantify the amount of ATP released to the media. The luminescence-based measurements were carried out in a Turner TD20/20 luminometer, with the following setup: delay time 5 s, integration time 20 s, sensitivity 50%.

Mosquito Larvae

Neonate larvae of *A. aegypti* from the Rockefeller strain were provided by Embrapa Recursos Genéticos e Biotecnologia (Brasília, Brazil). The larvae were reared at room temperature (25°C) in autoclaved distilled water and fed on cat biscuit until they reached second instar.

Bioassays

Sucrose-gradient isolated Cry11A and Cry4Aa crystals were added separately to 100 mL sterile tap water in disposable plastic

cups containing 20 second-instar *A. aegypti* larvae, and mosquito-cidal activities were determined after 24 h [56]. Serial dilutions of each crystal protein sample were used in the bioassays, and three replicates were performed for the seven doses tested, as well as for an untreated control group used as a negative control. The median lethal concentration for exposed populations was determined statistically by probit analysis using a program for PC (Polo Plus, LeOra Software). *A. aegypti* larvae were also exposed to a single dose of 20 $\mu\text{g}/\text{mL}$ of each GJ-activated toxin (Cry11A and Cry4Aa), following the same method of incubation described above. All bioassays were performed in duplicate.

Evaluation of Synergism

In order to identify potential synergistic effects, experimental LC_{50} (concentration of toxin responsible for 50% cell mortality) of each individual toxin and of the toxin mixtures (Cry4Aa/Cry11A, Cyt2Ba/Cry11A and Cyt2Ba/Cry4Aa) was estimated by probit analysis [57], using Polo Plus. Six doses ranging from 1 to 60 $\mu\text{g}/\text{mL}$ of each toxin were incubated with insect cell lines following the method used for the cytotoxicity assays described above. For the Cry toxin mixture the ratio used was 1:1, while for the Cry/Cyt mixtures the ratios were 0.8:0.2. Three replicates were assayed with each dose. Synergistic interactions between the Cry toxins

and between the Cry/Cyt were evaluated by the ratios of the theoretical LC_{50} , obtained by Tabashnik's equation [29], and the observed LC_{50} . When the ratio was >1 , the toxin interaction was considered to yield a synergistic effect as the toxicity was higher than the value predicted from individual additive toxicity. A ratio <1 meant that the interaction was antagonistic, whereas a ratio of 1 indicated only additive effects.

Acknowledgments

We thank Embrapa Recursos Genéticos e Biotecnologia-CENARGEM for kindly providing the *B. thuringiensis* subsp. *israelensis* strains; Érica Soares Martins for the advice on *B. thuringiensis* manipulation; Bruna Candido Guido for providing MCF-7 cells; Fernando Lucas Melo and Kjell-ove Holmström for the critical reading of the manuscript; Genevieve M Gwynne and Stefan T. Jaronski for kindly revising the manuscript for English writing style.

Author Contributions

Conceived and designed the experiments: RFTC BMR. Performed the experiments: RFTC DMAA. Analyzed the data: RFCT DMAA BMR. Contributed reagents/materials/analysis tools: RGM BMR. Wrote the paper: RFTC BMR.

References

- Estruch JJ, Carozzi NB, Desai N, Duck NB, Warren GW, et al. (1997) Transgenic plants: an emerging approach to pest control. *Nat Biotechnol* 15: 137–141.
- WHO (1996) Chapter 1: The state of world health. Insect-borne diseases. *In* The world health report 1996–Fighting Disease, Fostering Development. Geneva, Switzerland: WHO 46–56.
- Cook RJ (1993) Making greater use of introduced microorganisms for biological control of plant pathogens. *Ann Rev Phytopathol* 31: 53–80.
- Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, et al. (1998) *Bacillus thuringiensis* and pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62: 775–806.
- Bravo A, Gill SS, Soberon M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon Rev* 49: 423–35.
- Crickmore N (2005) Beyond the spore – past and future developments of *Bacillus thuringiensis* as a biopesticide. *J Appl Microbiol* 101: 616–619.
- de Maagd RA, Bravo A, Berry C, Crickmore N (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet* 17: 193–199.
- Li J, Koni PA, Ellar DJ (1996) Structure of the mosquito-cidal δ -endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. *J Mol Biol* 257: 129–152.
- Butko P (2003) Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. *Appl Environ Microbiol* 69: 2415–2422.
- Terra W, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. *Comp Biochem Physiol* 109B: 1–62.
- Chang C, Yu YM, Dai SM, Law SK, Gill SS (1993) High-level *cryIVD* and *cytA* gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. *Appl Environ Microbiol* 59: 815–821.
- Thomas WE, Ellar DJ (1983) *Bacillus thuringiensis* var. *israelensis* crystal endotoxin: Effects on insect and mammalian cells in vitro and in vivo. *J Cell Sci* 60: 181–197.
- Knowles BH, White PJ, Nicholls CN, Ellar DJ (1992) A broad-spectrum cytolytic toxin from *Bacillus thuringiensis* var. *kyushuensis*. *Proc R Soc* 248: 1–7.
- Gill SS, Cowles GJ, Pietrantoni PV (1992) The mode of action of *Bacillus thuringiensis* endotoxins. *Annu Rev Entomol* 37: 615–636.
- Mizuki E, Ohba M, Akao T, Yamashita H, Saitoh, et al. (1999) Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell-killing action on human cancer cells. *J Appl Microbiol* 86: 477–486.
- Wirth MC, Park HW, Walton WE, Federici BA (2005) Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. *Appl Environ Microbiol* 71: 185–189.
- Pérez C, Fernández LE, Sun J, Folch JL, Gill SS, et al. (2005) *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *PNAS* 102: 18303–18308.
- Beltrão HBM, Silva-Filha MHN (2007) Interaction of *Bacillus thuringiensis* var. *israelensis* Cry toxins with binding sites from *Aedes aegypti* (Diptera: Culicidae) larvae midgut. *FEMS Microbiol Lett* 266: 163–169.
- Berry C, O'Neil S, Bem-Dov E, Jones AF, Murphy L, et al. (2002) Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* 68: 5082–5095.
- Guerschicoff A, Ugalde RA, Rubinstein CP (1997) Identification and characterization of a previously undescribed *cyt* gene in *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* 63: 2716–2721.
- Ward ES, Ellar DJ (1987) Nucleotide sequence of a *Bacillus thuringiensis* var. *israelensis* gene encoding a 130 kDa delta-endotoxin. *Nucl Ac Res* 15: 7195.
- Xu Y, Nagai M, Bagdasarian M, Smith TW, Walker ED (2001) Expression of the p20 gene from *Bacillus thuringiensis* H-14 increases Cry11A toxin production and enhances mosquito larvicidal activity in recombinant gram-negative bacteria. *Appl Environ Microbiol* 67: 3010–3015.
- Höfte H, Whiteley HR (1989) Insecticidal crystal protein of *Bacillus thuringiensis*. *Microbiol Rev* 53: 242–255.
- Angsuthanasombat C, Uawithya P, Leetchewa S, Pornwiroon W, Ounjai P, et al. (2004) *Bacillus thuringiensis* Cry4Aa and Cry4Ba mosquito-larvicidal proteins: Homology-based 3D model and implications for toxin activity. *J Biochem Mol Biol* 37: 304–313.
- Yamagiwa M, Sakagawa K, Sakai H (2004) Functional analysis of two processed fragments of *Bacillus thuringiensis* Cry11A toxin. *Biosci Biotechnol Biochem* 68: 523–528.
- Chilcott CN, Ellar DJ (1988) Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. *J Gen Microbiol* 134: 2551–2558.
- Gringorten JL, Sohi SS, Masson L (1999) Activity spectra of *Bacillus thuringiensis* δ -endotoxins against eight insect cell lines *in vitro*. *Cell Dev Biol* 35: 299–303.
- Sauka DH, Sánchez J, Bravo A, Benintende GB (2007) Toxicity of *Bacillus thuringiensis* δ -endotoxins against bean shoot borer (*Epinotia aporema* Wals.) larvae, a major soybean pest in Argentina. *J Invertebr Pathol* 94: 125–129.
- Tabashnik BE (1992) Evaluation of synergism among *Bacillus thuringiensis* toxins. *Appl Environ Microbiol* 58: 3343–3346.
- Poncet S, Delécluse A, Klier A, Rapoport G (1995) Evaluation of synergistic interactions among the CryIVA, CryIVB and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. *J Invertebr Pathol* 66: 131–135.
- Promdonkoy B, Chewawiwat N, Tanapongpipat S, Luxanani P, Panyim S (2003) Cloning and characterization of a cytolytic and mosquito-cidal δ -endotoxin from *B. thuringiensis* subsp. *damstadensis*. *Curr Microbiol* 46: 94–98.
- Cheong H, Gill SS (1997) Cloning and characterization of a cytolytic and mosquito-cidal δ -endotoxin from *B. thuringiensis* subsp. *jegathesan*. *Appl Environ Microbiol* 63: 3254–3260.
- Wirth MC, Georghiou GP, Federici BA (1997) CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. *Proc Natl Acad Sci USA* 94: 10536–10540.
- Hughes PA, Stevens MM, Park HW, Federici BA (2005) Response of larval *Chironomus tentheri* (Diptera: Chironomidae) to individual *Bacillus thuringiensis* var. *israelensis* toxins and toxin mixtures. *J Invertebr Pathol* 88: 34–39.
- Carmona D, Rodríguez-Almazán C, Portugal L, Pérez C, et al. (2011) Dominant negative phenotype of *Bacillus thuringiensis* Cry1Ab, Cry11Aa and Cry4Ba mutants suggest hetero-oligomer formation among different Cry toxins. *PLoS One* 6(5): e19952.
- Uawithya P, Tuntitppawan T, Katzenmeier G, Panyim S, Angsuthanasombat C (1998) Effects on larvicidal activity of single proline substitutions in alpha 3 or alpha 4 of the *Bacillus thuringiensis* Cry4B toxin. *Biochem Mol Int* 44: 825–832.

37. Gerber D, Shay Y (2000) Insertion and organization within membranes of the delta-endotoxin pore-forming domain, helix 4-loop-helix 5, and inhibition of its activity by a mutant helix 4 peptide. *J Biol Chem* 275: 23602–23607.
38. Christeller JT, Laing WA, Shaw BP, Burgess EPJ (1990) Characterization and partial purification of the digestive protease of the black field cricket *Teleogryllus commodus* (Walker): elastase is a major component. *Insect Biochem* 20: 157–164.
39. Rausell C, Garcia-Robles I, Sanchez J, Muñoz-Garay C, Martínez-Ramírez AC, et al. (2004) Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* (Say). *Biochim. Biophys. Acta* 166: 99–105.
40. Pootanakit K, Angsuthanasombat C, Panyim (2003) Identification of two isoforms of aminopeptidase N in *Aedes aegypti* larval midgut. *J Biochem Mol Biol* 36: 508–513.
41. Mizuki E, Ohba M, Akao T, Yamashita H, Saitoh, et al. (1999) Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell-killing action on human cancer cells. *J Appl Microbiol* 86: 477–486.
42. Mizuki E, Park YS, Saitoh H, Yamashita S, Akao T, et al. (2000) Parasporin, a human leukemic cell-recognizing parasporal protein of *Bacillus thuringiensis*. *Clin Diagn Lab Immunol* 7: 625–634.
43. Ohba M, Mizuki E, Uemori A (2009) Parasporin, a new anticancer protein group from *Bacillus thuringiensis*. *Anticanc Res* 29: 427–434.
44. Vaughn JL, Goowin RH, Tompkins GJ, McCawley P (1977) The establishment of two cell lines from insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *In Vitro* 13: 213–217.
45. McClintock T, Dougherty EE, Weiner RM (1986) Semipermissive replication of a polihedrosis virus of *Autographa californica* in a gypsy moth cell line. *J Virol* 57: 197–204.
46. Grace TD (1967) Establishment of a line of cells from the silkworm *Bombyx mori*. *Nature London* 216: 613.
47. Igarashi A (1978) Isolation of Singhs *Aedes albopictus* cell clone sensitive to dengue and chinkugunya viruses. *J Gen Vir* 40: 531–544.
48. Soule HD, Vazquez J, Long A, Albert S, Brennan M (1973) A human cell line from a pleural effusion derived from a breast carcinoma. *J Nat Can Ins* 51: 1409–1416.
49. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor, New York.
50. Crickmore N, Nicholls C, Earp DJ, Hodman C, Ellar DJ (1990) The construction of *Bacillus thuringiensis* strains expressing novel entomocidal δ -endotoxins combinations. *J Biochem* 270: 133–136.
51. Crickmore N, Ellar DJ (1992) Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism *in vivo*. *FEMS Microbiol Lett* 94: 63–68.
52. Peng D, Luo Y, Guo S, Zeng H, Ju S, et al. (2009) Elaboration of an electroporation protocol for large plasmids and wild-type strains of *Bacillus thuringiensis*. *J Appl Microbiol* 106: 1849–1858.
53. Yousten AA (1984) *Bacillus sphaericus*: Microbiological factors related to its potential as a mosquito larvicide. *Adv In Biotechnol Proc* 3: 315–343.
54. Bravo A, Arrieta G, Benintende G, Real MD, Espinoza AM, et al. (2001) Metodologías utilizadas en investigación sobre bacterias entomopatógenas, Unam, México.
55. Vilchez S, Jacoby J, Ellar DJ (2004) Display of biological functional insecticidal toxin on the surface of lambda phage. *Appl Env Microbiol* 70: 6587–6594.
56. Ben-Dov E, Boussiba S, Zaritsky A (1995) Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. *J Bacteriol* 177: 2851–2857.
57. Finney DJ (1971) *Probit analysis*, third ed. Cambridge: Cambridge University Press.