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Journal of Invertebrate Pathology 88 (2005) 154–162
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Evolution of resistance toward *Bacillus sphaericus* or a mixture of *B. sphaericus* + Cyt1A from *Bacillus thuringiensis*, in the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae)

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Received 29 July 2004; accepted 10 January 2005

Abstract

The 2362 strain of *Bacillus sphaericus* (Bs) Neide is a highly mosquitocidal bacterium used in commercial bacterial larvicides primarily to control mosquitoes of the genus *Culex*. Unfortunately, Bs is at high risk for selecting resistance in mosquito populations, because its binary toxin apparently only binds to a single receptor type on midgut microvilli. A potential key strategy for delaying resistance to insecticidal proteins is to use mixtures of toxins that act at different targets within the insect, especially mixtures that interact synergistically. We tested this hypothesis for delaying the phenotypic expression of resistance by exposing *Culex quinquefasciatus* Say larvae to Bs alone or in combination with Cyt1A from *Bacillus thuringiensis* subsp. *israelensis*. Two laboratory lines of *Cx. quinquefasciatus*, one sensitive to Bs and the other containing Bs resistance alleles, were subjected to intensive selection pressure for 20 generations with either Bs 2362 or a 3:1 mixture of Bs 2362 + Cyt1A. At the end of the study, the sensitive line had evolved >1000-fold resistance when selected with Bs alone, whereas the parallel line selected with Bs + Cyt1A exhibited only low resistance toward this mixture (RR_{95}, 1.4). Similar results were observed in the lines containing Bs resistance alleles. Both lines selected with Bs + Cyt1A exhibited substantial resistance to Bs in the absence of Cyt1A. Although selection with Bs + Cyt1A did not prevent the underlying evolution of resistance to Bs, these results suggest that a mixture of Bs with other endotoxins, particularly one like Bs + Cyt1A in which the components interact synergistically, will provide longer lasting and more effective mosquito control than Bs alone.

Keywords: *Bacillus sphaericus*; Cyt1A; Resistance; Resistance management

1. Introduction

The 2362 strain of *Bacillus sphaericus* (Bs) Neide is an aerobic, spore forming bacterium that produces a highly mosquitocidal binary (Bin) toxin. Bs Bin is composed of two proteins, a 51-kDa binding component (BinB) and a 42-kDa toxin component (BinA). These are synthesized as protoxins during sporulation and co-crystallize forming a parasporal body attached to the inner face of the exosporium membrane. Both components are necessary for optimal activity and, after ingestion by mosquito larvae, undergo proteolytic cleavage and then associate to form the active toxin. In sensitive mosquito species, such as those belonging to the genus *Culex*, the BinB component binds to a specific receptor, a 60-kDa α-glucosidase, which is held to the epithelial membrane by a glucosylphosphatidylinositol (GPI) anchor (Darboux et al., 2001; Nielsen-LeRoux and Charles, 1992; Silva-Filha et al., 1999). Most evidence suggests the toxin

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0022-2011/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2005.01.003

is internalized by endocytosis, followed by midgut cells lysis and larval death 24–48 h later (Davidson et al., 1987; Oei et al., 1992; Silva-Filha and Peixoto, 2003).

Insecticides based on Bs were developed for commercial mosquito control, where its residual activity is important for controlling mosquito larvae that breed in polluted water. However, the target spectrum of Bs is strictly limited to mosquitoes, primarily *Culex* and certain *Anopheles* species (Delécluse et al., 2000). It is much less effective against other important vector species such as *Aedes aegypti* and the blackfly, *Simulium damnosum* (Becker, 2000). Importantly, Bs is at a risk for selecting insecticide resistance because it targets a single receptor type in the midgut (Darboux et al., 2001; Nielsen-LeRoux et al., 1995; Silva-Filha et al., 1999). Resistance was shown to evolve rapidly in laboratory lines of *Culex quinquefasciatus* Say selected with Bs (Rodcharoen and Mulla, 1994; Wirth et al., 2000a), and significant levels of field resistance, greater than 1000-fold, have been reported in *Culex* populations in China and Thailand (Mulla et al., 2003; Yuan et al., 2000).

One approach to improving host-range and reducing the risk of resistance to Bs is to develop recombinant bacteria that express Bin toxins in combination with mosquitocidal toxins from other bacteria (Federici et al., 2000, 2003). For example, combining Bin with insecticidal proteins from *Bacillus thuringiensis* subsp. *israelensis* could potentially expand host-range to include insects sensitive to both bacteria species. Such recombinants should be less prone to selecting insecticide resistance due to the presence of toxins that act at different target sites in the mosquito midgut. A natural model for this is *B.t.* subsp. *israelensis*, which produces a crystalline parasporal body that contains a complex of four major insecticidal proteins, Cry4A, Cry4B, Cry11A, and Cyt1A (Crickmore et al., 1998). This bacterium has a broader host-range than Bs, being active against a wider spectrum of mosquito species, blackflies, and some chironomid midges (Becker, 2000). The Cyt1A toxin is of particular interest because of its affinity for unsaturated fatty acids in the lipid layer of the membrane, whereas Cry proteins and Bs Bin each target specific receptor molecules. Cyt1A is considered to be only weakly insecticidal (Crickmore et al., 1995; Wu and Federici, 1993), with an estimated LC_{50} in recent tests against *Culex* species of 11.7 μg/ml compared to 0.01 μg/ml for Bs (data not shown). Despite its relatively low mosquitocidal activity, Cyt1A was demonstrated to increase Cry protein toxicity in *B.t.* subsp. *israelensis* through synergism (Crickmore et al., 1995; Ibarra and Federici, 1986; Wu and Chang, 1985). Although the precise mechanism of Cyt1A synergy remains unclear, it plays an important role in *B.t.* subsp. *israelensis* activity and, perhaps more importantly, can help retard the evolution of resistance. Selection studies demonstrated that, in the absence of Cyt1A, high levels of resistance evolved in *Cx. quinquefasciatus* to the Cry proteins of *B.t.* subsp. *israelensis*, whereas little or no resistance was apparent when Cyt1A was present (Georghiou and Wirth, 1997). More recently, we showed that resistance of approximately 1000-fold evolved in *Cx. quinquefasciatus* selected with Cry11A, whereas less than 15-fold resistance evolved when this species was selected with a 3:1 mixture of Cry11A + Cyt1A (Wirth et al., 2005). These results support the hypothesis that Cyt1A is a key factor in delaying the evolution of phenotypic expression of resistance to *B.t.* subsp. *israelensis* in mosquitoes. In addition to delaying resistance, Cyt1A can suppress resistance once it develops. Greater than 1000-fold resistance to Cry11A and 10,000-fold resistance to *B. sphaericus* 2362 were suppressed through synergy when bacterial strains producing these proteins were combined with Cyt1A in a 3:1 ratio (Wirth et al., 1997, 2000b). Cyt1A synergy with Bs resembles that with Cry toxins and thus suggests a common mechanism of interaction. If so, then Bs and Cyt1A combinations may help delay the evolution of resistance to that bacterium as well.

To test this hypothesis, two laboratory lines of *Cx. quinquefasciatus*, one sensitive to Bs and one containing Bs resistance alleles, were subjected to intensive selection pressure for 20 generations with either *B. sphaericus* 2362 or a 3:1 mixture of Bs + Cyt1A from *B.t.* subsp. *israelensis*. By generation 20, mosquitoes selected with Bs evolved more than 1000-fold resistance, whereas the two mosquito lines selected with Bs + Cyt1A exhibited only 1.4–6.7-fold resistance to the mixture at the LC_{95}, although resistance levels were higher at the LC_{50} level. Furthermore, Bs + Cyt1A retained very high activity against mosquitoes resistant to Bs. These data show that the Bs + Cyt1A mixture is persistently insecticidal to sensitive and Bs-resistant mosquitoes and that the phenotypic expression of resistance was highly constrained in mosquitoes selected with this mixture. Consequently, Bs insecticides with increased toxin complexity and synergy should provide more effective and longer lasting mosquito control than Bs alone.

2. Materials and methods

2.1. Mosquito lines

A sensitive line of *Cx. quinquefasciatus* was established in the laboratory from approximately 1000 larvae and 50 egg rafts collected from three dairies in San Bernardino County, California, in September 2000. Adults were combined in a rearing cage and maintained following standard laboratory procedures for five months before selection was initiated. This parental susceptible line was named SynC.

A second line of *Cx. quinquefasciatus* was established that contained Bs resistance alleles derived from an

existing laboratory resistant line, Bs-R (Wirth et al., 2000a), and a second susceptible parental line that was field-collected in 1995 and previously identified as SynP (Wirth et al., 2004). The Bs-R line is homozygous for Bs resistance due to the loss of the toxin receptor in the midgut epithelium that is controlled by a recessive allele (Darboux et al., 2002; Wirth et al., 2000a). Approximately, 3000 larvae from each line were pooled and emerging adults were placed into a rearing cage. The new line was named BsRX and was reared under standard laboratory conditions for five months before selection began.

2.2. Bacterial strains and toxins

Lyophilized powders of lysed bacterial cultures were used for bioassays and selections. Technical powder of the 2362 strain of Bs was obtained from Valent Biosciences (Libertyville, IL). Cyt1A was produced in a recombinant strain of *B.t.* subsp. *israelensis* (Wu and Federici, 1993) and herein referred to as Cyt1A. The estimated percentage of powder that was toxin, based on SDS–PAGE analysis, was 10% for *B. sphaericus* and 20% for Cyt1A (Wirth et al., 1997). For the selections, larval lines were exposed either to Bs or to Bs + Cyt1A in a 3:1 ratio based on weight.

2.3. Selections

Two alternative approaches for applying Bs + Cyt1A were tested for their capacity to delay or manage insecticide resistance. One approach targeted a susceptible line to evaluate the mixture's potential to prevent or delay the evolution of resistance. The second approach targeted a line containing Bs resistance alleles to evaluate the capacity of the mixture to reverse or retard further evolution of resistance. Early fourth instars were selected in groups of 1000 and treated with aqueous suspensions of lyophilized powders of the bacterial strains in enameled metal pans containing 1000 ml of deionized water. Surviving larvae were fed after 48 h and reared to pupation in the selection pan. Mortality estimates were based on the total number of pupae collected for each generation.

2.4. Bioassays

Selected lines and their respective unselected parental lines were bioassayed with Bs every fourth generation. Assays with Bs + Cyt1A began in generation 8 and generation 12 for the SynC and BsRX lines after significant Bs resistance was detected. For those bioassays, groups of 20 early fourth instars were exposed in 100 ml of deionized water in 250 ml plastic cups to 8–15 different concentrations that yielded mortality between 2 and 98% after 48 h. Control lines were not treated. Due to the lack of a normal distribution of Bs susceptibility in the BsRX-unsel line and the very high level of resistance that precluded application of probit analysis, bioassays for the line selected with Bs used groups of 20 larvae treated with a discriminating concentration of 100,000 ng/ml of Bs in 100 ml deionized water in 250 ml plastic cups, and mortality was evaluated after 48 h. This concentration is about 100-fold the LC_{95} for Bs against SynC, and killed all susceptible larvae, whereas homozygous-resistant BsR larvae survived. The same procedure was used for the SynC line selected with Bs in generations 16 and 20.

All bioassay treatments were replicated five times on five different days. Dose–response lines were analyzed by Probit (Finney, 1971) using a program written for the PC (Raymond et al., 1995). Lethal concentration values with overlapping fiducial limits were not considered significantly different. Resistance ratios (RR) were calculated by dividing the LC_{50} or the LC_{95} of the selected line with the concurrently determined LC_{50} or LC_{95} of the unselected parental line, i.e., SynC or BsRX. Resistance ratios with Bs for the highly resistant lines were conservatively estimated by dividing the discriminating concentration 100,000 ng/ml by the LC_{50} of SynC (33.0 ng/ml).

3. Results

Approximately, 8000 larvae were treated each generation in the SynC lines selected with Bs or Bs + Cyt1A. Mortality averaged 75.7% (SD = 14.9) for the SynC-Bs-selected and 68% (SD = 24.8) for SynC-Bs + Cyt1A-selected lines. Approximately, 7000–8000 larvae on average were selected with Bs or Bs + Cyt1A in the BsRX derived lines. Mortality averaged 76.6% (SD = 12.3) through generation 13 for BsRX-Bs-selected line and 86.8% (SD = 5.1) for the BsRX-Bs + Cyt1A-selected line. After generation 13, a concentration of 160,000 ng/ml gave virtually no mortality in the BsRX-Bs-selected line. Consequently, selection pressure was not increased due to the large quantities of Bs powder that would be required. Selection concentrations for the other lines increased steadily throughout the study (Figs. 1 and 2).

Dose–response results or estimates of the frequency of individuals resistant to Bs are presented in Tables 1–4. For simplified comparison among the selected lines and toxins tested, the resistance ratios for all the dose–response data are summarized in Table 5.

In the SynC populations, the line selected with Bs evolved very low levels of resistance to Bs, 2.5 and 6.8 at the LC_{50} and LC_{95} in generation 8, and resistance increased to 4.3 and 0.4 at the LC_{50} and LC_{95}, respectively, in generation 12 (Tables 1 and 5). However, resistance ratios exceeded 1000 in generations 16 and 20. The unselected line did not develop resistance. The SynC line selected with Bs + Cyt1A evolved 13.8-fold resistance at

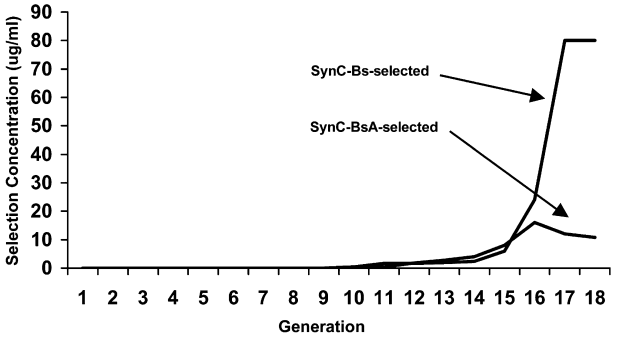

Fig. 1. Change in the selection concentration over 20 generations of selection pressure producing an average of 68–75.7% mortality against the SynC lines selected with Bs or with Bs + CytA at a 3:1 ratio.

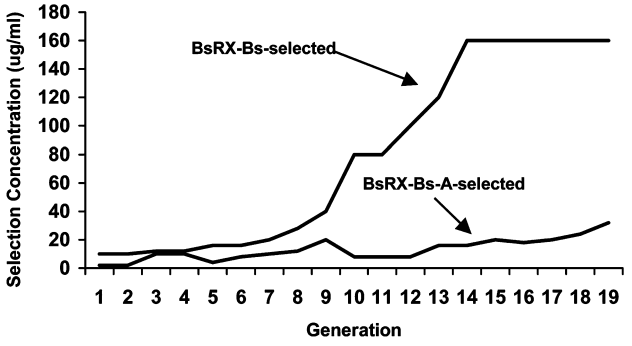

Fig. 2. Change in the selection concentration over 20 generations of selection pressure producing an average of 76.6–86.8% mortality against the BsRX lines selected with Bs or with Bs + CytA at a 3:1 ratio.

the LC_{95} toward the toxin mixture in generation 8, but no significant resistance was observed at the LC_{50} (Tables 2 and 5). Both resistance ratios regressed to 1.2 in generation 12. In generations 16 and 20, resistance ratios increased to 148 and 161 at the LC_{50}, but levels were considerably lower, 4.2 and 1.4 at the LC_{95}, for those same generations.

When the BsRX colony was selected with Bs, the frequency of resistant mosquitoes, defined as the percentage of mosquitoes surviving the diagnostic concentration of Bs, rapidly increased from an initial level of 18 to 79% after four generations of selection (Tables 3 and 5). By generation 8, the frequency of Bs-resistant mosquitoes reached 98–100%. In contrast, BsRX mosquitoes selected with Bs + Cyt1A evolved lower resistance levels toward the toxin mixture, 49.8- and 2.8-fold resistance at the LC_{50} and LC_{95}, respectively, after 12 generations of selection (Tables 4 and 5). Resistance ratios subsequently reached 205 and 43.2 at the LC_{50} and LC_{95}, respectively, in generation 16, and 85.4 and 6.7 at the LC_{50} and LC_{95}, respectively, in generation 20.

Mosquitoes selected with Bs were evaluated for their susceptibility to Bs + Cyt1A, whereas mosquitoes selected with Bs + Cyt1A were evaluated for their susceptibility to Bs. In the SynC line selected with Bs + Cyt1A, resistance to Bs first appeared in generation 4 with resistance ratios of 2.7 and 20 at the LC_{50} and LC_{95}, respectively (Tables 1 and 5). Resistance ratios increased to 3.6 and 213 in generation 8, declined to 3.2 and 3.8 in generation 12, and subsequently rebounded to >1000-fold by generations 16 and 20, reaching significantly higher resistance to Bs than was detected in that same generation toward Bs + Cyt1A. The parallel SynC line selected with Bs exhibited significantly lower resistance toward Bs + Cyt1A than it showed toward Bs,

Table 1
Evolution of resistance to *B. sphaericus* in strains of *Cx. quinquefasciatus* selected with either *B. sphaericus* 2363 or a 3:1 mixture of *B. sphaericus* 2362 + Cyt1A over 20 generations

Mosquito line[a]	Generation	LC_{50} (ng/ml)	Fiducial limits	LC_{95} (ng/ml)	Fiducial limits	Resistance Ratio		Slope
						LC_{50}	LC_{95}	
SynC-U	4	33.0	18.8–59.4	825	203–3,010	1.0	1.0	0.8
	8	22.0	13.3–47.3	735	359–1,580	1.0	1.0	0.9
	12	26.5	16.9–41.5	1,550	624–3,960	1.0	1.0	0.9
	16	5.7	4.41–7.28	459	286–828	1.0	1.0	0.9
	20	44.0	174–111	7,320	1,140–4,900	1.0	1.0	0.7
SynC-Bs-S	4	37.4	31.2–45.0	497	336–837	1.1	0.6	1.5
	8	62.4	27.5–144.0	5,020	458–60,000	2.5	6.8	0.9
	12	115.0	101.0–132.0	573	447–793	4.3	0.4	2.4
	16	NA[b]				>1000		
	20	NA[b]				>1000		
SynC-Bs + A-S	4	90.4	58.3–142.0	16,500	5,470–54,900	2.7	20.0	0.7
	8	79.9	31.8–199.0	157,000	$19{,}800–1.2 \times 10^6$	3.6	213.0	0.5
	12	84.5	462–154	5,890	1,860–19,000	3.2	3.8	0.9
	16	NA[c]				>1000		
	20	NA[c]				>1000		

[a] Mosquito strains were SynC-U, a control strain not subjected to selection pressure; SynC-Bs-S, the strain selected with *B. sphaericus* 2362; and SynC-Bs + A-S, the strain selected with a 3:1 ratio of *B. sphaericus* 2362 to Cyt1A of *B.t.* subsp. *israelensis*.

[b] NA, not applicable. At concentrations between 10,000–100,000 ng/ml, maximal mortality at generation 16 was 2% and at generation 20, 1%.

[c] NA, not applicable. At concentrations between 10,000–100,000 ng/ml, maximal mortality at generation 16 was 5.8% and at generation 20, 6%.

Table 2
Evolution of resistance to *B. sphaericus* + Cyt1Aa in strains of *Cx. quinquefasciatus* selected with either *B. sphaericus* 2363 or a 3:1 mixture of *B. sphaericus* 2362 + Cyt1A over 20 generations

Mosquito line[a]	Generation	LC_{50} (ng/ml)	Fiducial limits	LC_{95} (ng/ml)	Fiducial limits	Resistance Ratio		Slope
						LC_{50}	LC_{95}	
SynC-U	8	60.3	36.2–100	4,740	1,750–13,100	1.0	1.0	0.9
	12	79.3	45.0–144	2,480	646–10,100	1.0	1.0	1.1
	16	17.7	10.0–31.2	3,570	989–13,400	1.0	1.0	0.7
	20	17.1	11.2–25.5	9,390	4,560–23,200	1.0	1.0	0.6
SynC-Bs-S	8	74.4	45.2–123	19,200	5,890–65,400	1.2	4.1	0.7
	12	44.6	24.9–800	1,110	329–3,820	0.6	0.4	1.2
	16	2,530	1,240–5,170	13,300	3,480–51,700	143	3.7	2.3
	20	10,900	7,240–16,500	86,800	38,400–204,000	641	9.2	1.8
SynC-Bs + A-S	8	117	74.9–184	65,200	20,000–227,000	1.9	13.8	0.6
	12	97.3	78.7–121	3,040	2,060–4,840	1.2	1.2	1.1
	16	2,630	1,210–5,710	14,900	3,860–58,100	148	4.2	2.2
	20	2,760	1,420–5,350	13,100	3,900–44,600	161	1.4	2.4

[a] Mosquito strains were SynC-U, a control strain not subjected to selection pressure; SynC-Bs-S, the strain selected with *B. sphaericus* 2362; and SynC-Bs + A-S, the strain selected with a 3:1 ratio of *B. sphaericus* 2362 to Cyt1A of *B.t.* subsp. *israelensis*.

Table 3
Survival of BsRX strain of *Cx. quinquefasciatus* treated with 100,000 ng/ml *B. sphaericus* 2362 over 20 generation of selection with *B. sphaericus* or a 3:1 ratio of *B. sphaericus* 2362 to Cyt1A

Generation	Percent survival		
	BsRX	BsRX-Bs	BsRX-Bs + A
1	18	18	18
4	9	79	100
8	16	100	96
12	8	98	99
16	8	100	100
20	6	100	100

Mosquito strains were BsRX, a control strain developed by crossing Bs-R and Syn-P, not subjected to selection pressure; BsRX-Bs, the strain selected with *B. sphaericus* 2362; and BsRX-Bs + A, the strain selected with a 3:1 ratio of *B. sphaericus* 2362 to Cyt1A of *B.t.* subsp. *israelensis*.

with resistance ratios of 143 and 641 at the LC_{50} in generations 16 and 20. Concurrent LC_{95} values were even lower and were significant only in generation 20, at 9.2-fold (Tables 2 and 5).

In the BsRX line selected with Bs + Cyt1A but assayed with Bs, the frequency of Bs-resistant larvae was 96% or greater after four generations of selection and remained high for the remainder of the study (Tables 3 and 5). The resistance ratio was estimated to exceed 1000-fold and was significantly higher than the concurrent level of resistance in that line against Bs + Cyt1A. The BsRX line selected with Bs but assayed with Bs + Cyt1A showed 95-fold resistance at the LC_{50} to that mixture in generation 12 (Tables 4 and 5). The resistance ratios at the LC_{50} in generations 16 and 20 were 149 and 44.8. Resistance ratios were 7.3, 15.0, and 3.3 at the LC_{95} in generations 12, 16, and 20, respectively, and were considerably lower than the levels detected at the LC_{50}. Resistance levels were significantly lower toward the Bs + Cyt1A mixture than toward Bs alone in that Bs-selected line.

The insecticide concentrations needed for selection in the two series followed similar patterns. Concentrations of Bs rose from 3.2 to 80,000 ng/ml over 20 generations

Table 4
Evolution of resistance to *B. sphaericus* + Cyt1A in BsRX strains of *Cx. quinquefasciatus* selected with either *B. sphaericus* 2363 or a 3:1 mixture of *B. sphaericus* 2362 + Cyt1A over 20 generations

Mosquito line[a]	Generation	LC_{50} (ng/ml)	Fiducial limits	LC_{95} (ng/ml)	Fiducial limits	Resistance Ratio		Slope
						LC_{50}	LC_{95}	
BsRX-U	12	44.5	35.3–55.8	4,220	2760–6950	1.0	1.0	0.8
	16	20.6	16.2–26.1	847	519–1580	1.0	1.0	1.0
	20	95.0	77.1–117	4,740	3200–7500	1.0	1.0	1.0
BsRX-Bs	12	4230	2330–7680	30,500	9160–103,000	95	7.3	1.9
	16	3070	2660–3530	12,700	10,200–16,900	149	15.0	2.7
	20	4260	3780–4780	15,700	447–793	44.8	3.3	2.9
BsRX-Bs + A	12	2220	1211–4420	11,700	3450–40,300	49.8	2.8	2.3
	16	4240	2380–7530	36,600	12,800–107,000	205	43.2	2.0
	20	8110	7160–9150	31,700	25,800–41,400	85.4	6.7	2.8

[a] Mosquito strains were BsRX, a control strain developed by crossing Bs-R and Syn-P, not subjected to selection pressure; BsRX-Bs, the strain selected with *B. sphaericus* 2362; and BsRX-Bs + A, the strain selected with a 3:1 ratio of *B. sphaericus* 2362 to Cyt1A of *B.t.* subsp. *israelensis*.

Table 5
Summary of data on the evolution of resistance in strains of *Cx. quinquefasciatus* larvae exposed to *B. sphaericus* or *B. sphaericus* + Cyt1A over 20 generations

Generation	SynC strain				BsRX strain			
	Bs-sel[a]		Bs + Cyt1A-sel[a]		Bs-sel[b]		Bs + Cyt1A-sel[b]	
	RR_{50}	RR_{95}	RR_{50}	RR_{95}	RR_{50}	RR_{95}	RR_{50}	RR_{95}
Tested with B. sphaericus								
4	1.1	0.6	2.7	20	—	—	>1000	
8	2.5	6.8	3.6	213	>1000	—	>1000	
12	4.3	0.4	3.2	3.8	>1000		>1000	
16	>1000		>1000		>1000		>1000	
20	>1000		>1000		>1000		>1000	
Tested with B. sphaericus + Cyt1A								
8	1.2	4.1	1.9	13.8	—	—	—	—
12	0.6	0.4	1.2	1.2	95	7.3	49.8	2.8
16	143	3.7	148	4.2	149	15	205	43.2
20	641	9.2	161	1.4	44.8	3.3	85.4	6.7

[a] Mosquito strains were SynC-U, a control strain not subjected to selection pressure; SynC-Bs-S, the strain selected with *B. sphaericus* 2362; and SynC-Bs + A-S, the strain selected with a 3:1 ratio of *B. sphaericus* 2362 to Cyt1A of *B.t.* subsp. *israelensis*.
[b] Mosquito strains were BsRX, a control strain developed by crossing Bs-R and Syn-P, not subjected to selection pressure; BsRX-Bs, the strain selected with *B. sphaericus* 2362; and BsRX-Bs + A, the strain selected with a 3:1 ratio of *B. sphaericus* 2362 to Cyt1A of *B.t.* subsp. *israelensis*.

of selection against the SynC colony (Fig. 1). Concentrations of Bs + Cyt1A rose from 2.0 to 11,000 ng/ml in that same period. Against the BsRX colony, concentrations of Bs rose from 10,000 to 160,000 ng/ml by generation 13, where they were maintained to conserve powder (Fig. 2). Concentrations of Bs + Cyt1A rose from 2000 to 32,000 ng/ml over 20 generations of selection against mosquitoes derived from the BsRX colony.

4. Discussion

After 20 generations of intensive selection pressure, mosquitoes selected with Bs alone evolved more than 1000-fold resistance, regardless of the presence or absence of Bs resistance alleles in the original parental population. In contrast, mosquitoes selected with Bs + Cyt1A evolved more moderate levels of resistance toward that mixture; resistance ratios reached a maximum of 161 at the LC_{50} in the SynC line and 149 at the LC_{50} in the BsRX line. These results show that the phenotypic expression of resistance was constrained in mosquitoes selected with the Bs + Cyt1A mixture.

Although the levels of resistance that evolved toward the mixture were significantly lower in mosquitoes selected with Bs + Cyt1A, the mixture did not prevent the underlying evolution of resistance to Bs, as demonstrated by the high levels of Bs resistance detected in the absence of Cyt1A. However, the lower resistance that evolved to Bs + Cyt1A over 20 generations of selection with the toxin mixture does illustrate the benefit of the increased toxin complexity and synergy contributed by Cyt1A. These results are consistent with resistance evolution in *Cx. quinquefasciatus* selected with the Bs strain IAB59, which contains a 49.4 kDa component in addition to the Bin toxins (Pei et al., 2002). Mosquitoes selected with Bs IAB59 evolved much lower resistance than those selected with a Bs strain lacking this additional protein. The Bs + Cyt1A mixture in this study was less effective at avoiding resistance evolution than a mixture of Bs and *B.t.* subsp. *israelensis*. In combination, mixtures of these two wild-type species were moderately effective at reversing resistance in a laboratory colony of *Cx. quinquefasciatus* previously selected for resistance to Bs (Zahiri et al., 2002). Moreover, the Bs/*B.t.* subsp. *israelensis* mixture avoided the evolution of Bs resistance when it was used proactively against a susceptible laboratory line for more than 30 generations (Zahiri and Mulla, 2003). These results, in conjunction with the results from the present study, support the hypothesis that Bs insecticides containing greater toxin complexity can effectively retard the evolution of resistance.

A distinctive characteristic of the dose–response lines of mosquitoes selected with Bs + Cyt1A was a decline in variation to that mixture, apparent from the 2- to 3-fold increase in slope of dose–response lines (Tables 2 and 4). Consequently, LC_{50} values increased more dramatically than corresponding values at the LC_{95} and resistance ratios at the LC_{50} were 1–2 orders of magnitude larger than the resistance ratios at the LC_{95}. The implication is that mosquitoes exposed to Bs + Cyt1A remained very sensitive to higher insecticide concentrations of the toxin mixture. These results also suggest that the Bs + Cyt1A selected larvae incurred significant selection pressure which, over time, caused an overall reduction in population variation toward the toxin mixture. Consequently, the Bs + Cyt1A selected lines were constrained from evolving higher resistance because the population lacked sufficient genetic heterogeneity to further respond to directional selection pressure with the mixture. This lack

of heterogeneity was not evident in mosquitoes originating from the same gene pool and selected solely with Bs; those larvae evolved significantly higher resistance. The key factor constraining the evolution of resistance was the presence of the Cyt1A toxin from *B.t.* subsp. *israelensis*.

Another interesting observation was the failure of the unselected parental BsRX colony to regain full susceptibility in the absence of Bs selection pressure. One potential outcome to the removal of insecticide selection pressure was the eventual loss of the resistance allele from that population. Bs resistance in BsR colony is controlled by a monofactorial, recessive allele (Wirth et al., 2000a), and in the absence of insecticide selection pressure, might have reduced fitness. After 25 generations of standard rearing without insecticide exposure, the frequency of homozygous-resistant larvae declined to 6%. Assuming that Hardy–Weinberg equilibrium conditions existed, the estimated frequency of Bs alleles would be 0.245, a 42% reduction in frequency from its level (0.424) in generation 5. Fitness influence of a recessive trait may be minimal in the heterozygous insects, which are fully susceptible to the action of Bs. Bin toxins have an intracellular mode of action, and although the α-glucosidase receptor for Bin is absent from the epithelial membrane in resistant mosquitoes because of premature truncation of the gene for its GPI membrane anchor, the α-glucosidase is expressed within the cytoplasm and thus can contribute to metabolism in the insect (Darboux et al., 2002). Consequently, little reduction in fitness would be expected. This hypothesis is supported by the relatively slow decline in the frequency of resistance alleles observed in the unselected BSRX line. However, it is important to consider that the fitness of this resistance allele may be quite different under field conditions.

It was not unexpected that mosquitoes would evolve high levels of resistance under intensive laboratory selection pressure with Bs and the results herein concur with reports from previous laboratory selection studies (Rodcharoen and Mulla, 1994; Wirth et al., 2000a) and with the reports of resistance in field-treated lines (Rao et al., 1995; Silva-Filha et al., 1995; Sinégre et al., 1994; Yuan et al., 2000). However, the rapid evolution of Bs resistance in the two lines selected with Bs + Cyt1A was somewhat unexpected. Resistance ratios toward Bs exceeded 1000-fold in generation 16 of SynC selected with Bs + Cyt1A. In BsRX line selected with Bs + Cyt1A, the frequency of Bs resistance increased from 18% to greater than 99% after only four generations of selection and the frequency of Bs-resistant individuals was higher than in the parallel line selected only with Bs, which had a frequency of 79%. A similar pattern of high Bs resistance in the absence of the 49.4 kDa component was reported in the Bs IAB59-selected mosquitoes (Pei et al., 2002). One inference from this observation is that mosquitoes apparently experienced greater Bs selection pressure when exposed to Bs + Cyt1A than when selected solely with Bs, which raises some interesting questions about the mechanism of Bs and Cyt1A synergy.

Evidence for a potential mechanism driving the rapid evolution of Bs resistance in mosquitoes selected with Bs + Cyt1A may be found in recent in vivo binding studies with *Cx. quinquefasciatus*. Mosquito larvae were fed Bs and Cyt1A toxins labeled with different fluorescent tags. Bs fluorescence was observed in the cytosol of epithelial midgut cells of susceptible larvae, whereas Cyt1A fluorescence was limited primarily to the microvilli. Resistant mosquitoes failed to bind or incorporate Bs toxin into epithelial cells. However, in the presence of Cyt1A, Bs was rapidly detected in the cytosol of midgut epithelial cells of resistant larvae; much more quickly than in susceptible mosquitoes (Federici et al., 2003). Cyt1A's effect on the epithelial membrane apparently accelerates the passage of Bs to the cytosol of targeted cells where it can exert toxicity, and this phenomenon may explain both the earlier reports of Bs/Cyt1A synergy in Bs-resistant mosquitoes (Wirth et al., 2000b) and the increased Bs selection pressure experienced by mosquitoes treated with the toxin mixture. Despite the rapid onset and the high level of Bs resistance that evolved in Bs + Cyt1A-selected mosquitoes, the toxin mixture retained high activity and those same insects expressed low resistance (<15-fold at the LC_{95}) to Bs + Cyt1A.

The Bs resistance levels observed in mosquitoes selected with Bs + Cyt1A are not consistent with the resistance levels that evolved against Cry11A from *B.t.* subsp. *israelensis* in mosquitoes selected with a 3:1 mixture of Cry11A + Cyt1A (Wirth et al., 2005). Resistance was only 9-fold toward Cry11A in mosquitoes selected with Cry11A + Cyt1A, whereas >1000-fold resistance evolved in the line selected solely with Cry11A. The contrast in the level of Cry11A-resistance that evolved when mosquitoes were selected with Cry11A + Cyt1A with the level of resistance that evolved to Bs in mosquitoes selected with Bs + Cyt1A may be due to fundamental differences in the interaction of Cyt1A with these two materials or to differences in the mode of action of Cry11A, which acts through colloid-osmotic lysis (Knowles and Ellar, 1987), versus Bs, which acts within the cytosol of midgut epithelial cells (Davidson et al., 1987; Oei et al., 1992; Silva-Filha and Peixoto, 2003).

Bs is an important microbial agent for mosquito control, yet its practical field life is limited by the propensity for mosquitoes to evolve resistance. Various strategies have been proposed to manage this problem, however recent evidence suggests that increased toxin diversity may be the more effective approach. Toxin diversity could be increased by formulating insecticides to contain both Bs and *B.t.* subsp. *israelensis*, or through recombinant bacteria engineered to express toxins from both microbes (Federici et al., 2003; Zahiri and Mulla, 2003). Although a cautious approach to adopting novel insecti-

cides is needed, they may provide the tools needed for controlling mosquito populations.

Acknowledgments

Assistance from Hyun-Woo Park and Jeffrey J. Johnson is gratefully acknowledged. This project was supported by US Public Health Service Grant AI45817 and the University of California Mosquito Control Research Program.

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