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Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* Enhances Mosquitocidal Activity of *B. thuringiensis* subsp. *kurstaki* HD-1 Against *Aedes aegypti* but not *Culex quinquefasciatus*

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The Cyt1Aa protein of *Bacillus thuringiensis* subsp. *israelensis* is known to synergize mosquitocidal proteins of *B. thuringiensis* and *Bacillus sphaericus* strains. Cyt1Aa is highly lipophilic, and after binding *in vivo* to the midgut microvillar membrane serves as a "receptor" for mosquitocidal Cry proteins, which subsequently form cation channels that kill mosquito larvae. Here we report that Cyt1Aa can serve a similar function for lepidopteran-specific Cry proteins of *B. thuringiensis* in certain mosquito larvae. Engineering Cyt1Aa into the HD-1 isolate of *B. thuringiensis* subsp. *kurstaki* enhanced toxicity against 4th instars of *Aedes aegypti*, but not against 4th instars of *Culex quinquefasciatus*.

Key words: Cyt1Aa, *Bacillus thuringiensis* subsp. *kurstaki* HD-1, *Aedes aegypti*, synergism

Cyt1Aa is the major mosquitocidal protein produced by the most widely used commercial bacterial mosquito larvicide, *Bacillus thuringiensis* subsp. *israelensis* [5]. Although its toxicity is low, Cyt1Aa is a potent synergist that significantly (i) increases the toxicity of mosquitocidal Cry proteins of *B. thuringiensis* [1, 17], (ii) expands the host range of the binary toxin of *Bacillus sphaericus* [14], (iii) has toxicity against lepidopteran [13] and coleopteran [4] larvae, and (iv) masks resistance in insect populations [15, 16].

Lepidopteran-specific strains of *B. thuringiensis* (Bt) typically lack Cyt1Aa as part of their crystalline inclusion complements. For example, commercial formulations of *B. thuringiensis* subsp. *kurstaki* HD-1 such as Dipel and Thuricide lacking Cyt1Aa have been used worldwide to control agricultural insect pests for more than five decades. More recently, Cry proteins of this bacterium have been the dominant type used to construct genetically engineered crops, such as Bt cotton and Bt maize [5]. The high toxicity of *B. thuringiensis* subsp. *kurstaki* HD-1 against lepidopterans is due to four major insecticidal proteins synthesized during sporulation (Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa), among which Cry1Aa, Cry1Ab, and Cry1Ac co-crystallize to form large bipyramidal crystal, whereas Cry2Aa forms a much smaller cuboidal crystal [5]. The Cry1 and Cry2Aa proteins of HD-1 are toxic to lepidopterans, but Cry2Aa is known to be toxic to lepidopterans as well as mosquito larvae, although only weakly toxic to the latter [2, 3, 9, 18].

In the present study, our primary aim was to determine whether Cyt1Aa could significantly synergize or enhance the potency of toxins produced by *B. thuringiensis* subsp. *kurstaki* HD-1 against mosquito larvae, in essence expanding or enhancing the host range of this bacterium. Therefore, we engineered the HD-1 isolate of *B. thuringiensis* subsp. *kurstaki* to produce Cyt1Aa and assayed its effect against *Aedes aegypti* and *Culex quinquefasciatus*, vectors of important parasitic diseases such as malaria and filariasis, and viral diseases including dengue and yellow fever. Our results show statistically significant enhanced toxicity of the recombinant strain against *Aedes aegypti*, but not *Culex quinquefasciatus*.

For our study, the *B. thuringiensis* subsp. *israelensis* acrystalliferous strain 4Q7 was obtained from the Institut Pasteur, Paris, France, and *B. thuringiensis* subsp. *kurstaki* HD-1 was obtained from the *Bacillus* Genetic Stock Center

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at Ohio State University, Columbus, OH, USA. These are referred to hereafter as 4Q7 and HD-1, respectively. Plasmid pWF45 [17] containing the *cyt1Aa* gene with the 20 kDa chaperone-like protein gene was introduced into 4Q7 and HD-1 strains by electroporation as described by Park *et al.* [10]. 4Q7 harboring pWF45 (4Q7/pWF45) and HD-1 harboring pWF45 (HD-1/pWF45) were selected on Brain Heart Infusion (BHI) agar supplemented with erythromycin (25 μg/ml) at 28°C.

The HD-1, 4Q7/pWF45, and HD-1/pWF45 strains were grown in NBG broth [11] with erythromycin (25 μg/ml) in a shaker incubator (250 rpm) for 5 days at 28°C, at which time >95% of cells had sporulated and lysed. Microscopic examination of *B. thuringiensis* subsp. *kurstaki* HD-1, 4Q7/pWF45, and HD-1/pWF45 showed crystalline inclusions resembling those of, respectively, Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, and Cry1Aa; Cyt1Aa; and Cry1Ab, Cry1Ac, Cry2Aa, and Cyt1Aa (Fig. 1A). The protein profiles of these strains were confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B). Spore/crystal mixtures were collected by centrifugation in a Beckman Coulter Allegra 25R centrifuge at 5,000 ×*g* at 4°C for 30 min using the TS-5.1-500 rotor. Mixtures were resuspended with double-distilled water and collected using the same centrifugation protocol. Pellets were frozen at −80°C for 2 h and lyophilized for 24 h under vacuum using the Virtronic Vacuum with refrigeration set at −60°C.

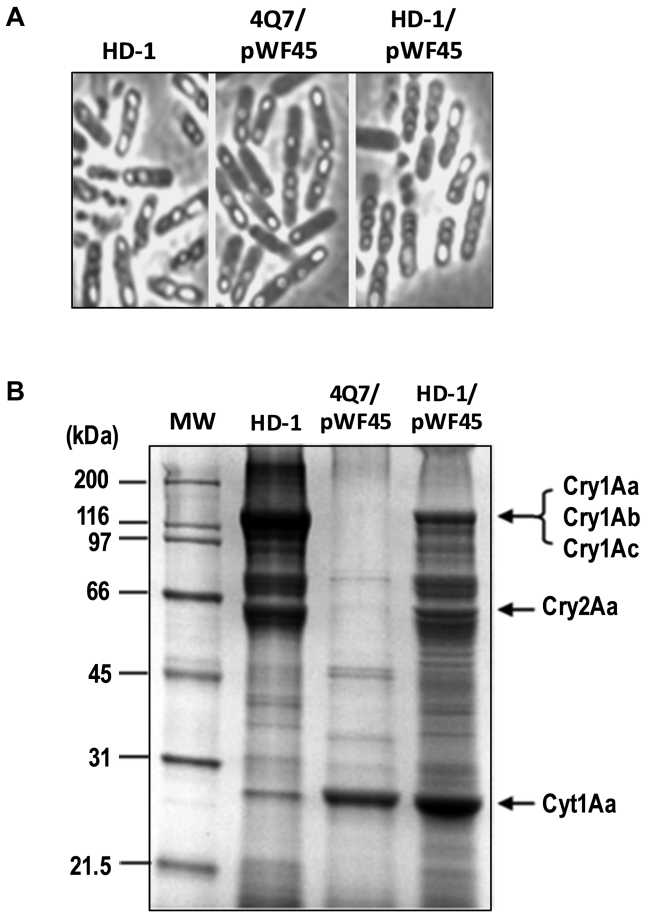

Fig. 1. (**A**) Micrographs of sporulating cells, and (**B**) SDS-PAGE protein profiles of *B. thuringiensis* subsp. *kurstaki* HD-1 (HD-1) [Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa]; *B. thuringiensis* subsp. *israelensis* 4Q7 (4Q7/pWF45), [Cyt1Aa]; and the recombinant strain of *B. thuringiensis* subsp. *kurstaki* HD-1 harboring pWF45 (HD-1/pWF45) [Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, and Cyt1Aa]. Magnification, 1,000×; kDa, kilodalton; MW, molecular mass standards.

Laboratory colonies of early 4th instars of *Ae. aegypti* and *Cx. quinquefasciatus*, kindly provided by Dr. Margaret C. Wirth (Department of Entomology, University of California, Riverside), were used in bioassays, which were replicated three times on three different days using three different lyophilized powder preparations, as described previously [10]. The data were analyzed using Probit software [7].

HD-1 lacked toxicity against *Cx. quinquefasciatus* 4th instars, even at the highest concentration of the spore/crystal mixture used. However, we were able to calculate an LC_{50} (88.6 μg/ml) for HD-1 against *Ae. aegypti* (Table 1).

Cyt1Aa is known to be only weakly toxic to mosquitoes [5], with LC_{50} values against *Ae. aegypti* and *Cx. quinquefasciatus* reported at, respectively, 1.2 μg/ml [1] and 22–26 μg/ml [15]. In the present study, the 4Q7 strain producing Cyt1Aa (4Q7/pWF45) had LC_{50} values of 551.7 μg/ml and 50.3 μg/ml against, respectively, *Cx. quinquefasciatus* and *Ae. aegypti* (Table 1). Although our results showed a similar pattern (*i.e.*, toxicity of Cyt1Aa was higher against *Ae. aegypti* than *Cx. quinquefasciatus*), the LC_{50} values were significantly higher than those reported previously. This could be due to different bioassay procedures or differences in the mosquito strains, but is most likely due to our use of 4th instars, whereas 1st and 2nd instars were used in the earlier studies.

The bioassay results using spore/crystal mixtures of the recombinant HD-1 strain (HD-1/pWF45) producing the normal complement of Cry proteins plus Cyt1Aa inclusions showed increases in toxicity (*i.e.*, decreases in LC_{50}s) against *Cx. quinquefasciatus* (285.7 μg/ml) and *Ae. aegypti* (15.2 μg/ml) when compared with those of HD-1 or 4Q7/pWF45 (Table 1). However, the fiducial limits of the value against *Cx. quinquefasciatus* overlapped with that of 4Q7/pWF45 (551.7 μg/ml), suggesting that there is no significant improvement in toxicity between 4Q7/pWF45 and HD-1/pWF45 against this species. In contrast to this, HD-1/pWF45 showed significantly enhanced toxicity against *Ae. aegypti*. Spore/crystal preparations from this strain showed a 5.8-fold and 3.3-fold enhancement in toxicity when compared with, respectively, HD-1 and 4Q7/pWF45.

Table 1. Mosquitocidal activities of wild-type and recombinant *Bacillus thuringiensis* strains.

Mosquito larvae/bacterial strain (toxin combination)	LC_{50} (fiducial limits)[a]	Slope
Culex quinquefasciatus		
HD-1 (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa)	N/T[b]	--
4Q7/pWF45 (Cyt1Aa)	551.7 (206.9–5,113.5)	0.8 ± 0.2
HD-1/pWF45 (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cyt1Aa)	285.7 (127.8–1,441.6)	0.7 ± 0.1
Aedes aegypti		
HD-1 (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa)	88.6 (58.1–164.3)	0.8 ± 0.1
4Q7/pWF45 (Cyt1Aa)	50.3 (38.1–71.2)	1.1 ± 0.1
HD-1/pWF45 (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cyt1Aa)	15.2 (8.9–26.1)	1.3 ± 0.2

[a]μg/ml, 48 h mortality.

[b]Unable to reliably calculate LC values because variable results were consistently observed at the highest concentration (200 μg/ml) of spore/crystal mixture used in bioassays.

The present study demonstrates that Cyt1Aa from *B. thuringiensis* subsp. *israelensis* enhances the mosquitocidal activity of *B. thuringiensis* subsp. *kurstaki* HD-1 against *Ae. aegypti* but not *Cx. quinquefasciatus*. However, the HD-1/pWF45 strain is markedly less toxic than *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* currently used in mosquito abatement programs. The molecular basis for the difference in toxicity between the two species is not known, but it could be due to factors related to the presence or absence of "receptors" (*i.e.*, the microvillar proteins to which the toxins bind) for the Cry proteins and differential binding of lipophilic Cyt1Aa to the midgut microvilli. In other studies, we observed that Cyt1Aa binds Cry2Aa, both *in vitro* and *in vivo* (Bideshi *et al.* unpublished data), similar to the interaction that has been reported for Cyt1Aa and Cry11Aa of *B. thuringiensis* subsp. *israelensis* [12]. Interestingly, among Cry proteins, Cry2Aa is structurally most similar to Cry11Aa [6]. Thus, the enhancement in toxicity against *Ae. aegypti* could be due to intermolecular interactions between Cyt1Aa and Cry2Aa following their proteolytic activation in the midgut. Further studies are required to determine if Cyt1Aa could function as an additional binding site for the Cry1 proteins of HD-1, and whether Cyt1Aa in the recombinant strain (HD-1/pWF53) enhances or synergizes the activity of the native toxins against lepidopteran larvae.

Finally, it is known that Cry2Aa is toxic to both lepidopteran and mosquito larvae [2, 3, 9, 18], whereas the Cry1A proteins are only toxic to lepidopteran larvae. Therefore, it is likely that the *Cx. quinquefasciatus* strain used in this study lacks receptors for Cry2Aa, whereas midgut binding sites for this protein are present in *Ae. aegypti*.

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