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Intermolecular Interaction Between Cry2Aa and Cyt1Aa and Its Effect on Larvicidal Activity Against *Culex quinquefasciatus*

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The Cyt1Aa protein of *Bacillus thuringiensis* susbp. *israelensis* elaborates demonstrable toxicity to mosquito larvae, but more importantly, it enhances the larvicidal activity of this species Cry proteins (Cry11Aa, Cry4Aa, and Cry4Ba) and delays the phenotypic expression of resistance to these that has evolved in *Culex quinquefasciatus*. It is also known that Cyt1Aa, which is highly lipophilic, synergizes Cry11Aa by functioning as a surrogate membrane-bound receptor for the latter protein. Little is known, however, about whether Cyt1Aa can interact similarly with other Cry proteins not primarily mosquitocidal; for example, Cry2Aa, which is active against lepidopteran larvae, but essentially inactive or has very low toxicity to mosquito larvae. Here we demonstrate by ligand binding and enzyme-linked immunosorbent assays that Cyt1Aa and Cry2Aa form intermolecular complexes *in vitro*, and in addition show that Cyt1Aa facilitates binding of Cry2Aa throughout the midgut of *C. quinquefasciatus* larvae. As Cry2Aa and Cry11Aa share structural similarity in domain II, the interaction between Cyt1Aa and Cry2Aa could be a result of a similar mechanism previously proposed for Cry11Aa and Cyt1Aa. Finally, despite the observed interaction between Cry2Aa and Cyt1Aa, only a 2-fold enhancement in toxicity resulted against *C. quinquefasciatus*. Regardless, our results suggest that Cry2Aa could be a useful component of mosquitocidal endotoxin complements being developed for recombinant strains of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* aimed at improving the efficacy of commercial products and avoiding resistance.

Keywords: Cry2Aa, Cyt1Aa, *Bacillus thuringiensis* subsp. *israelensis*, synergy, mosquito larvicide

Introduction

The insecticidal bacterium *Bacillus thuringiensis* is a gram-positive aerobic spore-former found in many environmental niches [30]. This species is distinguished from other *Bacillus* species by the synthesis of crystalline inclusions that typically contain insecticidal proteins known as Cry (crystal) and Cyt (cytolytic) protoxins. Upon ingestion by a larval host, these protoxins dissolve in the midgut lumen, where they are cleaved by proteases to yield active toxins. The toxins then bind to microvillar receptors, forming cation channels that cause lysis of midgut epithelial cells, which erode from the epithelium, resulting in larval death [2, 3, 6, 7, 14, 19, 32]. The target spectrum of an isolate depends primarily on its complement of Cry and Cyt proteins. Cry1 and Cry3 toxins, for example, are active against, respectively, lepidopterans and coleopterans, whereas Cry4A, Cry4B, and Cry11A are toxic to nematoceran dipterans, which notably include many mosquito and blackfly vectors of important human diseases such as malaria, yellow fever, and filariasis [6, 7, 27, 28]. Cry2Aa, unlike other Cry proteins, is unusual in that its target spectrum includes both lepidopteran and

dipteran larvae [34]. Compared with Cry11A, Cry4A, and Cry4B, however, the toxicity of Cry2Aa against mosquito larvae is very low, being at least 10-fold less toxic than the former three proteins [21, 33]. Alternatively, Cyt1Aa, which is not related to Cry toxins, is moderately toxic to dipterans and certain coleopterans, but is not toxic to lepidopterans [20].

The most potent subspecies of *B. thuringiensis* active against mosquito larvae is *B. thuringiensis* subsp. *israelensis*. Its high toxicity is due to a combination of toxins, mainly Cry11Aa, Cry4Aa, Cry4Ba, and Cyt1Aa [7]. These occur together in a large parasporal body enveloped in a fibrous matrix, yielding high toxicity and a broad target host range, a structural combination that appears to have been selected for during evolution [13, 5]. Moreover, Cyt1Aa synergizes Cry4Aa, Cry4Ba, and Cry11Aa toxicity and delays the evolution of resistance to Cry11Aa [4, 26, 31, 36, 37, 38]. After decades of use in mosquito and blackfly abatement programs, no significant resistance to date has evolved to *B. thuringiensis* subsp. *israelensis*, with a primary reason apparently being its unique parasporal body.

More potent recombinant strains of *B. thuringiensis* subsp. *israelensis* have been constructed recently that also delay or prevent mosquito resistance. These recombinants include various strains of *B. thuringiensis* engineered to produce the binary toxin (Bin) of *Lysinibacillus* (previously *Bacillus*) *sphaericus* combined with Cyt1Aa and other mosquitocidal proteins such as Cry11Aa, and Cry11Ba [23, 24, 35], or Cry4Ba and Cry2Aa [40], all of which are significantly more potent than wild-type strains used in current commercial products. In the search for other toxins to add to these recombinants, we identified Cry2Aa owing to its potential activity against mosquito larvae, its structural similarity to Cry11Aa, and especially its potential for synergistic interaction with Cyt1Aa [8, 25]. Here we first demonstrate that purified Cry2Aa and Cyt1Aa formed intermolecular complexes *in vitro*, suggesting that such interactions could potentially facilitate an enhancement in mosquito larvicidal activity. Furthermore, we show that a combination of Cyt1Aa and Cry2Aa enhances larvicidal activity against a wild-type strain of *C. quinquefasciatus*, but not against a mutant strain of this species resistant to a combination of Cry4Aa and Cry4Ba.

Materials and Methods

Plasmids, Genes, Bacterial Strains, and Transformation

Plasmids pDBF69, pWF45, and pWF53, which are recombinant constructs of pHT3101 [16] containing, respectively, the *cry2Aa*, *cyt1Aa*, and *cry11Aa* genes, have been previously described [10, 39]. Plasmid pHTC-*cry2Aa* was generated by cloning the 4 kb *Sal*I/*Xba*I fragment with the *cry2Aa* gene in pDBF69 into the same sites in pHTC [23]. All plasmids were amplified in *Escherichia coli* DH5a and purified with the Wizard Plus Miniprep kit (Promega). The 4Q7 acrystalliferous strain of *B. thuringiensis* subsp. *israelensis* (*Bacillus* Genetic Stock Center at Ohio State University, Columbus, OH, USA) was transformed by electroporation as described by Wu and Federici [39]. Recombinants 4Q7/pDBF69, 4Q7/pWF45, 4Q7/pWF53, and pHTC-*cry2A* were selected on Luria-Bertani (LB) agar with, respectively, erythromycin (25 μg/ml) and chloramphenicol (5 μg/ml) at 28°C. Strain 4Q7/pWF45+pHTC-*cry2Aa* was selected on LB agar with erythromycin (12.5 μg/ml) and chloramphenicol (5 μg/ml) at 28°C.

Growth of Bacterial Strains, Microscopy, and Protein Profiles

Recombinant strains 4Q7/pDBF69, 4Q7/pWF45, 4Q7/pWF53, and 4Q7/pWF45+pHTC-*cry2Aa* were grown on nutrient agar (NA) with appropriate antibiotic selection for 4 days at 28°C when >95% of cells had sporulated and lysed, as determined by light microscopy with a Zeiss photomicroscope (USA) using a 100× oil-immersion objective. The protein profile of each strain was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% acrylamide gel [15]. Spores and crystals were harvested in 500 ml of sterile double-distilled water and collected by centrifugation in a Sorvall RC5C centrifuge at 5,000 rpm, 4°C for 30 min using the HS-4 rotor. Supernatants were discarded and pellets were resuspended and washed twice in 500 ml of water using the same centrifugation protocol. After the final wash, pellets were frozen at -80°C for 2 h and lyophilized for 48 h under vacuum using the Virtronic Vacuum with refrigeration set at -60°C. Powders were stored at room temperature prior to bioassays.

Mosquito Colonies

Two colonies of *Culex quinquefasciatus* were used for bioassays with various toxin powders. CqSyn, shown in this study to harbor putative receptor(s) for Cry2Aa, is a synthetic colony that was formed in 1995 by pooling multiple field collections and used to establish baseline toxicity values for all the toxin powders and combinations of powders [38]. Cq4AB, shown here to lack putative Cry2Aa receptors, is highly resistant to Cry4Aa+Cry4Ba of *Bacillus thuringiensis* subsp. *israelensis* and is also maintained under selection pressure [11].

Purification of Parasporal Crystalline Inclusions

Crystalline inclusions of Cyt1Aa and Cry2Aa were separated from spores through three successive rounds of purification by centrifugation at 18,000 ×*g* at 4°C for 1 h in discontinuous sucrose gradients (79%-72%-67% (w/v)) using the SW27 rotor and the Beckman L7-55 ultracentrifuge. Purified crystals were washed three times in phosphate-buffered saline (PBS, 0.01M phosphate buffer, pH 7.5; 120 mM NaCl) by resuspension and centrifugation as described above.

Ligand Blot Assays Using Solubilized Biotinylated Cyt1Aa and Cry2Aa

One milligram of purified Cyt1Aa was solubilized in 1.5 ml of alkaline buffer (50 mM Na_2CO_3, pH 11) overnight at 30°C. The solution was adjusted to pH 8.0 with 12.1 N HCl, and then centrifuged at 1,000 ×*g* for 5 min to pellet particulate undissolved crystals. The solution was dialyzed in PBS overnight, and total protein concentration was determined by the Bradford method [1]. Cyt1Aa in PBS (0.5 mg/ml) was labeled with Sulfo-NHS-Biotin, and the biotinylated-Cyt1Aa was purified and quantified according to the manufacturer's protocols (Pierce). One or 2 µg of Cry2Aa, Cry11Aa, and Cyt1Aa was fractionated by SDS-PAGE in a 10% gel and electroblotted onto nitrocellulose membrane using the SemiPhor electroblotter (Hoeffer Scientific Instruments) set at 8 volts for 30 min. The membrane was air-dried overnight, soaked in 0.05% PBS-Tween 20 (PBST) for 30 min, and blocked in PBST + 1% BSA for 2 h. The blocking reagent was removed and replaced with 50 ml of PBST containing biotinylated Cyt1Aa (~1 µg/ml) for 2 h. Labeled Cyt1Aa was removed and the membrane was washed three times, 10 min per wash, with 50 ml of PBST. The secondary antibody, anti-biotin-horse radish peroxidase (HRP) (Sigma) in PBS (1:4000), was added for 1 h, after which the antibody was removed and the membrane was washed three times, 10 min per wash, with 50 ml of PBS. Detection of binding with HRP-streptavidin conjugate and the 3,3′,5,5′′-tetramethylbenzidine substrate was performed as described in the manufacturer's protocol (Promega). The reaction was terminated by rinsing the membrane in distilled water.

Enzyme-Linked Immunosorbent Assay (ELISA)

For these assays, solubilized toxins were activated by proteolytic cleavage as determined by SDS-PAGE (data not shown). Purified Cry11Aa inclusions were solubilized in 100 mM NaOH and activated with 1:50 (w/w) trypsin (Sigma) for 2 h at 25°C to yield fragments of 36 and 32 kDa. Purified Cyt1Aa inclusions were solubilized in 50 mM Na_2CO_3, pH 10.5, for 2 h at 30°C, and activated with 1:30 (w/w) proteinase K (Sigma) for 1 h at 30°C to obtain the activated 25 kDa toxin. Purified Cry2Aa inclusions were solubilized in 50 mM Na_2CO_3, pH 10.5, for 2 h at 30°C, and activated with 1:10 (w/w) trypsin (Sigma) for 1 h at 30°C to obtain the activated 60 kDa toxin. ELISA plates (96 wells, Fisher Scientific) were incubated for 12 h at 4°C with 10 µg/ml Cry2Aa, Cry11Aa, or bovine serum albumin (BSA) in PBS. After incubation, the wells were washed five times with 200 µl of PBS/0.2% Tween 20. Fifty microliters of PBS/0.5% BSA/0.2% Tween 20 was added to each well and the plates were incubated for 1 h at 37°C, followed by five washes with PBS/0.1% Tween 20. Cyt1Aa was biotinylated and quantified using the ECL Biotinylation Module kit (Amersham), according to the manufacturer's protocol. Different concentrations of biotinylated-Cyt1Aa (0.25, 0.5, 1, 2, 4, 8 µg/ml) were added to the wells and the plates were incubated for 2 h at 37°C, and then washed five times with PBS/0.1% Tween 20. Binding of biotinylated Cyt1Aa to Cry2Aa, Cry11Aa, or BSA was detected with an anti-biotin antibody conjugated to horseradish peroxidase (1:4,000; Sigma) and the chromogenic *o*-phenylenediamine substrate (Sigma) according to the manufacturer's protocol. The enzymatic reaction was terminated by adding 50 µl of 6 M HCl, and the absorbance was read spectrophotometrically at 490 nm. Assays were repeated in triplicate.

***In Vivo* and *In Vitro* Interaction Between Fluorescently Labeled Cyt1Aa and Cry2Aa**

From 1-3 mg of purified Cyt1Aa and Cry2Aa crystals were solubilized for 12 h in 1 ml of 50 mM sodium carbonate (Na_2CO_4, pH 12). Particulate components were removed from the mixture by centrifugation at 16,300 ×*g* for 30 s. Solubilized Cyt1Aa and Cry2Aa proteins were then labeled, purified, and quantified using, respectively, the FluoReporter Rhodamine Red-X (F-6161) and FluoReporter Oregon Green 488 (F-6153) protein fluorescence labeling kits, according to the manufacturer's protocols (Molecular Probes). Fourth-instar *C. quinquefasciatus* larvae were placed in wells, three larvae per well, containing a total of 3 ml of water with diet (20-25 mg of a 3:1 mixture of mouse meal and brewer's yeast) supplemented with labeled Cyt1Aa or Cry2Aa, or a combination of both labeled toxins, each at a concentration of 1.1 µg/ml. After feeding for 2, 4, or 12 h, the larvae were removed from the well, placed in PBS in a petri dish on ice, and covered with aluminum foil. Individual larvae were removed from the plate, and the head and thorax were severed using a razor blade. The posterior midgut after segment 5 was also severed and discarded. The peritrophic membrane and food column were removed as an intact unit by immobilizing the midgut segment with jeweler's forceps while gently grasping the food column with forceps and slowly pulling it free. The remaining tissue was gently washed throughly and perfused with PBS. Individual microvillar cells were obtained after a longitudinal incision of the isolated midgut, by teasing apart isolated midgut tissue in PBS and visually searching the slide for isolated, intact, fluorescing microvillar cells. Cells were also treated with trypan blue (Sigma) at a final concentration of 0.2% to confirm that they excluded the dye and thus possessed intact membranes. At least 10 cells obtained from each replicate assay were selected for further microscopic analysis. Tissues and isolated cells were observed with the Nikon SM7800 and Leica DMRE fluorescent microscopes, respectively. At least three replicate assays were performed in each of these studies.

Bioassays

Toxin stocks were prepared using lyophilized crystal/spore powders by suspending weighed powder in deionized water with 20-25 glass beads to promote homogenization during shaking. Stocks were prepared monthly and 10-fold serial dilutions were prepared weekly. Stocks and dilutions were stored at -20°C when not in use. Different concentrations were fed to groups of 20 early-fourth instars, placed in 100 ml of deionized water in 250 ml plastic cups. Five or more different concentrations yielding mortality

between 0 and 100% were used for each replicate, and at least five replicates on five different days were prepared. A water control was used in each replicate. Larvae were provided with a few drops of food suspension after 24 h and mortality was determined after 48 h. Tests with mixtures of different recombinant powders were prepared based on the weight of each respective powder. Data were analyzed using Probit [9]. The activity enhancement for the mixtures was calculated using methods developed by Tabashnik [31], where the activity of Cry2Aa was assumed to be low because even the high concentrations tested yielded mortality of less than 15%.

Results

Recombinant Strains and Protein Profiles

Recombinant acrystalliferous 4Q7 strains of *B. thuringiensis* subsp. *israelensis* harboring plasmids pWF45, pDBF69, pWF45 and pHTC69, and pWF53 with genes encoding, respectively, Cyt1Aa (27 kDa), Cry2Aa (65 kDa), Cyt1Aa and Cry2Aa, and Cry11Aa (72 kDa) showed the presence of characteristic crystalline inclusions that formed during the sporulation phase of growth, as determined by phase contrast microscopy (data not shown). The identity of the crystal toxin proteins was confirmed by SDS-PAGE, in which each protein toxin migrated to a position corresponding to its predicted molecular mass (Fig. 1).

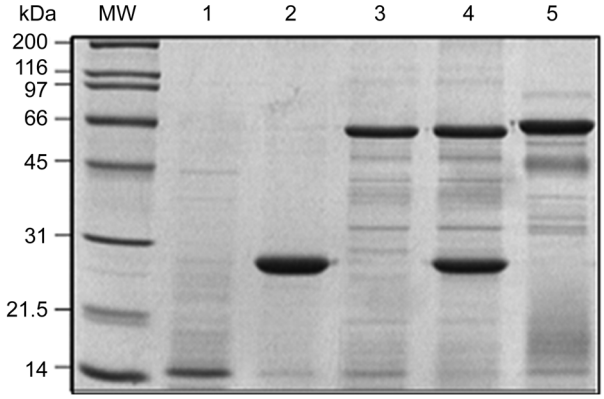

Fig. 1. Protein profiles of recombinant *Bacillus thuringiensis* subsp. *israelensis* strain 4Q7 producing crystal toxins.

Strains were grown on nutrient agar with appropriate antibiotics for 72 h, and sporulation and crystal synthesis were monitored by phase contrast microscopy. Proteins were fractionated on a 10% polyacrylamide gel by sodium dodecylsulfate polyacrylamide gel electrophoresis. Strains of 4Q7 are acrystalliferous (lane 1); 4Q7/pWF45 (Cyt1Aa, lane 2); 4Q7/pDBF69 (Cry2Aa, lane 3); 4Q7/pWF45 + pHTC-*cry2Aa* (Cyt1Aa + Cry2Aa, lane 4); and 4Q7/pWF53 (Cry11Aa, lane 5). MW, protein molecular mass standards; kDa, kilodaltons.

Cyt1Aa Binds Cry2Aa *In Vitro*

Ligand blot assays demonstrated that solubilized biotinylated Cyt1Aa was capable of binding to purified immobilized Cry2Aa (Fig. 2). In this assay, purified Cry11Aa was used as a positive control, as it has been established that intermolecular interactions occur between this protein and Cyt1Aa [25]. Interestingly, little or no binding occurred between labeled Cyt1Aa and purified unlabeled Cyt1Aa, which suggested the toxin has little or no affinity for itself, at least under the conditions used in the assay.

The ELISA analysis using proteolytically activated toxins confirmed the results observed in the ligand blots assays. Activated biotinylated Cyt1Aa bound immobilized activated Cry2Aa and Cry11Aa, the latter of which was previously described [25]. No binding of the cytolytic toxin occurred with BSA, which was used in this study as the negative control (Fig. 3).

Potential Interaction Between Cyt1Aa and Cry2Aa *In Vivo*

To further investigate the potential for *in vivo* interaction between Cry2Aa and Cyt1Aa, the CqSyn and Cq4AB strains were fed purified labeled Cry2Aa alone or a combination of purified labeled Cry2Aa and Cyt1Aa toxins. Fluorescence microscopy showed that labeled Cry2Aa localized in the posterior midgut, distal from the gastric caecae in CqSyn (Fig. 4A), suggesting that putative receptor(s) for the toxin is not evenly distributed along the midgut in this mosquito strain. No fluorescence of labeled Cry2Aa was observed in the digestive tract of Cq4AB (Fig. 4B), a result that suggested that Cq4AB lacked putative functional Cry2Aa receptor(s). Fluorescence from labeled Cry2Aa, when administered

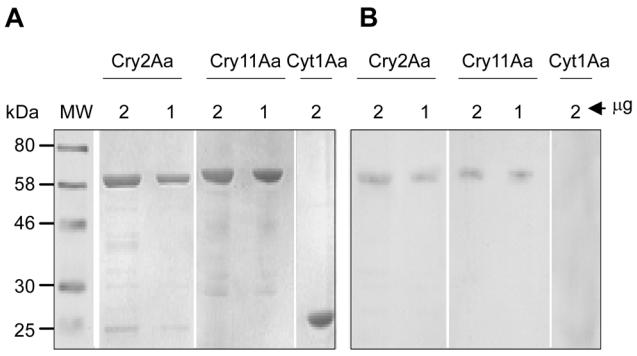

Fig. 2. Ligand blot analysis showing that Cyt1Aa binds Cry2Aa *in vitro*.

Purified crystal proteins were fractionated on a 10% polyacrylamide gel by sodium dodecylsulfate polyacrylamide gel electrophesis (**A**), electroblotted, and probed with biotin-labeled Cyt1Aa (**B**).

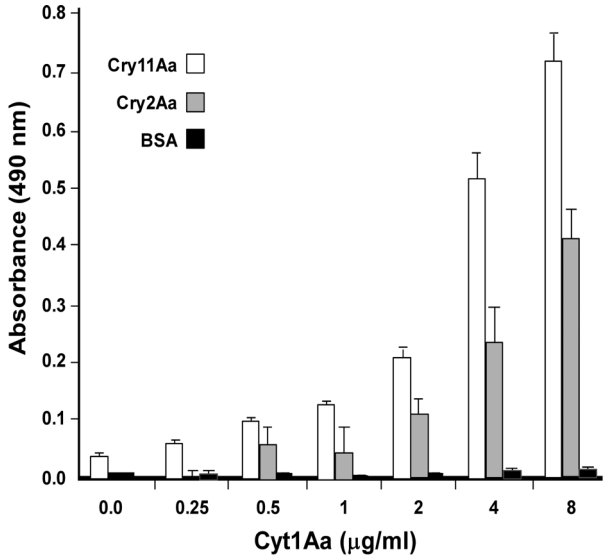

Fig. 3. Enzyme-linked immunosorbent assay showing that Cyt1Aa binds to Cry2Aa.

Cry11Aa was used as the positive control, as it is known that Cyt1Aa binds this protein with high affinity, whereas bovine serum albumin (BSA), which has no homology with Cyt and Cry proteins, was used a negative control.

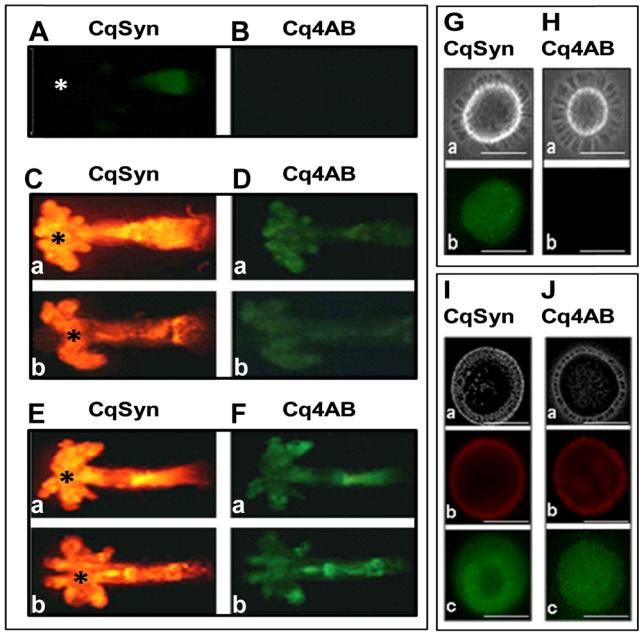

Fig. 4. Binding of fluorescent-labeled Cry2Aa (green) and Cyt1Aa (red) to the midgut of fourth-instar larvae and isolated midgut cells of *Culex quinquefasciatus* CqSyn and Cq4AB strains.

Larvae were fed solubilized labeled toxin for 2, 4, or 12 h before midgut dissection and imaging. Upon exposure to labeled Cry2Aa alone in CqSyn, the labeled toxin was detected in the midgut distal to the gastric caeca (asterisk) (**A**). Fluorescence was not observed in Cq4AB that was selected for resistance to Cry4Aa and Cry4Ba (**B**). When these strains were fed both labeled Cyt1Aa and labeled Cry2Aa (1:1), Cyt1Aa fluorescence was distributed in the gastric caecae and along the midgut in CqSyn (**C**) and Cq4AB (**E**). Labeled Cry2Aa localized in areas of the digestive tract where Cyt1Aa bound in both CqSyn (**D**) and Cq4AB (**F**). Two (**a**, **b**) independent samples are shown for each assay. (**G, H**) Examples showing *in vivo* interaction of fluorescent-labeled Cry2Aa with isolated midgut cells from fourth-instar CqSyn and Cq4AB**.** Larvae were fed solubilized labeled Cry2Aa for 2 h (shown here), 4, or 12 h before dissection of the midgut and separation of individual midgut cells. Similar results were observed in all samplings. Microscopy, phase contrast (**a**) and fluorescence emitted from labeled Cry2Aa (**b**); magnification, 640×; bar, 50 μm. (**I, J**) Examples showing *in vivo* interaction of fluorescent-labeled Cyt1Aa and Cry2Aa with isolated midgut cells from fourth-instar CqSyn and Cq4AB; larvae were fed solubilized labeled Cyt1Aa and Cry2Aa for 2 h (shown here), 4, or 12 h before dissection of the midgut and separation of individual midgut cells. Similar results were observed in all samplings. Microscopy, phase contrast (**a**) and fluorescence emission from labeled Cyt1Aa (**b**) and Cry2Aa (**c**); magnification, 640×; bar, 50 μm.

with Cyt1Aa, was observed along the entire midgut epithelium (*i.e.*, in the gastric caeca, anterior stomach, and posterior stomach), not only in CqSyn but, importantly, also in Cq4AB (Figs. 4C-4F).

At the cellular level, isolated midgut cells of CqSyn larvae showed binding of the labeled Cry2Aa toxin (Fig. 4G). We were unable to conclude whether the toxin penetrated the plasma membrane to translocate into the cytoplasm by the experimental protocol used in the study. No detectable fluorescence from labeled Cry2Aa was observed in midgut cells isolated from the Cq4AB strain (Fig. 4H). In contrast, labeled Cyt1Aa bound to epithelial cells of both CqSyn and Cq4AB (Figs. 4I and 4J), apparently primarily on the peripheral (microvillar) membrane of these cells. Moreover, binding of Cyt1Aa facilitated binding of labeled Cry2Aa to Cq4AB cells (Figs. 4Ic and 4Jc). Interestingly, the appearance of the fluorescence pattern emitted by labeled Cry2Aa on Cq4AB cells was markedly different. Whereas with the CqSyn cells, the green fluorescence of Cry2Aa was generally uniform in distribution, a speckled pattern of labeled Cry2Aa was observed with Cq4AB cells (Figs. 4I and 4J).

Bioassays

The results of the studies described above suggested that the physical interactions between Cyt1Aa and Cry2Aa could enhance their toxicity to mosquito larvae. In bioassays using spore/crystal mixtures, the recombinant strain that

Table 1. Toxicity of Cry2Aa and Cyt1Aa alone and in combination[a] to sensitive and resistant colonies of *Culex quinquefasciatus*.

Toxins	Colony[b,c]	LC_{50} (FL^{d}) (μg/ml)	LC_{95} (FL^{d}) (μg/ml)	Enhancement
Cry2A	CqSyn	> 200 μg/ml[e]	ND[g]	
	Cq4AB	> 200 μg/ml[f]	ND[g]	
Cyt1A	CqSyn	31.3 (26–37)	256 (187–387)	
	Cq4AB	19.6 (17–23)	170 (129–245)	
Cry2A + Cyt1A	CqSyn	15.2 (13–18)	127 (98–172)	2-fold ($LC_{50, 95}$)
(1:1 ratio)	Cq4AB	20.2 (14–29)	132 (67–266)	Minor

[a]Toxins were added in a 1:1 mass ratio based on dried spore/crystal mixtures.

[b]CqSyn, sensitive strain.

[c]Cq4AB, resistant strain.

[d]FL, Fiducial Limits.

[e]Only 2% average mortality at 200 μg/ml.

[f]Approximately 12.5% mortality at 200 μg/ml.

[g]Could not be determined.

produced Cry2Aa or Cyt1Aa alone showed very low toxicity to CqSyn and Cq4AB larvae (Table 1). The toxicity of 4Q7/pDBF69 strain producing Cry2Aa was especially low, essentially non-toxic, yielding only 2% mortality at a spore/crystal concentration of 200 μg/ml for the wild-type CqSyn strain and only 12.5% mortality for the Cq4AB resistant mosquito strain, making it impossible to calculate accurate LC_{50} and LC_{95} values. The toxicity of the recombinant strain producing Cyt1Aa was also low, but much better than the Cry2Aa strain, with LC_{50} values of 31.3 μg/ml for CqSyn and 19.6 μg/ml for Cq4AB (Table 1). Data for the 1:1 combination of the Cry2Aa and Cyt1Aa strains showed a statistically significant 2-fold enhancement of toxicity when the LC_{50} (15.2 μg/ml versus 31.2) and LC_{95} (127 versus 256 μg/ml) values were compared for the CqSyn mosquito strain. No significant enhancement was observed against the Cq4AB resistant mosquito strain.

Discussion

The present study provides evidence that Cyt1Aa can interact directly with Cry2Aa, and a combination of the two toxins enhances their combined activity against the CqSyn strain of *C. quinquefasciatus*. Although we do not have a clear explanation for the observed enhancement in toxicity, the structural characteristics of Cyt1Aa and its synergistic interaction with Cry11Aa [25] could be informative in identifying possible mechanisms related to this activity. Cyt toxins comprise two highly related groups, Cyt1 and Cyt2, which are primarily found in dipteran-specific strains of *B. thuringiensis* [3, 12] and contain a single α-β domain consisting of two outer layers of α-helical hairpins that surround a β-sheet [17, 18]. The synergistic activity of Cyt1Aa is thought to result from its binding to the midgut microvilli and subsequent interactions with Cry proteins [25, 36]. Perez *et al*. [25] identified motifs that could facilitate interactions between Cry11Aa and Cyt1Aa. Based on ligand binding assays, these authors demonstrated that peptides corresponding to loop β6-αE and part of β7 of Cyt1Aa bind to Cry11Aa. Complementary binding to Cyt1Aa involved domain II-loop α-8 and β-4 of Cry11Aa. As molecular modeling of Cry11Aa showed the highest level of structural similarity with Cry2Aa, although these sequences show significant divergence [8], it is possible that corresponding secondary structures mediate interaction(s) between Cyt1Aa and Cry2Aa.

Although it is tempting to speculate that the 2-fold increase in toxicity against CqSyn (Table 1), shown here to contain putative "receptor(s)" for Cry2Aa in an area posterior to the gastric caeca (Fig. 4A), is a result of direct interaction(s) between Cry2Aa and Cyt1Aa, similar to that proposed for Cry11Aa and Cyt1Aa [25], other viable alternatives must be considered. A second possibility is that the interaction is indirect, where perturbation of the midgut membrane by the lipophilic Cyt1Aa facilitates enhanced binding of Cry2Aa to its putative receptor(s); that is, affinity of Cyt1Aa for the midgut microvillar membrane enhances the exposure of the Cry2Aa receptor(s), thereby increasing its affinity for its toxin ligand. Third, it is also possible that the binding of Cry2Aa to midgut receptor(s) in CqSyn enhances the activity of Cyt1Aa, an enhancement not observed in Cq4AB, shown here to lack putative receptor(s) for Cry2Aa (Fig. 4). Fourth, it is also possible that the presence of independent Cry2Aa and Cyt1Aa

lesions in the midgut microvillar of CqSyn produced an additive lethal effect well below the threshold of the level required for these toxins when used independently. Finally, a combination of all of these possible mechanisms should also be considered as a viable explanation. However, our observation that Cyt1Aa binds Cry2Aa, as determined *in vitro* by ligand blot and ELISA analyses (Figs. 2 and 3), and the apparent *in vivo* co-distribution of the differentially labeled toxins (Fig. 4) suggest that the enhancement in toxicity in CqSyn could be a result of direct interactions between these toxins, where Cyt1Aa functions as a surrogate receptor for Cry2Aa. This conclusion is further supported by data showing Cry2Aa's binding to Cq4AB only when provided in combination with Cyt1Aa (Fig. 4J).

The lack of binding of Cry2Aa on the midgut microvilli of Cq4AB that was selected for high-level resistance to Cry4Aa and Cry4Ba toxins of *B. thuringiensis* subsp. *israelensis* suggests that these three toxins could share identical or similar receptor(s), or that the binding sites on different putative receptors are topologically similar. It remains to be determined whether this is the case, but if so, the utilization of a common receptor or different receptors that contain topologically similar ligand-binding sites would not be surprising, as Cry toxins share common structural characteristics. Cry proteins show considerable variations in their amino acid sequences, but are nevertheless remarkably similar in their predicted three-dimensional structure. All of the Cry proteins have a highly conserved similar three-domain (I, II, and III) structure, including the dipteran-lepidopteran specific Cry2Aa that shares only ~20-23% identity with the other Cry proteins [29]. Among the three domains, domain II is the most variable, particularly in length and conformation in its apex loops and β-strands, especially in Cry2Aa and Cry4Ba, which are extreme examples. As such, it is thought that domain II is a major determinant of toxin specificity and, by extension, target host range [34, 22]. Putative natural receptors for Cry toxin ligands could include aminopeptidase N, cadherins, and alkaline phosphatases [29]. Whether these are utilized by Cry2Aa in mosquito larvae remains to be resolved.

With respect to improvement in toxicity, although the interaction between Cyt1Aa and Cry2Aa enhanced the potency by only 2-fold, the inclusion of the latter in recombinant mosquitocidal strains, such as *Bacillus thuringiensis* subsp. *israelensis* or the PG14 isolate of *B. thuringiensis* subsp. *morrisoni*, with combinations of Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa toxins [7] could potentially produce a higher additive lethal effect against other susceptible mosquito larvae. Because Cry2Aa is capable of interacting with at least two entomotoxins, Cyt1Aa and the Cry4Ba-type toxin of *Bacillus thuringiensis* BUPM97, the latter which results in a comparable level of activity improvement of 2-fold toward *Cx. pipiens* [40], and Cry toxins are known to interact synergistically with one another as well as Cyt1Aa [36], it would not be surprising if the addition of Cry2Aa contributes to significant improvements in the larvicidal activity of recombinant strains. It is expected that the ratio of protoxin to spore yield in such recombinants would be higher, in contrast to when different strains producing toxins are combined. Regardless, further toxicologic studies against a wider variety of mosquito species and strains are required to clarify whether the production of a complement of diverse protoxins, including Cry2Aa, Cry4Ba, and Cyt1Aa, synthesized in a single mosquito larvicidal bacterial host produces the desired enhancement or synergistic interactions required for applied use.

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