

# An Intramolecular Salt Bridge in *Bacillus thuringiensis* Cry4Ba Toxin Is Involved in the Stability of Helix $\alpha$ -3, Which Is Needed for Oligomerization and Insecticidal Activity

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**ABSTRACT** *Bacillus thuringiensis* three-domain Cry toxins kill insects by forming pores in the apical membrane of larval midgut cells. Oligomerization of the toxin is an important step for pore formation. Domain I helix  $\alpha$ -3 participates in toxin oligomerization. Here we identify an intramolecular salt bridge within helix  $\alpha$ -3 of Cry4Ba (D111-K115) that is conserved in many members of the family of three-domain Cry toxins. Single point mutations such as D111K or K115D resulted in proteins severely affected in toxicity. These mutants were also altered in oligomerization, and the mutant K115D was more sensitive to protease digestion. The double point mutant with reversed charges, D111K-K115D, recovered both oligomerization and toxicity, suggesting that this salt bridge is highly important for conservation of the structure of helix  $\alpha$ -3 and necessary to promote the correct oligomerization of the toxin.

**IMPORTANCE** Domain I has been shown to be involved in oligomerization through helix  $\alpha$ -3 in different Cry toxins, and mutations affecting oligomerization also elicit changes in toxicity. The three-dimensional structure of the Cry4Ba toxin reveals an intramolecular salt bridge in helix  $\alpha$ -3 of domain I. Mutations that disrupt this salt bridge resulted in changes in Cry4Ba oligomerization and toxicity, while a double point reciprocal mutation that restored the salt bridge resulted in recovery of toxin oligomerization and toxicity. These data highlight the role of oligomer formation as a key step in Cry4Ba toxicity.

**KEYWORDS** *Bacillus thuringiensis*, Cry toxins, intramolecular salt bridge

The Cry toxins produced by *Bacillus thuringiensis* (Bt) bacteria are toxic to diverse insect species and nematodes. These proteins have been used to control insect pests in agriculture and in the control of dipteran insects that are vectors of human diseases (1, 2). Specifically, the proteins produced by Bt serovar israelensis (*B. thuringiensis* subsp. *israelensis*) have been used worldwide to control different mosquito species, such as *Aedes aegypti*, *Anopheles* spp., and *Culex* spp. *B. thuringiensis* subsp. *israelensis* produces two different types of toxins: the cytolytic toxin Cyt1Aa and the three-domain Cry toxins (3d-Cry), such as Cry4Aa, Cry4Ba, and Cry11Aa. The Cyt toxins are part of a small family of proteins that are present in Bt strains that kill mosquitoes. These are pore-forming toxins that are able to synergize the toxicity of some Cry toxins (3). The 3d-Cry toxin family is the largest family of proteins produced by Bt, with more than 200 members showing high specificity against different insect orders (see *Bacillus thuringiensis* Toxin Nomenclature, <http://www.btnomenclature.info/>). The three-dimensional structures of several 3d-Cry proteins show similar folds, suggesting similar modes of action of these proteins (4–10). Domain I is a seven- $\alpha$ -helix bundle

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involved in pore formation and oligomerization, while domains II and III are mainly composed of  $\beta$ -sheets and are involved in recognition of toxin receptors. In the case of domain I, it was shown that helix  $\alpha$ -3 is important for toxin oligomerization since single point mutations in this helix affected toxin oligomerization and toxicity of Cry1Ab and Cry11Aa toxins (11–13).

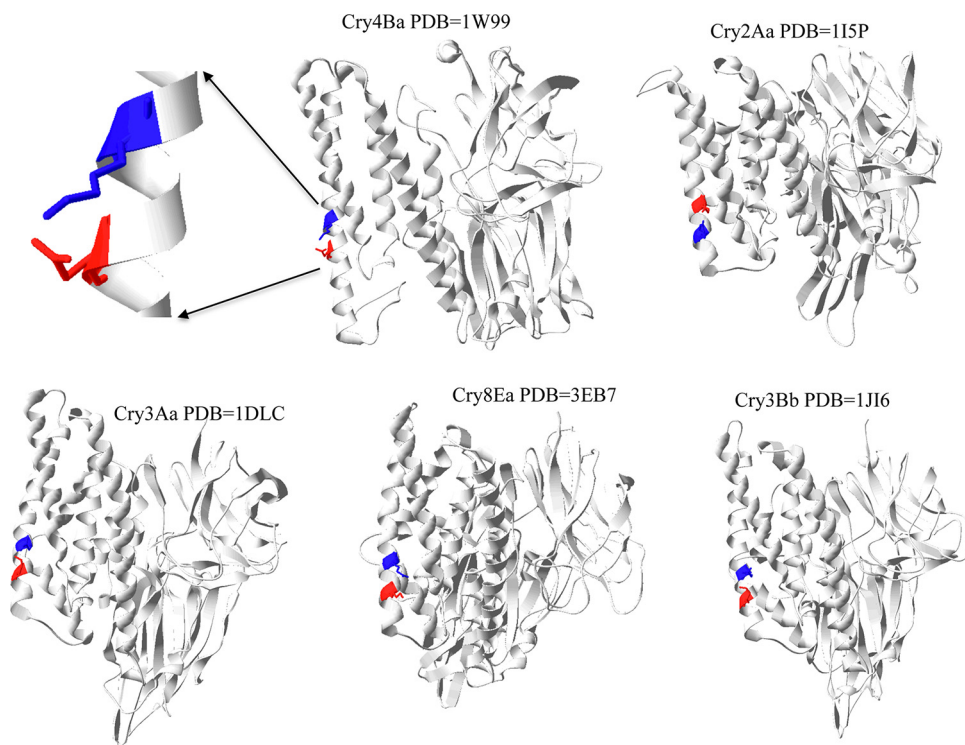
The 3d-Cry toxins form pores in insect gut cells, leading to osmotic shock, cell burst, and death of the larvae. Pore formation is a complex mechanism that involves several steps, resulting in toxin oligomerization and membrane insertion of the toxin to form a pore. Different insect proteins have been identified as Cry toxin receptors, such as aminopeptidase (APN) (14, 15), alkaline phosphatase (ALP) (15, 16), and cadherin-like (CAD) receptor (17). The CAD receptor is a key component, since binding to this receptor is a high-affinity interaction and induces toxin oligomerization (18). The binding of 3d-Cry toxins to CAD involves three epitopes in CAD that interact with three exposed loops on the toxin, located in domain II. This complex interaction promotes the proteolytic cleavage of the N-terminal end, including helix  $\alpha$ -1 of domain I (19, 20). It was proposed that this cleavage exposes buried hydrophobic regions of domain I and induces the formation of an oligomeric structure of the toxin (19). However, it was shown that Cry4Ba is a special protein since it is able to oligomerize *in vitro* after activation with proteases in the absence of the CAD molecule, in contrast with other proteins, such as Cry11Aa, that require binding to *A. aegypti* CAD to oligomerize (21). It was also shown that Cry4Ba binds to CAD with lower affinity ( $K_d$  [dissociation constant] = 154 nM) than does Cry11Aa, which has much higher affinity for CAD ( $K_d$  = 17 nM) (21). In addition, it was shown that Cry4Ba does not compete with the binding of Cry11Aa to the CAD protein from *A. aegypti* (22).

The  $\alpha$ -helices found in proteins are frequently stabilized by electrostatic interactions; this is achieved by pattern repeats with alternating charged residues positioned at a helix face to form salt bridges between side chains (23, 24). In this work, we identify a putative intramolecular salt bridge in Cry4Ba helix  $\alpha$ -3. Helix  $\alpha$ -3 has a role in toxin oligomerization and toxicity of this protein (11–13). Single point mutations in this putative salt bridge affected toxicity and oligomerization, while a double point mutant with reversed charges recovered both the capacity to oligomerize and toxicity against mosquito larvae, supporting the hypothesis that these two residues are involved in formation of a salt bridge.

## RESULTS

**Construction of single and double point mutants.** Inspection of the Cry4Ba three-dimensional structure (8) revealed the presence of a putative intermolecular salt bridge (D111-K115) located in helix  $\alpha$ -3 (Fig. 1). To analyze the role of this putative salt bridge in Cry4Ba toxicity and oligomerization, single point mutants were constructed: namely, aspartate 111 was changed to lysine (D111K) and lysine 115 was changed to aspartate (K115D). In addition, a double point mutant with reversed charges (D111K-K115D) was also constructed. The wild-type and mutant proteins were produced in Bt transformant strains, and parasporal crystals were purified and solubilized by suspension at an alkaline pH. Figure 2 shows the Coomassie blue staining of Cry4Ba proteins after boiling of samples for 5 min and separation by SDS-PAGE. This figure shows that all mutant proteins were produced as 130-kDa protoxins, although the amount of the K115D mutant protein was lower than that of the other proteins (Fig. 2A). After alkaline solubilization of crystals and 5 min of boiling, the protoxins were analyzed by SDS-PAGE. The K115D mutant protein demonstrated a lower concentration of solubilized protoxin (Fig. 2B) and was also shown to be highly sensitive to protease treatment during its activation with chymotrypsin (Fig. 2C), indicating that this substitution severely affected protein stability and led to protein degradation.

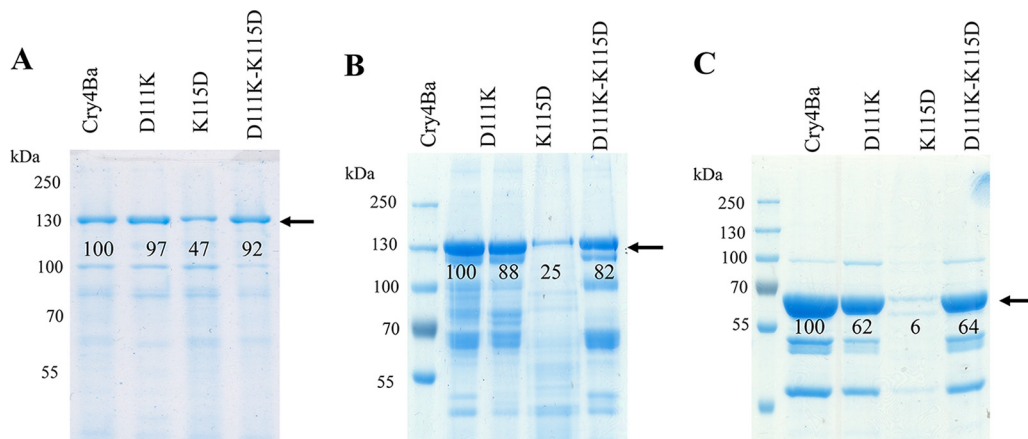
**Effects of single and double point mutations of Cry4Ba.** Bioassays were conducted with *A. aegypti* fourth-instar larvae. Table 1 shows that the single point mutants D111K and K115D were greatly affected in their toxicity against *A. aegypti* since no mortality of the larvae was observed even when exposed at the highest toxin concen-



**FIG 1** Structures of monomeric 3d-Cry toxins showing the putative salt bridge in domain I helix  $\alpha$ -3. Basic and acid residues are labeled in blue and red colors, respectively.

tration (10,000 ng/ml), in contrast to the wild-type Cry4Ba, which exhibited a 50% lethal concentration ( $LC_{50}$ ) of 455 ng/ml. The double point mutant D111K-K115D recovered full toxicity since the confidence limits of the  $LC_{50}$ s overlap the  $LC_{50}$ s of the wild-type toxin.

To determine if these mutations affected oligomer formation, the mutant protoxins were incubated with 2.5% chymotrypsin for 30 min and then samples were heated for 3 min at 50°C before being loaded in a gel for SDS-PAGE (Fig. 3). This lower heating temperature was necessary in order to avoid the disassembly of the oligomeric



**FIG 2** SDS-PAGE analysis of the Cry4Ba wild-type and mutant proteins produced in *B. thuringiensis*. (A) Spore/crystal suspension directly loaded in the SDS-PAGE gel, (B) Cry4Ba and mutant protoxins after solubilization in alkaline buffer, and (C) Cry4Ba and mutants after chymotrypsin activation for 30 min at 37°C. All samples were boiled for 5 min before being loaded into the SDS-PAGE gel, and the gel was stained with Coomassie brilliant blue. The arrows point to protoxins with a size of 130 kDa or to the activated 60-kDa toxins; the optical density of the bands was measured by using the ImageJ program (<http://imagej.nih.gov/ij/>).

**TABLE 1** Toxicity assays of crystal/spore suspension of the different Bt strains against 4th-instar larvae of *Aedes aegypti*

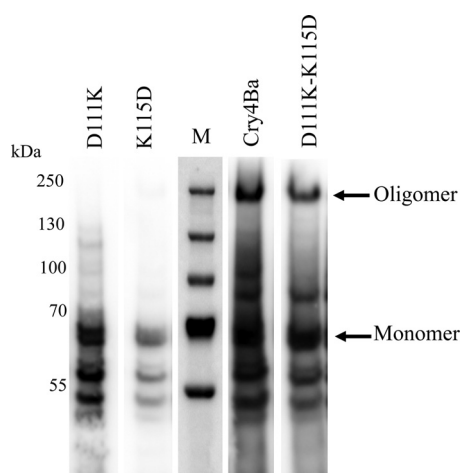
Toxin	LC <sub>50</sub> in ng/ml (confidence limits)
Cry4Ba	455 (75–702)
D111K	>10,000
K115D	>10,000
D111K-K115D	865 (546–1,126)

structure, since it was previously shown that Cry oligomers are resistant to SDS but disassemble after boiling (18); that is the reason why no oligomers were observed in Fig. 2C, where samples were boiled. The Cry4Ba oligomers were revealed by Western blotting with an anti-Cry4Ba polyclonal antibody. Figure 3 shows that both D111K and K115D mutants were severely affected in oligomer formation, in contrast to the double point mutant D111K-K115D protein, which produced an oligomeric structure of 250 kDa similar to the wild-type protein. Figure 3 also shows that the K115D mutant was more susceptible to degradation with chymotrypsin, since a lower concentration of the protein was observed after proteolysis.

## DISCUSSION

Electrostatic interactions are of fundamental importance in protein structure and stabilization (23–25). It has been proposed that salt bridges within the same  $\alpha$ -helix may have a stabilizing role (23–25). One useful strategy to demonstrate that two charged residues may be forming a salt bridge is to make single point mutations that affect the protein activity and a double point mutation with reversed charges that restores such activity. Several examples showing the participation of salt bridges by analysis of single point mutations and restoration of protein function by reversing the charges of the putative salt bridges have been previously described (26–29).

In this work, we identify a putative salt bridge that is present within helix  $\alpha$ -3 of the Cry4Ba toxin. In order to analyze the role of these charged residues of helix  $\alpha$ -3, we analyzed the effects of single point mutations that would destroy the salt bridge and compared the results with those for a double point reciprocal mutation that potentially may restore the salt bridge formation. Here we show that mutations in residues D111K and K115D of domain I helix  $\alpha$ -3 result in proteins severely affected in toxicity and toxin oligomerization. The mutation K115D resulted in an unstable protein, since this point mutation results in a smaller amount of total protoxin produced and increased sus-



**FIG 3** *In vitro* oligomer formation of Cry4Ba toxin and mutants. Oligomers were formed, and the samples were analyzed by Western blotting. Mass (M) markers of 250 kDa and 70 kDa are labeled with arrows. All samples were heated for 3 min at 50°C before loading into the SDS-PAGE gel. The proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes and analyzed by Western blot assays using polyclonal anti-Cry4Ba antibody.

ceptibility to degradation with chymotrypsin treatment. However, our data indicate that the double mutant D111K-K115D exhibits oligomer formation and toxicity comparable to the wild-type toxin, indicating that the D111-K115 electrostatic interaction has a fundamental role in maintaining the helix  $\alpha$ -3 structure important for proper toxin oligomerization. It was proposed that helix  $\alpha$ -3 plays an important role in oligomerization and pore formation activity of 3d-Cry toxins (11–13). Analysis *in silico* of the homo-oligomerization tendencies of the individual  $\alpha$ -helices of domain I also supports the proposal that helix  $\alpha$ -3 participates in Cry toxin oligomerization (30).

Alignment of 3d-Cry toxin family sequences shows that the corresponding salt bridge is not present in all members of this family. We analyzed a total of 91 sequences, including one representative sequence of each 3d-Cry toxin type, with the subindex “a” in the third range of the nomenclature (<http://www.btnomenclature.info/>), and found that this salt bridge of helix  $\alpha$ -3 is present in 36 of 91 sequences analyzed. Besides the Cry4Ba protein, this salt bridge is also found in Cry11a, Cry1Ma, Cry2Aa, Cry3Aa, Cry3Ba, Cry3Ca, Cry7Aa, Cry7Ca, Cry7Da, Cry7Ja, Cry7Ka, Cry7La, Cry8Aa, Cry8Ba, Cry8Ca, Cry8Ea, Cry8Fa, Cry8Ga, Cry8Ha, Cry9Da, Cry17Aa, Cry18Aa, Cry18Ba, Cry18Ca, Cry26Aa, Cry29Aa, Cry32Da, Cry32Ea, Cry41Aa, Cry43Aa, Cry47Aa, Cry53Aa, Cry56Aa, Cry62Aa, and Cry68Aa toxins (see Fig. S1 in the supplemental material). In addition, the corresponding negative and positive residues of this putative salt bridge were localized in the three-dimensional structures of Cry2Aa, Cry3Aa, Cry3Bb, and Cry8Ea at a position in helix  $\alpha$ -3 similar to that of Cry4Ba (Fig. 1).

It was proposed that all proteins of the 3d-Cry family shared a similar mechanism of action that involves the toxin oligomerization necessary for formation of the ionic pore in the target cell. However, the oligomerization step has been studied in a limited number of toxins (Cry1, Cry3A, Cry4, and Cry11). It was described that the CAD receptor plays an important role in inducing oligomerization of Cry1A toxins (18, 31, 32). CAD also induced oligomerization of Cry11Aa (33) and Cry3Aa (34). The requirement of receptors for inducing oligomerization has not been analyzed for the rest of the 3d-Cry toxins. Cry4Ba is a special toxin since this is the only toxin that has been shown to be able to form oligomers *in vitro* without interaction with the CAD receptor (21, 22). Some studies have analyzed the oligomeric structures formed by Cry4B in the presence of synthetic lipids but in the absence of CAD or any other toxin receptor. Atomic force microscopy studies showed that Cry4Ba preferentially inserts into the membrane as a self-assembled structure with a 4-fold symmetry (35), while negative-stain electron microscopy of two-dimensional (2D) crystals of Cry4Ba suggested a trimeric array (36). In addition, it was reported that the silencing of the CAD gene in *A. aegypti* by double-stranded RNA feeding (22) or in transgenic mosquitoes (33) did not affect Cry4Ba toxicity, supporting the theory that CAD protein is not necessary to induce oligomerization of Cry4Ba. In contrast, the same CAD-silenced mosquitoes showed reduced susceptibility to Cry11Aa, supporting the hypothesis that Cry11Aa relies on the CAD interaction for its oligomerization and thus was less toxic to CAD-silenced larvae (22) or to the transgenic mosquitoes with reduced CAD expression (33). However, different molecules (such as APN and ALP) are still involved in Cry4Ba binding and are necessary for Cry4Ba toxicity, explaining the high specificity of Cry4Ba toxin to *A. aegypti* larvae (37).

We do not know exactly why Cry4Ba toxin is able to oligomerize in the absence of receptors, nor do we know whether other Cry proteins show similar behavior. It is interesting that in the crystallization studies of this protein, it was reported that during the chymotrypsin activation and crystallization process the N-terminal region of the Cry4Ba protein was proteolyzed, resulting in the loss of  $\alpha$ -helices 1 and 2 in the final structure that showed a trimeric array (8). This cleavage was confirmed by mass spectroscopy after dissolving the three-dimensional (3D) crystals; the cleaved protein was fully active against mosquito larvae (8). One possible explanation could be that during activation with chymotrypsin this toxin is truncated and helices  $\alpha$ -1 and  $\alpha$ -2a are cleaved out, resulting in a protein that is ready to oligomerize.

Our data indicate that the salt bridge identified is important to stabilize helix  $\alpha$ -3 of



Cry4Ba. It is an intramolecular salt bridge formed within the same helix  $\alpha$ -3 and thus is most probably not involved in the interaction of different monomers of toxin during oligomerization. However, we cannot conclude that the presence of this salt bridge is a requirement for oligomerization in the absence of receptors. Cry3Aa also has this salt bridge within helix  $\alpha$ -3, and this protein requires the interaction with CAD for its oligomerization (34). It will be interesting to analyze the oligomerization of other 3d-Cry toxins that also contain this salt bridge and determine if they can form oligomers *in vitro* in the absence of receptor interaction.

Overall, our results show that a salt bridge within helix  $\alpha$ -3 of Cry4Ba stabilizes the structure of this helix and is necessary for Cry4B insecticidal activity. These data are consistent with previous work that highlighted the importance of helix  $\alpha$ -3 in the oligomerization and pore formation activity of these important biotechnological proteins (11–13, 30–34).

## MATERIALS AND METHODS

**Site-directed mutagenesis.** Plasmid pHT315-*cry4Ba*, containing the wild-type *cry4Ba* gene, was used as the template to construct D111K, K115D, and D11K-K115 mutants by site-directed mutagenesis. Mutagenesis was performed using the Quick-Change mutagenesis kit from Stratagene (La Jolla, CA) following the manufacturer's instructions. The mutagenic oligonucleotides were as follows: for D111K, 5' GTA ACA GCT TAT GTA CGA ACA AAA GCA AAT GCA AAA ATG ACG G 3'; for K115D, 5' GTA CGA ACA GAT GCA AAT GCA GAT ATG ACG GTT GTG AAA GAT TA 3'; and for D111K/K115D, 5' GTA ACA GCT TAT GTA CGA ACA AAA GCA AAT GCA GAT ATG ACG GTT GTG AAA GAT TA 3'.

After mutagenesis, plasmids were transformed into *Escherichia coli* DH5 $\alpha$  cells. Point mutations were confirmed by automated DNA sequencing at Instituto de Biotecnología-Universidad Nacional Autónoma de México (UNAM) facilities. Plasmids were transformed into the acrySTALLIFEROUS *B. thuringiensis* strain 407 as reported elsewhere (38). Transformant strains were selected in Luria broth at 30°C supplemented with erythromycin (10  $\mu$ g ml<sup>-1</sup>), and single-colony lysates were used to amplify the *cry4Ba* gene by PCR and confirm the mutation expressed in Bt strain 407 by DNA sequencing.

**Purification of Cry4Ba protoxins and activation with chymotrypsin.** Nutrient broth sporulation medium, as reported by Schaeffer et al. (39), supplemented with erythromycin (10  $\mu$ g ml<sup>-1</sup>) was used for the expression of Cry4Ba wild-type or mutant proteins. After 3 days at 30°C, the sporulation process was complete and spores and crystals were harvested by centrifugation at 12,857  $\times$  g for 10 min at 10°C and washed twice with a mixture of 300 mM NaCl and 10 mM EDTA. The crystal inclusions were purified by using discontinuous sucrose gradients (40). The crystals were solubilized by suspension in alkaline buffer (50 mM Na<sub>2</sub>CO<sub>3</sub>, 0.2%  $\beta$ -mercaptoethanol, pH 10.5) for 2 h, and insoluble material was removed by centrifugation for 20 min at 15,000  $\times$  g. The pH of the protoxin solution was lowered to pH 8.5 by adding 1:4 (wt/wt) 1 M Tris buffer at pH 8.5, and the concentration of protein was determined by the Bradford assay using bovine serum albumin as a standard. For protoxin activation, a sample of 50  $\mu$ g of soluble protoxin was incubated with 2.5  $\mu$ g of chymotrypsin for 30 min at 37°C. The samples of spore/crystal suspension, soluble protoxin, and activated toxins were boiled for 5 min and analyzed by SDS-PAGE using Coomassie blue stain.

For oligomerization assays, 2.5  $\mu$ g of crystal suspension was incubated in alkaline buffer, as described above, with 2.5% chymotrypsin for 30 min at 37°C. Phenylmethylsulfonyl fluoride (PMSF) (1 mM final concentration) was added to stop proteolysis. The samples were heated for 3 min at 50°C before being loaded in SDS-PAGE gels, electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA), and analyzed by Western blot assays as described below.

**Western blot assays.** PVDF membranes were blocked for 1 h at room temperature with 5% skimmed milk in phosphate-buffered saline (PBS) buffer (pH 7.4) plus 0.1% Tween 20. The membranes were rinsed once with the same buffer. The Cry4Ba oligomeric or monomeric structures were detected after 1 h of incubation with polyclonal anti-Cry4Ba (diluted 1/30,000) and then 1 h of incubation with goat anti-rabbit secondary antibody coupled to horseradish peroxidase (Santa Cruz) (diluted 1/20,000), followed by incubation with SuperSignal West Pico chemiluminescent substrate (Pierce), according to the instructions of the manufacturer.

**Toxicity assays against *Aedes aegypti* larvae.** Bioassays were performed with *A. aegypti* 4th-instar larvae using five different doses of spore/crystal suspensions (from 0 to 10,000 ng/ml) directly applied to 100 ml dechlorinated H<sub>2</sub>O. We used 10 larvae per toxin concentration in triplicate. Positive (*B. thuringiensis* subsp. *israelensis*) and negative (dechlorinated water) controls were included in the bioassay, and mortality of the larvae was examined after 24 h of treatment. The 50% lethal concentration (LC<sub>50</sub>) was calculated with Probit (PoloPlus, LeOra Software, Berkeley, CA). Three repetitions of these bioassays were performed.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01515-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

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