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Mtx toxins from *Lysinibacillus sphaericus* enhance mosquitocidal cry-toxin activity and suppress cry-resistance in *Culex quinquefasciatus*

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ABSTRACT

The interaction of Mtx toxins from *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) with *Bacillus thuringiensis* subsp. *israelensis* Cry toxins and the influence of such interactions on Cry-resistance were evaluated in susceptible and Cry-resistant *Culex quinquefasciatus* larvae. Mtx-1 and Mtx-2 were observed to be active against both susceptible and resistant mosquitoes; however varying levels of cross-resistance toward Mtx toxins were observed in the resistant mosquitoes. A 1:1 mixture of either Mtx-1 or Mtx-2 with different Cry toxins generally showed moderate synergism, but some combinations were highly toxic to resistant larvae and suppressed resistance. Toxin synergy has been demonstrated to be a powerful tool for enhancing activity and managing Cry-resistance in mosquitoes, thus Mtx toxins may be useful as components of engineered bacterial larvicides.

1. Introduction

The bacteria *Lysinibacillus sphaericus* comb. nov. (formerly *Bacillus sphaericus* Neide) (Ahmed et al., 2007) and *Bacillus thuringiensis* subsp. *israelensis* are significant components of mosquito control programs worldwide. Both species are highly specific to mosquito larvae and safe for mammals, fish, birds and nondipteran insects, (Siegel, 2001; Berry, 2012) and both can be used effectively against mosquito larvae that are resistant to other classes of insecticides (Sun et al., 1980; Liu et al., 2004; Akiner et al., 2009; Marcombe et al., 2011). However each bacterium has characteristics that can be considered disadvantageous. *L. sphaericus* lacks activity against *Aedes aegypti*, an important vector of yellow fever and dengue viruses, and carries a high risk for selecting insecticide resistance in larval mosquito populations that are repetitively treated, as it targets a single receptor class in the larval midgut, a GPI-anchored α-glucosidase (Rao et al., 1995; Silva-Filha et al., 1995; Yuan et al., 2000; Darboux et al., 2002; Nielsen-LeRoux et al., 2002; Su and Mulla, 2004). *B. t.* subsp. *israelensis* has demonstrated reduced activity in water with high organic content, a frequent larval development site for medically important species such as *Culex quinquefasciatus* Say (Lacey, 2007).

Safe and effective insecticides such as *L. sphaericus* and *B. t.* subsp. *israelensis* constitute valuable resources that require conservation, particularly because so few effective alternatives exist. One strategy to prolong the useful life of both *L. sphaericus* and *B. t.* subsp. *israelensis*, and to overcome their perceived limitations, is the use of recombinant DNA techniques to enhance their activity through the synthesis of novel combinations of endotoxins from different bacteria into a single bacterium (Federici et al., 2003). This approach can draw upon endotoxins from a variety of mosquitocidal bacterial strains for expression in *B. t.* subsp. *israelensis* or an alternative species, which can be engineered with the goal of producing microbial larvicides that circumvent these limitations. A critical step in such an endeavor is identifying mosquitocidal toxins with the potential to interact with other such toxins in order to increase toxicity, to extend the host range of the end product, and to suppress or delay the evolution of resistance.

A number of mosquitocidal toxins from different strains of *L. sphaericus* and *B. thuringiensis* have been investigated, and toxins with beneficial characteristics have been identified with potential for use in this strategy. Included among these toxins are the Cyt toxins and Cry toxins (Cyt1Aa, Cyt2A, Cry11Ba, Cry19A and others) from various mosquitocidal strains of *B. thuringiensis,* and the Cry48/Cry49 toxins and Mtx toxins from *L. sphaericus.* Many of these toxins have been evaluated for their mosquitocidal activity (Sun et al., 1980; Chang et al., 1992; Wu et al., 1994; Crickmore et al., 1995; Kawalek et al., 1995; Poncet et al., 1995; Sirichatpakorn et al., 2001; Promdomkoy et al., 2005; Jones et al., 2007) their cross-resistance spectra, and their toxin interactions (Wirth and Georghiou, 1997; Wirth et al., 1998, 2001, 2004, 2007; Jones et al., 2008). Of particular interest are toxins that interact synergistically with the major toxins from both *L. sphaericus* and *B. t.* subsp.

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israelensis, as such synergy greatly increases the number of potential toxin combinations with the characteristics necessary to delay and suppress resistance and the potential to extend host range (Wirth et al., 1997, 2000a; Wirth et al., 2000b; Park et al., 2005). Our earlier investigation of Mtx toxins revealed that both Mtx-1 and Mtx-2 interacted synergistically and suppressed resistance when combined with *L. sphaericus* as well as with Cry11Aa from *B. t.* subsp. *israelensis* (Wirth et al., 2007). Recently we extended this study and tested a wider spectrum of *B. thuringiensis*-Cry toxins to determine whether Mtx-1 and Mtx-2 also interact with Cry4Aa, Cry4Ba, and combinations of Cry toxins from *B. t. israelensis*, and consequently have greater potential utility in engineered bacterial strains. Here we show that Mtx-1 and Mtx-2 interact with these Cry toxins, that such interactions are predominantly synergistic, though variable, and that synergy in these combinations suppressed Cry-resistance in Cry-resistant *C. quinquefasciatus*.

2. Materials and methods

2.1. Mosquito colonies

Five laboratory colonies of *C. quinquefasciatus* were tested in this study. CqSyn is a synthetic laboratory susceptible colony formed in 1995 by pooling multiple field collections of that species (Wirth et al., 2005). Colonies Cq11A, CqAB, CqAB11A, and CqAB11AcytA were derived from a single synthetic population of *C. quinquefasciatus* but each colony was selected for resistance using different combinations of toxins from *B. t.* subsp. *israelensis* (Georghiou and Wirth, 1997). Cq11A was selected with Cry11Aa, CqAB was selected with Cry4Aa and Cry4Ba, CqAB11A was selected with Cry4Aa, Cry4Ba, and Cry11Aa, and CqAB11AcytA was selected with wild-type *B. thuringiensis* subsp. *israelensis* (IPS 80, Pasteur Institute).

2.2. Bacterial strains and toxins

Crystal/spore powders from lyophilized cultures of wild type and recombinant bacteria were used for selections and bioassays. The *B. t.* subsp. *israelensis* preparation was IPS 80 (Institut Pasteur Standard 1980; potency 10,000 IU/mg) (Dulmage et al., 1990), which produces the major toxins Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa. Strains of *B. thuringiensis* that synthesize Cry4Aa + Cry4Ba (Délécluse et al., 1993), Cry4Aa + Cry4Ba + Cry11Aa (Délécluse et al., 1991), Cry4Aa (Délécluse et al., 1993), Cry4Ba (Délécluse et al., 1993), or Cry11Aa (Chang et al., 1992) were also used. Mtx-1 and Mtx-2 were produced as fusion proteins with glutathione *S*-transferase in *E. coli* and induced with IPTG (isopropyl-β-$_D$-thiogalactopyranoside) as described previously (Thanabalu et al., 1991; Yang et al., 2007). Cells were harvested by centrifugation and cell pellets were washed twice in distilled water before lyophilization.

2.3. Selection and bioassay procedure

Stocks of lyophilized powders were prepared by weight in deionized water in 125 ml flasks containing approximately 25 glass beads and shaken vigorously on a vortex mixer to produce a homogeneous suspension. Stocks were prepared monthly and 10-fold serial dilutions were prepared weekly. Stocks and dilutions were stored at −20 °C when not in use.

Selections exposed 1000 early-fourth instars of the resistant *C. quinquefasciatus* colonies to the appropriate concentrations of spore/crystal suspension in 1000 ml of deionized water in enameled metal pans for 24 h. The survivors were added to the respective colonies to maintain population densities. Bioassay tests involved exposing groups of 20 early-fourth instars to different concentrations of crystal/spore suspension in 100 ml of deionized water in 250 ml plastic cups, with mortality determined after 48 h. Test suspensions for mixtures of Mtx-1 or Mtx-2 with the various Cry-toxins were made at a 1:1 ratio by weight. Tests were replicated 5 times over 5 different days and the data were analyzed using a Probit program for the computer (Raymond et al., 1993). Resistance ratios (RR_{50}, RR_{95}) were determined from concurrent tests on CqSyn and the resistant colonies using the same bacterial suspensions, and were calculated by dividing the respective LC_{50} or LC_{95} of the selected colony by that of the susceptible colony. Dose response values with overlapping fiducial limits were not considered significantly different.

Synergism factors (SF) were calculated as described by Tabashnik (1992), where an SF value of 1 indicates an additive interaction, SF values >1 indicate synergy, and SF values <1 indicates an antagonistic interaction. For this study, SF values of 1.5 or greater were classified as synergistic because this value represents a 50% increase in activity, whereas values of 1.1–1.4 were classified as weakly synergistic.

3. Results

Mtx-1 showed significantly lower toxicity against the Cry-resistant colonies than toward CqSyn (Table 1). The LC_{50} for CqSyn was 0.245 μg/ml, whereas CqAB, the least susceptible colony, showed an LC_{50} of 4.64 μg/ml and a RR_{50} of 18.9. The remaining colonies in order of increasing susceptibility were: CqAB11Acyt (RR_{50} of 9.9), CqAB11A (RR_{50} of 9.8), and Cq11A (RR_{50} of 4.2). The 1:1 mixture of Mtx-1 and Cry11Aa was weakly synergistic against CqSyn at the LC_{95} (SF_{95} = 1.3) but was not synergistic at the LC_{50}, nor was the mixture synergistic against colony Cq11A. Mtx-1 combined with Cry4Aa showed no increased activity against CqSyn but was synergistic against CqAB with SF values of 3.9 and 2.2 at the LC_{50} and LC_{95} respectively, and resistance was suppressed from approximately 40-fold to 2-fold. The Mtx-1 + Cry4Ba mixture showed no synergy toward either CqSyn or CqAB and was strongly antagonistic. However Mtx-1 combined with Cry4Aa + Cry4Ba was synergistic toward both CqSyn and CqAB. SF values for CqSyn were 2.7 and 6.5 at the LC_{50} and LC_{95}, respectively. Higher synergy factors were observed for CqAB, 11.9 and 5.6 at the LC_{50} and the LC_{95}, respectively, and resistance was reduced from 2334-fold to 10.4 at the LC_{95}. When mixed with Mtx-1, the 3- toxin recombinant, Cry4Aa + Cry4Ba + Cry11Aa showed no synergy toward CqSyn at the LC_{50}, but significant synergy (SF 9.4) was observed at the LC_{95}. Significant synergy was also measured against the resistant colony CqAB11A. The resistance ratio declined from 213-fold at the LC_{95} to 14.8 and SF values were 3.9 and 7.1 at the LC_{50} and the LC_{95}, respectively. When Mtx-1 was combined with native Bti expressing the major toxins Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa, moderate synergy was detected against CqSyn (SF values, 1.6 and 2.4) whereas no synergy was detected against the resistant colony CqAB11Acyt.

Mtx-2 was active against CqSyn with an LC_{50} of 4.13 μg/ml and an LC_{95} of 347.9 μg/ml (Table 2). The Cry-resistant colonies showed reduced susceptibility toward Mtx-2 with the lowest resistance noted in CqAB (RR_{50} of 5.4). Higher resistance was measured in Cq11A (RR_{50} = 12.0), CqAB11A (RR_{50} = 23.7), and CqAB11Acyt (RR_{50} = 25.9). Mixtures of Mtx-2 and the various Cry toxins were generally synergistic. Mtx-2 combined with Cry11Aa showed synergy against CqSyn at the LC_{50} (SF_{50} = 2.2) but not at the LC_{95}, and no synergy was detected with the mixture toward Cq11A. However Mtx-2 was strongly synergistic in combination with Cry4Aa. Against CqSyn, SF values were 16.8 and 392 at the LC_{50} and the LC_{95}, respectively and the mixture was also strongly synergistic

Table 1
Toxicity, resistance levels, and synergy in *Culex quinquefasciatus* tested with Mtx-1 combined with various Cry toxins from *Bacillus thuringiensis* at a 1:1 ratio.

Toxin(s)	Colony	LC_{50} (FL) (μg/ml)	LC_{95} (FL) (μg/ml)	Resistance ratio		Synergy factor	
				LC_{50}	LC_{95}	LC_{50}	LC_{95}
Mtx-1	CqSyn	0.245 (0.207–0.288)	2.40 (1.81–3.41)	1.0	1.0		
	Cq11A	1.02 (0.621–1.67)	6.72 (2.30–21.1)	4.2	2.8		
	CqAB	4.64 (4.11–5.23)	18.5 (15.4–23.8)	18.9	7.7		
	CqAB11A	2.39 (2.10–2.70)	8.86 (7.22–11.6)	9.8	3.7		
	CqAB11AcytA	2.40 (2.09–2.76)	12.30 (9.59–17.0)	9.9	5.1		
Cry11Aa	CqSyn	1.30 (0.792–2.12)	14.2 (5.73–35.6)	1.0	1.0		
	Cq11A	Average mortality 47% at 200 μg/ml		154	na		
Cry4Aa	CqSyn	5.04 (1.89–13.4)	983 (52.5–19219)	1.0	1.0		
	CqAB	Average mortality 14% at 200 μg/ml		>40	na		
Cry4Ba	CqSyn	Average 6% mortality at 200 μg/ml					
	CqAB	Average 1% mortality at 200 μg/ml					
Cry4A, Cry4B	CqSyn	1.49 (0.961–2.30)	15.2 (6.78–34.5)	1.0	1.0		
	CqAB	315 (188–722)	35481 (8245–442709)	211	2334		
Cry4A, Cry4B, Cry11A	CqSyn	0.00820 (0.00586–0.0115)	0.0549 (0.0297–0.105)	1.0	1.0		
	Cq4AB11A	0.590 (0.409–0.849)	11.7 (5.78–24.5)	80.0	213		
Cry4A, Cry4B, Cry11A, Cyt1A	CqSyn	0.020 (0.0174–0.0229)	0.108 (0.0840–0.148)	1.0	1.0		
	CqAB11Acyt	0.0753 (0.0648–0.0877)	0.582 (0.437–0.837)	3.8	5.4		
Mtx-1, Cry11A	CqSyn	0.664 (0.584–0.758)	3.13 (2.45–4.29)	1.0	1.0	0.62	1.3
	Cq11A	3.03 (2.65–3.44)	13.8 (11.2–18.1)	4.6	4.4	0.67	0.97
Mtx-1, Cry4A	CqSyn	1.06 (0.608–1.84)	8.37 (2.95–24.3)	1.0	1.0	0.44	0.57
	CqAB	2.37 (2.03–2.76)	16.6 (12.5–23.8)	2.2	2.0	3.9	2.2
Mtx-1, Cry4B	CqSyn	18.2 (12.2–27.2)	215 (91.4–532)	1.0	1.0	0.03	0.02
	CqAB	29.0 (17.8–47.4)	147 (56.6–389)	1.6	0.7	0.32	0.25
Mtx-1, Cry4A, Cry4B	CqSyn	0.181 (0.161–0.204)	0.642 (0.528–0.826)	1.0	1.0	2.7	6.5
	CqAB	0.769 (0.658–0.901)	6.66 (4.91–9.85)	4.2	10.4	11.9	5.6
Mtx-1, Cry4A, Cry4B, Cry11A	CqSyn	0.0192 (0.0167–0.0219)	0.0956 (0.0767–0.126)	1.0	1.0	0.83	9.4
	CqAB11A	0.244 (0.102–0.583)	1.42 (0.174–11.6)	12.7	14.8	3.9	7.1
Mtx-1, Cry4A, Cry4B, Cry11A, Cyt1A	CqSyn	0.0228 (0.0171–0.0304)	0.0878 (0.0519–0.155)	1.0	1.0	1.6	2.4
	CqAB11Acyt	0.0643 (0.0554–0.0742)	0.433 (0.340–0.586)	2.8	4.9	0.77	0.72

toward the resistant colony CqAB, with SF values of 37.0 and 122 at the LC_{50} and the LC_{95}, respectively. Resistance ratios declined from >40-fold to 4.5–6.2 fold. Cry4Ba was not improved by combining it with Mtx-2 and the mixture was slightly antagonistic. However the combination of Cry4Aa + Cry4Ba and Mtx-2 was strongly synergistic to both the susceptible and resistant colonies. Against CqSyn, the SF values were 19.2 and 51.3 at the LC_{50} and the LC_{95}, respectively. SF values were higher against CqAB at 129 and 570 at the LC_{50} and the LC_{95}, and resistance declined from 211 and 2334 at the LC_{50} and the LC_{95}, to 2.8 and 3.1-fold. Mtx-2 mixed with Cry4Aa + Cry4Ba + Cry11Aa was not synergistic toward CqSyn but showed strong synergy toward the resistant colony CqAB11A with SF values of 20.7 and 127.5 at the LC_{50} and the LC_{95}, respectively. Resistance ratios declined from 80 and 213 to 1.9 and 1.7 at the LC_{50} and the LC_{95}, respectively. However Mtx-2 mixed with Bti (Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa) showed no synergy against the susceptible CqSyn colony or against the resistant colony CqAB11Acyt.

4. Discussion

Mtx-1 and Mtx-2 from *L. sphaericus* were found to interact synergistically with a variety of Cry-toxins from *B. thuringiensis* subsp. *israelensis* against susceptible and Cry-resistant *C. quinquefasciatus.* Interestingly, cross-resistance was detected toward both Mtx toxins in the 4 Cry-resistant colonies, particularly at the LC_{50}. This result confirms our previous report of Mtx-toxin cross-resistance in Cq11Aa (Wirth et al., 2007) and extends that cross-resistance to mosquitoes resistant to the other major *B. t.* subsp. *israelensis* Cry-toxins and toxin combinations, including Cry4Aa, Cry4Ba, Cry4Aa + Cry4Ba, Cry4Aa + CryBa + Cry11Aa, and Cry4Aa + CryBa + Cry11Aa + Cyt1Aa. The presence of cross-resistance in Cry-toxin selected *C. quinquefasciatus* is intriguing because of the absence of any such cross-resistance in *L. sphaericus* resistant mosquitoes (Wirth et al., 2007; Wei et al., 2007). In view of the significant cross-resistance shown among the various Cry toxins from *B. thuringiensis* subsp. *israelensis* by the Cry-resistant colonies (Wirth and Georghiou, 1997), this result suggests that Mtx-1 and Mtx-2 modes of action share some common features with those of Cry-toxins in mosquitoes. Cross-resistance between Mtx and Cry toxins is unexpected since Mtx-1 is an ADP-ribosyl transferase toxin (Thanabalu et al., 1993), whereas Mtx-2 appears to be a pore forming toxin (Thanabalu and Porter, 1996) and thus, acts by a distinct mechanism. However, the non-enzymatic 70-kDa moiety of the activated Mtx-1 toxin appears to have direct effects on *C. quinquefasciatus* cells *in vitro* and is expected to interact with the cell membrane to form a pore to allow entry of the 27 kDa moiety into the cell (Thanabalu et al., 1993). It is possible, therefore, to speculate that the resistance phenotype produces an uncharacterized effect on membrane interactions.

Mtx-1 was more toxic than Mtx-2 when the toxins were assayed individually, however higher synergy factors were measured for Mtx-2 when those toxins were combined with Cry toxins. The synergy resulting from the interaction of Mtx-2 and the various Cry toxins and toxin combinations resulted in activity that was similar to, or better than, that observed in Mtx-1 combinations. Although both Mtx toxins enhanced activity when combined with Cry toxins, Mtx-2 appeared to have the greatest benefit based on

Table 2
Toxicity, resistance levels, and synergy in *Culex quinquefasciatus* tested with Mtx-2 combined with various Cry toxins from *Bacillus thuringiensis* at a 1:1 ratio.

Toxin(s)	Colony	LC_{50} (FL) (μg/ml)	LC_{95} (FL) (μg/ml)	Resistance ratio		Synergy factor	
				LC_{50}	LC_{95}	LC_{50}	LC_{95}
Mtx-2	CqSyn	4.13 (2.37–7.20)	347.9 (102–1216)	1.0	1.0		
	Cq11A	49.6 (31.9–77.6)	702.7 (251.4–2053)	12.0	2.0		
	CqAB	22.4 (18.3–27.5)	500 (326–867)	5.4	1.4		
	CqAB11A	97.9 (78.3–128)	1696 (951–3869)	23.7	4.9		
	CqAB11Acyt	107 (59.6–194)	696 (139–3736)	25.9	2.0		
Cry11Aa	CqSyn	1.30 (0.792–2.12)	14.2 (5.73–35.6)	1.0	1.0		
	Cq11A	Average mortality 47% at 200 μg/ml		154	na		
Cry4Aa	CqSyn	5.04 (1.89–13.4)	983 (52.5–19219)	1.0	1.0		
	CqAB	Average mortality 14% at 200 μg/ml		>40	na		
Cry4Ba	CqSyn	Average 6% mortality at 200 μg/ml					
	CqAB	Average 1% mortality at 200 μg/ml					
Cry4A, Cry4B	CqSyn	1.49 (0.961–2.30)	15.2 (6.78–34.5)	1.0	1.0		
	CqAB	315 (188–722)	35481 (8245–442709)	211	2334		
Cry4A, Cry4B, Cry11A	CqSyn	0.00820 (0.00586–0.0115)	0.0549 (0.0297–0.105)	1.0	1.0		
	CqAB11A	0.590 (0.409–0.849)	11.7 (5.78–24.5)	80.0	213		
Cry4A, Cry4B, Cry11A, Cyt1A	CqSyn	0.020 (0.0174)	0.0229	1.0	1.0		
	CqAB11Acyt	0.0753 (0.0648–0.0877)	0.582 (0.437–0.837)	3.8	5.4		
Mtx-2, Cry11A	CqSyn	0.904 (0.447–1.83)	40.8 (9.97–169)	1.0	1.0	2.2	0.67
	Cq11A	Average mortality 53.8% at 200 μg/ml	222	na	none		
Mtx-2, Cry4A	CqSyn	0.268 (0.200–0.357)	1.31 (0.783–2.28)	1.0	1.0	16.8	392
	Cq4AB	1.21 (0.781–1.87)	8.18 (3.04–24.1)	4.5	6.2	37.0	122
Mtx-2, Cry4B	CqSyn	85.7 (72.0–104)	673 (448–1190)	1.0	1.0	1.0	0.5
	CqAB	Average mortality 29% at 200 μg/ml	na	na	na	na	
Mtx-2, Cry4A, Cry4B	CqSyn	0.114 (0.0997–0.130)	0.567 (0.454–0.750)	1.0	1.0	19.2	51.3
	CqAB	0.324 (0.281–0.371)	1.73 (1.39–2.29)	2.8	3.1	129	570
Mtx-2, Cry4A, Cry4B, Cry11A	CqSyn	0.0295 (0.0261–0.0332)	0.105 (0.0875–0.134)	1.0	1.0	0.6	1.0
	CqAB11A	0.0564 (0.0505–0.0629)	0.182 (0.153–0.228)	1.9	1.7	20.7	127.5
Mtx-2, Cry4A, Cry4B, Cry11A, Cyt1A	CqSyn	0.0517 (0.0450–0.0592)	0.298 (0.237–0.395)	1.0	1.0	0.77	0.72
	CqAB11Acyt	0.163 (0.141–0.189)	1.09 (0.854–1.46)	3.2	3.7	0.9	1.1

the synergy factor values, the lethal concentration values, and the suppression of resistance in the Cry-resistant colonies.

Mtx-1 and Mtx-2 varied in their interactions with the different Cry toxins. For example, Mtx-1 failed to show any synergy with Cry4Aa against CqSyn, whereas Mtx-2 + Cry4Aa interacted quite strongly. Mtx-1 combined with Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa was synergistic when tested against CqSyn, but when Mtx-2 was used, no synergy was detected. The combination of Mtx-1 and Cry4Ba against both CqSyn and CqAB was strongly antagonistic, while no interaction was observed for combination with Mtx-2. These data are intriguing because both Mtx-1 and Mtx-2 interacted strongly and synergistically with the combination of Cry4Aa + Cry4Ba. Cry4Ba is known to be poorly active toward *Culex pipiens* complex mosquitoes but shows significant synergy in combination with the other *B. thuringiensis* subsp. *israelensis* Cry-toxins (Poncet et al., 1995). The synergy observed between Mtx-1 with Cry4Aa + Cry4Ba may be primarily due to the interaction between the Mtx toxin with Cry4Aa, in addition to the interaction between Cry4Aa and Cry4Ba. Alternatively, the interaction between Cry4Aa and Cry4Ba may facilitate the activity of Mtx-1. The differences may also be related to the different putative modes of action of Mtx-1 and Mtx-2 that were mentioned above. A definitive answer will not be obtained until the specific modes of action of Mtx-1 and Mtx-2 are elucidated and the mechanisms of resistance in the Cry-selected colonies are fully understood.

In previous work with Mtx-1 and Mtx-2, we reported that Cry11Aa combined with either Mtx toxin was synergistic and suppressed Cry11Aa resistance (Wirth et al., 2007). A 3:1 Cry11Aa toxin to Mtx-1 or Mtx-2 toxin ratio was used in that study whereas a 1:1 ratio was used in this study. In the first study, positive synergy factors were detected for both Mtx-1 and Mtx-2 against the susceptible and the resistant colony, whereas very weak or no synergy was detected in these later tests. These results suggest that the relative proportions of the toxins, which differed in the 2 experiments, may be important in the determining the interactions between Cry11Aa and Mtx toxins. Unfortunately, the scope of this study did not permit further investigation of the effect of toxin ratios on activity and synergy levels; therefore this remains an unresolved issue.

Although the precise modes of action of Mtx-1 and Mtx-2 are not known, their capacity to interact with a variety of mosquito-active proteins raises broader questions about mosquitocidal activity, and the mechanisms of synergy. Experiments carried out *in vitro* showed that Cyt1A synergy with Cry11A is a consequence of the cytolytic toxin acting as an additional receptor for the Cry toxin (Pérez et al., 2005) and facilitating oligomerization prior to pore formation (Pérez et al., 2007). Mtx-1 and Mtx-2 might act in a similar fashion, or they may have an alternative mechanism(s) for synergy. The diverse toxins that synergize Cry and Bin toxins raise the possibility that more mosquito-active materials with activity in the midgut may be capable of enhancing activity. Furthermore, the distinctive character of the mosquito midgut appears to facilitate these types of interaction, which are not commonly reported in other insects, and consequently the physiology of this area should be better investigated.

To-date, Mtx-1 and Mtx-2 toxins have been shown to interact with a wide variety of mosquitocidal toxins, including *L. sphaericus*

(Wirth et al., 2007), Cry4Aa, Cry11Aa, Cry4Aa + Cry4Ba, Cry4Aa + Cry4Ba + Cry11Aa (this study), Cyt1Aa (Zang et al., 2009), and each other (Rungrod et al., 2009). Mtx toxins are naturally expressed during vegetative growth, and the major mosquitocidal toxins of *L. sphaericus* and *B. t.* subsp. *israelensis* are expressed during sporulation. In addition, Mtx toxins are highly vulnerable to proteases in both *L. sphaericus* and *B. t.* subsp. *israelensis* (Yang et al., 2007). These limitations may not preclude their inclusion in recombinant bacteria as our understanding of regulatory and signal sequences in the host bacteria is increasing. Thus Mtx-1 and Mtx-2 should be included in the arsenal of mosquitocidal toxins with potential for inclusion in recombinant bacteria considered for development as alternative bacterial insecticides.

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