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Mtx Toxins Synergize *Bacillus sphaericus* and Cry11Aa against Susceptible and Insecticide-Resistant *Culex quinquefasciatus* Larvae[▽]

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Two mosquitocidal toxins (Mtx) of *Bacillus sphaericus*, which are produced during vegetative growth, were investigated for their potential to increase toxicity and reduce the expression of insecticide resistance through their interactions with other mosquitocidal proteins. Mtx-1 and Mtx-2 were fused with glutathione *S*-transferase and produced in *Escherichia coli*, after which lyophilized powders of these fusions were assayed against *Culex quinquefasciatus* larvae. Both Mtx proteins showed a high level of activity against susceptible *C. quinquefasciatus* mosquitoes, with 50% lethal concentrations (LC_{50}) of Mtx-1 and Mtx-2 of 0.246 and 4.13 μg/ml, respectively. The LC_{50}s were 0.406 to 0.430 μg/ml when Mtx-1 or Mtx-2 was mixed with *B. sphaericus*, and synergy improved activity and reduced resistance levels. When the proteins were combined with a recombinant *Bacillus thuringiensis* strain that produces Cry11Aa, the mixtures were highly active against Cry11A-resistant larvae and resistance was also reduced. The mixture of two Mtx toxins and *B. sphaericus* was 10 times more active against susceptible mosquitoes than *B. sphaericus* alone, demonstrating the influence of relatively low concentrations of these toxins. These results show that, similar to Cyt toxins from *B. thuringiensis* subsp. *israelensis*, Mtx toxins can increase the toxicity of other mosquitocidal proteins and may be useful for both increasing the activity of commercial bacterial larvicides and managing potential resistance to these substances among mosquito populations.

The taxon *Bacillus sphaericus* Neide corresponds to a complex of strains, some of which produce proteins that are toxic to mosquito larvae. The mosquitocidal strains are divided into two groups based on their degree of toxicity, those with high and low levels of activity (4). In highly active strains, such as 1593, 2297, 2362, and IAB 59, toxicity is due primarily to the production of the crystalline binary toxin Bin during sporulation. The two components of Bin, Bin A (41.9 kDa) and Bin B (51.4 kDa), are produced in equimolar amounts and are required for maximum activity (2, 4). Bin B is not toxic but is involved in binding to a specific receptor in *Culex pipiens*, a 60-kDa α-glucosidase (6) on the microvilli on the mosquito larval midgut. Bin A binds efficiently only in the presence of Bin B and is responsible for most insecticidal activity. The mode of action after binding is not well understood, but Bin is thought to form lipid membrane pores causing cellular osmotic disruption (1, 20).

The mosquitocidal activity of highly active strains of *B. sphaericus* resulted in their development as commercial larvicides, now used in many countries in various parts of the world to control vector and nuisance mosquito species. These larvicides are highly specific to mosquitoes, are safe for nontarget organisms, and retain good activity in polluted water, where other bacterial larvicides, such as *Bacillus thuringiensis* subsp. *israelensis*, do not provide the same efficacy or residual activity. However, field studies have demonstrated that routine and intensive use of these *B. sphaericus* formulations can result in the rapid evolution of resistance, especially in populations of the *C. pipiens* complex, thereby potentially limiting the long-term utility of these strains (13, 18, 21, 23, 40). This rapid evolution of resistance apparently resulted because the activity of these strains is due overwhelmingly to the Bin toxin. Consequently, it is important to develop strategies to maintain the effectiveness of *B. sphaericus* insecticides and manage the potential for resistance.

In contrast to the high-activity strains, the low-activity strains of *B. sphaericus* lack Bin. In these strains, mosquitocidal activity is due to proteins known as Mtx toxins (mosquitocidal toxins) that are synthesized during vegetative growth. Several Mtx toxins in both high- and low-activity *B. sphaericus* strains have been identified previously (11, 28, 29). The low and/or unstable activity associated with Mtx toxins probably results from low levels of production and proteolytic degradation during sporulation (29, 39). Consequently, Mtx proteins are not detectable in whole cultures of highly active strains, including 2362. Importantly, purified Mtx-1 was shown to be as toxic to mosquito larvae as the highly active strains of sporulated *B. sphaericus* in the vegetative and sporulation phases (27, 29). When Mtx-1 was expressed in *Escherichia coli* E-TH21 in a previous study, the sporulated and lyophilized powder was highly toxic to *Culex quinquefasciatus* and higher cumulative mortality among larvae exposed to E-TH21 than among those exposed to Bin was observed (31). The activity of Mtx toxins and their unique modes of action suggest that Mtx toxins may be useful for mosquito control.

Models for the evolution of insecticide resistance suggest that increasing toxin complexity to include toxins with different modes of action should help slow the evolution of resistance (5, 12, 24). For example, *B. thuringiensis* subsp. *israelensis* synthe-

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sizes at least four major toxins from three distinct toxin families, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa (10, 17), and resistance in laboratory and field populations has been extraordinarily slow to evolve (9). The Cyt1Aa toxin, which apparently has a detergent-like mode of action rather than forming cation channels like Cry toxins, was previously found to be critical to the low risk of resistance to *B. thuringiensis* subsp. *israelensis* (9). Furthermore, subsequent studies showed that Cyt1Aa enhances *B. sphaericus* toxicity. Mixtures of *B. sphaericus* and Cyt1Aa, for example, are synergistic and suppress resistance (32), and selection with a mixture of *B. sphaericus* and Cyt1Aa was found to delay resistance (34).

The results obtained with Cyt1A suggested that other proteins might be useful for increasing the toxicity of *B. sphaericus* and reducing the potential for the evolution of resistance. Thus, in the present study we investigated the interaction of Mtx proteins with other mosquitocidal proteins. We first determined the toxicities of two recombinant *E. coli* strains expressing either *mtx-1* or *mtx-2* toward susceptible and insecticide-resistant *C. quinquefasciatus* larvae. We then combined Mtx powders with *B. sphaericus* 2362 or recombinant strains expressing Cry11Aa or Cyt1Aa from *B. thuringiensis* subsp. *israelensis* to determine whether these components interacted synergistically. Here we show that Mtx-1 and Mtx-2 were highly toxic to susceptible and resistant mosquitoes and that synergistic interactions occurred when Mtx toxins were mixed with *B. sphaericus* 2362 or with Cry11Aa, resulting in reductions in resistance. Thus, Mtx toxins may be useful for both increasing the insecticidal activity of *B. sphaericus* strains and managing the evolution of resistance to the Bin toxins in mosquito populations.

MATERIALS AND METHODS

Bacterial strains and toxins. The production of Mtx-1 and Mtx-2 as fusion proteins with glutathione *S*-transferase in *E. coli* was induced as described previously (27, 39). Following induction for 3 h by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside), cells were harvested by centrifugation and cell pellets were washed twice in sterile distilled water. The final cell pellets were lyophilized for the production of powders for use in bioassays. *B. sphaericus* 2362 technical powder was provided by Valent Biosciences (Libertyville, IL). Cyt1Aa and Cry11Aa were produced independently in a recombinant strain of *B. thuringiensis* subsp. *israelensis* (37, 38). All *Bacillus* and *E. coli* materials were in the form of sporulated, lyophilized powders.

Mosquito colonies. Three laboratory colonies of *C. quinquefasciatus* mosquitoes were used in this study. The colony Syn-P (35) is a susceptible parental colony maintained in the laboratory since 1995 and used to establish baseline dose response values for the tested materials. The insecticide-resistant colonies included Bs-R, resistant to *B. sphaericus* 2362 with a resistance ratio (the ratio of the lethal concentration [LC] for the resistant mosquitoes to that for the susceptible mosquitoes) at the 95% LC (LC_{95}) of >1,000 (36), and Cq11A, resistant to Cry11Aa from *B. thuringiensis* subsp. *israelensis* with a resistance ratio at the LC_{95} of >1,000 (9, 33). The resistant colonies have been reared in the laboratory under selective pressure since 1990.

Bioassay and selection procedures. Stocks were prepared with the sporulated, lyophilized bacterial powders by suspending weighed powder in deionized water with 20 to 25 glass beads to promote homogenization. Different concentrations of the suspensions were fed to groups of 20 early-fourth-instar larvae in 250-ml plastic cups containing 100 ml of deionized water. Eight or more concentrations that produced mortality between 0 and 100% plus an untreated control were used for each dose response test, and each test was replicated a minimum of five times on five different days. No mortality from the control preparation was observed. Susceptible and resistant mosquito colonies were tested concurrently using the same test materials and stock suspensions. Mortality was determined at 24 and/or 48 h, depending on the material tested. Larvae tested for 48 h received a small amount of food at 24 h. Test mixtures of recombinant bacterial powders were produced based on the weight of each respective powder. Data were analyzed using probit analysis (8).

Interactions between toxins were evaluated using the method described by Tabashnik (25). The theoretical toxicity (50% LC [LC_{50}] or LC_{95}) of a mixture was predicted from the weighted harmonic mean of the toxicities of the individual materials. The synergy factor (SF) at the LC_{50} or the LC_{95} was calculated by dividing the theoretical toxicity value by the observed toxicity value. According to Tabashnik (25), a mixture with an SF equal to 1 is additive, that with an SF of >1 is synergistic, and a mixture with an SF of <1 is antagonistic. For this study, mixtures with an SF of 1.5 or higher were classified as synergistic because this value represented a 50% increase in activity. Mixtures with SFs from 1.1 to 1.4 were classified as weakly synergistic.

Selection procedures for the Cq11A and Bs-R resistant mosquito colonies were similar to the bioassay procedures except that groups of 1,000 early-fourth-instar larvae were exposed to a suspension of either Cry11Aa lyophilized spore/crystal recombinant bacterial powder or *B. sphaericus* technical powder in 1,000 ml of deionized water for 24 or 48 h. Survivors were transferred into clean water, fed, and used to continue the colonies.

RESULTS

Mtx-1 powder was toxic to the susceptible and resistant mosquito larvae, with LC_{50}s ranging from 0.245 to 1.02 μg/ml (Table 1). The Cq11A mosquitoes were significantly less susceptible than either Syn-P or Bs-R mosquitoes, and a 4.2 resistance ratio at the LC_{50} was detected. When Mtx-1 powder was mixed with *B. sphaericus* 2362 at a 1:3 ratio, the LC_{50} for Syn-P larvae was 0.0043 μg/ml, a 50-fold improvement compared to the toxicity of *B. sphaericus* alone. That mixture was also synergistic; SFs at the LC_{50} and LC_{95} were 9.8 and 14.1, respectively. Against the *B. sphaericus*-resistant colony, Bs-R, the mixture was toxic, with an LC_{50} of 0.332 μg/ml, and the resistance ratio was reduced from >1,000 for *B. sphaericus* alone to 77.2. SFs were lower, 2.7 to 3.1, than those observed for Syn-P mosquitoes.

The mixture of Mtx-1 powder and Cyt1Aa (1:1) was the least active of the mixtures tested, with an LC_{50} for the Syn-P colony of 0.685 μg/ml. This mixture was also less active against Bs-R mosquitoes, with an LC_{50} of 2.11 μg/ml and resistance ratios at the LC_{50} and LC_{95} of 3.1 and 8.0, respectively. No synergy toward Syn-P or Bs-R larvae was detected, and the mixture was classified as antagonistic, since SFs were less than 1. When *B. sphaericus* 2362 was mixed with Mtx-1 and Cyt1Aa (8:1:1), the LC_{50}s were 0.0427 μg/ml for Syn-P mosquitoes and 0.884 μg/ml for Bs-R mosquitoes. Resistance ratios for Bs-R larvae at the LC_{50} and LC_{95} were 2.1 and 9.1. Positive SFs at the LC_{95} for Syn-P larvae (2.3) and at both the LC_{50} and the LC_{95} for Bs-R larvae (2.9 and 5.5, respectively) were detected.

The mixture of Cry11Aa and Mtx-1 (3:1) was active against Syn-P larvae with an LC_{50} of 0.396 μg/ml. When tested against the resistant colony, Cq11A, the mixture showed an LC_{50} of 4.45 μg/ml. Resistance ratios declined from >1,000 for Cry11Aa alone to 11.2 at the LC_{50} of the mixture. The mixture of Cry11Aa and Mtx-1 was synergistic at both the LC_{50} and the LC_{95} for Syn-P mosquitoes (SFs, 1.5 to 4.1) and at the LC_{95} for Cq11A mosquitoes (SF, 1.5).

Mtx-2 powder was found to be less active than Mtx-1 powder when assayed against the susceptible and resistant mosquitoes (Table 2). The LC_{50} was 4.13 μg/ml for the Syn-P colony, 3.77 μg/ml for the Bs-R colony, and 49.6 μg/ml for the Cq11A colony. Cq11A mosquitoes were significantly less susceptible to Mtx-2 than to Mtx-1, and a resistance ratio of 12.0 at the LC_{50} was detected. The mixture of *B. sphaericus* and Mtx-2 (3:1) was

TABLE 1. Toxicity, resistance ratios, and interactions of Mtx-1 toxin with *B. sphaericus* and Cry11Aa and Cyt1Aa from *B. thuringiensis* subsp. *israelensis* against susceptible and insecticide-resistant *C. quinquefasciatus* larvae

Toxin(s) assayed	Mosquito colony	LC_{50} (μg/ml) (fiducial limits) or relevant result	LC_{95} (μg/ml) (fiducial limits)	Resistance ratio at:		SF at:	
				LC_{50}	LC_{95}	LC_{50}	LC_{95}
Mtx-1	Syn-P	0.245 (0.207–0.288)	2.40 (1.81–3.41)	1.0	1.0	NA[a]	NA
	BS-R	0.259 (0.221–0.302)	2.17 (1.63–3.12)	1.1	0.9	NA	NA
	Cq11A	1.02 (0.621–1.67)	6.72 (2.30–21.1)	4.2	2.8	NA	NA
B. sphaericus	Syn-P	0.033 (0.0188–0.0594)	0.825 (0.203–3.01)	1.0	1.0	NA	NA
	BS-R	17% mortality at 1,000 μg/ml		>1,000	NA	NA	NA
Cry11Aa	Syn-P	1.14 (0.452–2.89)	33.5 (6.96–163)	1.0	1.0	NA	NA
	Cq11A	1,181 (487–6,563)	223,893 (25,642–17.5 × 10^6)	1,035	6,683	NA	NA
Cyt1Aa	Syn-P	14.1 (10.5–18.2)	504 (336–854)	1.0	1.0	NA	NA
	BS-R	27.8 (14.8–52.2)	674 (189–2,432)	1.9	1.4	NA	NA
B. sphaericus + Mtx-1 (3:1)	Syn-P	0.00430 (0.00310–0.00594)	0.0702 (0.0379–0.137)	1.0	1.0	9.8	14.1
	BS-R	0.332 (0.278–0.395)	3.16 (2.27–4.89)	77.2	45.0	3.1	2.7
Mtx-1 + Cyt1Aa (1:1)	Syn-P	0.685 (0.578–0.811)	10.1 (7.45–14.3)	1.0	1.0	0.7	0.5
	BS-R	2.11 (1.37–3.23)	80.1 (36.1–184)	3.1	8.0	0.2	0.05
B. sphaericus + Mtx-1 + Cyt1Aa (8:1:1)	Syn-P	0.0427 (0.0265–0.0689)	0.436 (0.169–1.14)	1.0	1.0	0.1	2.3
	BS-R	0.884 (0.649–1.20)	3.97 (2.27–7.20)	2.1	9.1	2.9	5.5
Cry11Aa + Mtx-1 (3:1)	Syn-P	0.396 (0.259–0.605)	1.93 (0.908–4.17)	1.0	1.0	1.5	4.1
	Cq11A	4.45 (3.90–5.12)	18.2 (14.5–24.0)	11.2	9.4	0.9	1.5

[a] NA, not applicable.

very active against Syn-P larvae; the LC_{50} was 0.00406 μg/ml, and the LC_{95} was 0.0828 μg/ml. SFs were 10.8 and 13.2 at the LC_{50} and LC_{95}, respectively. This mixture was also quite toxic to Bs-R larvae, with an LC_{50} and an LC_{95} of 0.102 and 0.298 μg/ml, respectively. Resistance ratios declined from >1,000 to 25.1 and 3.5 at the LC_{50} and LC_{95}, respectively. SFs were also very high, 147 and 1,409 at the LC_{50} and LC_{95}, respectively.

The mixture of Mtx-2 and Cyt1Aa was less toxic, and the LC_{50} was 2.83 μg/ml for Syn-P larvae and 5.81 μg/ml for Bs-R larvae. This mixture was synergistic for Syn-P mosquitoes at the LC_{50} and LC_{95} (SFs, 2.3 and 5.2) and less so for Bs-R mosquitoes (SFs, 1.1 and 2.6). Very low levels of resistance among Bs-R mosquitoes were detected, and resistance ratios at the LC_{50} and LC_{95} were 2.1 and 0.9, respectively.

When a mixture of 8:1:1 of *B. sphaericus*, Mtx-2, and Cyt1Aa was tested, the LC_{50} was 0.0637 μg/ml for Syn-P mosquitoes and 0.755 μg/ml for Bs-R mosquitoes. Bs-R larvae continued to show resistance to the mixture, although the resistance ratio was reduced from >1,000 to 11.9 at the LC_{50}. The mixture was synergistic at the LC_{95} for Syn-P mosquitoes (SF, 2.3). However, the mixture was strongly synergistic when tested against Bs-R larvae, and SFs were 44 and 255 at the LC_{50} and LC_{95}.

A mixture of *B. sphaericus*, Mtx-1, and Mtx-2 (8:1:1) was highly active against Syn-P larvae and demonstrated an LC_{50} of 0.00730 μg/ml. The mixture was less active toward Bs-R larvae, with an LC_{50} of 0.195 μg/ml and a resistance ratio of 26.7. The mixture of *B. sphaericus*, Mtx-1, and Mtx-2 was synergistic against both Syn-P and Bs-R mosquitoes, with SFs ranging from 5.6 to 23.1.

When Cry11Aa was combined with Mtx-2 (3:1), the mixture was active against Syn-P mosquitoes, with an LC_{50} of 0.140 μg/ml and an SF of 9.9. This mixture was less active against the resistant colony, Cq11A, and showed an LC_{50} of 6.82 μg/ml and a resistance ratio of 25.8. This mixture was synergistic at the LC_{50} (SF, 25.8) but was antagonistic at the LC_{95} (SF, 0.6).

DISCUSSION

These results demonstrate that Mtx-1 and Mtx-2 have potential for use in recombinant bacterial insecticides as a resistance management tool because of their toxicity and their synergistic interactions with other mosquitocidal toxins. Recombinant *E. coli* strains synthesizing Mtx-1 or Mtx-2 showed high levels of toxicity toward susceptible and resistant *C. quinquefasciatus* larvae, whereas *E. coli* alone shows no toxicity to these insects (28). Interestingly, the loss of the 60-kDa α-glucosidase from the microvillar membranes of Bs-R larvae, the mechanism of *B. sphaericus* resistance in these insects (7, 14, 22), had no apparent effect on activity. This result probably occurred because this resistance evolved against the *B. sphaericus* binary toxin. In contrast, low levels of cross-resistance to both Mtx powders were detected in Cq11A larvae. This finding is unexpected since the two Mtx toxins are likely to have quite distinct mechanisms of action (Mtx-1, ADP-ribosylation; Mtx-2, pore formation). Other Cry-resistant mosquitoes should be tested to determine whether cross-resistance is present.

Mixtures of Mtx-1 or Mtx-2 with *B. sphaericus* 2362 were highly toxic to susceptible and resistant mosquitoes. Furthermore, these mixtures were strongly synergistic and dramatically reduced the levels of *B. sphaericus* resistance. Again, as noted above, this result probably occurred because resistance evolved by selection against the binary toxin, the dominant toxin in

TABLE 2. Toxicity, resistance ratios, and interactions of Mtx-2 toxin with *B. sphaericus* and Cry11Aa and Cyt1Aa from *B. thuringiensis* subsp. *israelensis* against susceptible and insecticide-resistant *C. quinquefasciatus* larvae

Toxin(s) assayed	Mosquito colony	LC_{50} (μg/ml) (fiducial limits) or relevant result	LC_{95} (μg/ml) (fiducial limits)	Resistance ratio at:		SF at:	
				LC_{50}	LC_{95}	LC_{50}	LC_{95}
Mtx-2	Syn-P	4.13 (2.36–7.20)	347.9 (102–1216)	1.0	1.0	NA[a]	NA
	BS-R	3.77 (2.26–6.27)	105.0 (35.9–320)	0.9	0.3	NA	NA
	Cq11A	49.6 (31.9–77.6)	702.7 (251.4–2053)	12.0	2.0	NA	NA
B. sphaericus	Syn-P	0.0330 (0.0188–0.0594)	0.825 (0.203–3.01)	1.0	1.0	NA	NA
	BS-R	17% mortality at 1,000 μg/ml		>1,000	NA	NA	NA
Cry11Aa	Syn-P	1.14 (0.452–2.89)	33.5 (6.96–163)	1.0	1.0	NA	NA
	Cq11A	1,181 (487–6,563)	223,893 (25,642–17.5 × 10^6)	1,035	6,683	NA	NA
Cyt1Aa	Syn-P	14.1 (10.5–18.2)	504 (336–854)	1.0	1.0	NA	NA
	BS-R	27.8 (14.8–52.2)	674 (189–2432)	1.9	1.4	NA	NA
B. sphaericus + Mtx-2 (3:1)	Syn-P	0.00406 (0.00337–0.00490)	0.0828 (0.0564–0.134)	1.0	1.0	10.8	13.2
	BS-R	0.102 (0.0913–0.113)	0.298 (0.237–0.383)	25.1	3.5	147	1409
Mtx-2 + Cyt1Aa (1:1)	Syn-P	2.83 (2.12–3.78)	78.8 (45.7–142)	1.0	1.0	2.3	5.2
	BS-R	5.81 (4.94–6.83)	70.6 (52.8–99.9)	2.1	0.9	1.1	2.6
B. sphaericus + Mtx-2 + Cyt1Aa (8:1:1)	Syn-P	0.0637 (0.0444–0.0915)	0.453 (0.234–0.895)	1.0	1.0	0.6	2.3
	BS-R	0.755 (0.666–0.856)	3.56 (2.89–4.69)	11.9	7.9	44.0	255
B. sphaericus + Mtx-1 + Mtx-2 (8:1:1)	Syn-P	0.00730 (0.00618–0.00862)	0.0800 (0.0595–0.115)	1.0	1.0	5.6	12.3
	BS-R	0.195 (0.137–0.278)	0.920 (0.486–1.77)	26.7	11.5	12.4	23.1
Cry11Aa + Mtx-2 (3:1)	Syn-P	0.140 (0.0430–0.454)	2.02 (0.440–9.43)	1.0	1.0	9.9	21.8
	Cq11A	6.82 (1.82–25.4)	4,642 (64.3–347,492)	48.7	2,298	25.8	0.6

[a] NA, not applicable.

sporulated preparations of *B. sphaericus*. The LC_{95}s of the *B. sphaericus*–Mtx-1 and *B. sphaericus*–Mtx-2 mixtures demonstrated that these mixtures were significantly more toxic than *B. sphaericus* toward Syn-P larvae. However, Mtx-2 appeared to have a small advantage over Mtx-1 when used against Bs-R larvae, as that mixture showed the lowest LC_{95} and the greatest resistance suppression.

The LCs and SFs of *B. sphaericus*-Mtx mixtures were remarkably similar to values previously reported for mixtures of *B. sphaericus* and Cyt1Aa from *B. thuringiensis* subsp. *israelensis* (32). These three types of toxins, Cyt1Aa, Mtx-1, and Mtx-2, have distinct modes of action. Cyt1Aa is lipophilic and lyses cells in vivo (30). Mtx-1 is an ADP-ribosyltransferase that can modify a number of proteins in target cells (3, 19, 26). Mtx-2 shows homology to the ε-toxin of *Clostridium perfringens* and the cytotoxin of *Pseudomonas aeruginosa*, both of which are believed to form pores on susceptible target cells (29). Thus, these data indicate that toxins with diverse modes of action can enhance the activity of *B. sphaericus* and suppress resistance.

The combination of Mtx-1 and Cyt1Aa was antagonistic toward susceptible and *B. sphaericus*-resistant mosquitoes. This observation is not consistent with the report of Zhang et al. (41) of synergy for a mixture of Mtx-1 and Cyt1Aa. There are, however, important differences in the materials tested in these two studies. First, two of our recombinant bacterial toxins (Cyt1Aa and Mtx-1) were more active than the comparable toxins tested by Zhang et al. For example, our recombinant Cyt1Aa toxin showed an LC_{50} of 14.1 μg/ml, whereas Zhang et al. (41) reported an LC_{50} of >100,000 μg/ml. Second, the Mtx-1 proteins, although each generated from a 3.8-kb fragment, were produced in different recombinant hosts, *E. coli* versus acrystalliferous *B. thuringiensis*. Consequently, comparisons between our results are difficult. Interestingly, we did observe synergy when Cyt1Aa was combined with Mtx-2, although that mixture showed only moderate toxicity. When *B. sphaericus* was combined with Cyt1Aa and Mtx-1 or Mtx-2, synergy was detected but the toxicity of the combination toward susceptible insects was no better than that of *B. sphaericus* combined with a single Mtx toxin only. The same mixtures were quite effective in reducing the resistance of Bs-R larvae. Further tests with a variety of toxin ratios may be informative but were beyond the scope of this study.

Although no significant difference in toxicity against susceptible mosquitoes was detected when Mtx-1 or Mtx-2 was combined with Cry11Aa, significant differences in larval mosquito mortality were apparent when those mixtures were assayed against Cry11Aa-resistant mosquitoes. The mixture with Mtx-1 was significantly more active and reduced resistance to a greater extent than the mixture with Mtx-2. These results are interesting because Cq11A mosquitoes showed cross-resistance to both materials, suggesting that these toxins share part of the same receptor. These differences in activity may be related to the individual mode of action of each Mtx toxin. It should be noted that no such differences were observed for the interactions of these toxins with *B. sphaericus.* The results also suggest that the mechanism of

synergy with *B. sphaericus* may be fundamentally different from that of synergy with Cry11Aa.

Another implication of these results for understanding the biology of mosquitocidal strains of *B. sphaericus* is that secondary toxins may play important, often subtle, roles in activity. These toxins have been of only minor interest until recently but, as we have shown here and elsewhere, have the potential to contribute to toxicity, synergy, and a reduced risk for insecticide resistance. Under natural conditions, interactions between the vegetative Mtx toxins and the spore-associated Bin toxin may occur if Mtx proteins are produced soon after the germination of spores in the larval gut. Thus, these toxins should be investigated further to evaluate their potential to delay the evolution of resistance in mosquito populations. While the sensitivity of Mtx toxins to proteolysis may be a problem in recombinant bacilli (39), different combinations of toxins chosen from the spectrum that occurs among *B. sphaericus*, *B. thuringiensis* subsp. *israelensis*, and other species may provide a continuing variety of recombinant bacteria, thereby enhancing the long-term success of these novel and environmentally compatible insecticides.

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