Evolution of Resistance in *Culex quinquefasciatus* (Say) Selected With a Recombinant *Bacillus thuringiensis* Strain-Producing Cyt1Aa and Cry11Ba, and the Binary Toxin, Bin, From *Lysinibacillus sphaericus*

MARGARET C. WIRTH,^{1,2} WILLIAM E. WALTON,¹ AND BRIAN A. FEDERICI^{1,3}

J. Med. Entomol. 52(5): 1028–1035 (2015); DOI: 10.1093/jme/tjv115

ABSTRACT Fourth instars of *Culex quinquefasciatus* (Say) (Diptera: Culicidae) were selected with a recombinant bacterial strain synthesizing the mosquitocidal proteins from *Lysinibacillus sphaericus* (Bin) and Cry11Ba and Cyt1Aa from *Bacillus thuringiensis*. Selection was initiated in Generation 1 with a concentration of 0.04 µg/ml, which rose to a maximum selection concentration of 8.0 µg/ml in Generation 14, followed by an unexpected, rapid increase in mortality in Generation 15. Subsequently, a selection concentration of 0.8 µg/ml was determined to be survivable. During this same period, resistance rose to nearly 1,000-fold (by Generation 12) and declined to 18.8-fold in Generation 19. Resistance remained low and fluctuated between 5.3 and 7.3 up to Generation 66. The cross-resistance patterns and interactions among the component proteins were analyzed to identify possible causes of this unusual pattern of evolution. Poor activity in the mid-range concentrations and lower-than-expected synergistic interactions were identified as potential sources of the early resistance. These findings should be considered in the development of genetically engineered strains intended to control nuisance and vector mosquitoes.

KEY WORDS recombinant *Bacillus thuringiensis*, mosquito, resistance

An estimated 2 billion people live in areas where mosquito-borne diseases are endemic (WHO 1999) and, consequently, controlling adult and larval populations of mosquitoes that vector disease pathogens can play an important role in improving human health. Historically, mosquito control relied on synthetic organic insecticides, which efficiently suppressed populations, but long-term use of these led to unanticipated consequences, namely, adverse effects on nontarget organisms and the evolution of insecticide resistance in populations of many mosquito species. The discovery of effective mosquito bacteria, specifically, *Bacillus thuringiensis* subsp. *israelensis* (Goldberg and Margalit 1977) and *Lysinibacillus sphaericus* (Kellen and Meyers 1964) (formerly *Bacillus sphaericus*; Ahmed et al. 2007), and their commercial development as larvicides enabled the safe and effective control of larval mosquito populations in many parts of the world (Porter et al. 1993). Unfortunately, products based on these two bacteria do not provide effective control against all mosquito species, nor do they function

optimally under all environmental conditions (Mulla 1990, Berry et al. 1993, Berry 2012). In addition, reliance on a small number of larvicides raises a serious concern for the evolution of insecticide resistance. This is apparent from the cases of resistance that have already been reported to *L. sphaericus* in France, India, Brazil, China, Thailand, and elsewhere (Sinègre et al. 1994, Rao et al. 1995, Silva-Filha et al. 1995, Yuan et al. 2000, Su and Mulla 2004).

Previous research has shown that a highly complex mosquitocidal bacterium like *B. thuringiensis* subsp. *israelensis* is less likely to select for resistance in treated mosquito populations (Becker and Ludwig 1993, Georgiou and Wirth 1997) than a bacterium such as *L. sphaericus* that acts at a single target site in the mosquito larval midgut (Rodcharoen and Mulla 1994, Silva-Filha et al. 1995, Wirth et al. 2000b). At sporulation, native *B. thuringiensis* subsp. *israelensis* produces a crystalline parasporal body that contains four major insecticidal proteins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, and laboratory studies demonstrated that the refractoriness to selecting resistance is a consequence of the complex interactions among these mosquitocidal proteins produced by *B. thuringiensis* subsp. *israelensis*. Individually, these proteins are mosquitocidal, but activity is poor to moderate (Crickmore et al. 1995, Poncet et al. 1995). However, when they are combined, as they naturally occur in the wild-type bacterium, activity is significantly increased owing to synergy to

¹ Department of Entomology, University of California, Riverside, CA 92521.

² Corresponding author, e-mail: mcwirth@ucr.edu.

³ Interdepartmental Graduate Programs in Microbiology, and Cell, Molecular and Developmental Biology, University of California, Riverside, CA 92521.

a level that exceeds their additive activity by 2- to 15-fold, depending on the mosquito species and toxin combination tested (Crickmore et al. 1995, Poncet et al. 1995). Moreover, and importantly, synergistic interactions occur between these *B. thuringiensis* subsp. *israelensis* toxins and those of other mosquitocidal bacterial strains, including the Bin toxins from *L. sphaericus* and Cry11Ba from *B. thuringiensis* subsp. *jegathesan* (Wirth et al. 2004a, b), and that synergy suppresses high levels of resistance to those (Wirth et al. 2000a, b). One proposed strategy to enhance and diversify commercial bacterial larvicides is to engineer strains to synthesize specific larvicidal proteins from a variety of different bacterial strains specifically chosen to avoid the known problems associated with commercial strains.

Toward this goal, a recombinant strain of *B. thuringiensis* that synthesizes the Cyt1Aa protein from *B. thuringiensis* subsp. *israelensis*, the Cry11Ba protein from *B. thuringiensis* subsp. *jegathesan*, and the Bin protein of *L. sphaericus* was constructed (Park et al. 2003), thus combining the high toxicity of the latter two proteins with the established capacity of Cyt1Aa to synergize the activity of these proteins and to delay the evolution of resistance in populations of mosquito larvae (Wirth et al. 1997, 2005a; Wirth and Georgiou 1997). The purpose of the present study was to test the effectiveness of this recombinant strain for its ability to delay the evolution of resistance in larvae of *Culex quinquefasciatus* (Say) (Diptera: Culicidae). Here, we report the results of long-term selection studies and show that, contrary to our initial hypothesis, resistance evolved rapidly in early generations, but abruptly declined and remained low thereafter. Analysis of resistance to the component toxins revealed high Bin resistance and low Cry11Ba resistance after 25 generations of selection. No resistance to Cyt1Aa was detected. These results are discussed in relation to the interactions among the components and their impact on the suppression and/or evolution of resistance.

Materials and Methods

Bacterial Strains. Four bacterial strains were used for this study. A highly active, recombinant strain was synthesized using acrySTALLIFEROUS *B. thuringiensis* to synthesize the binary protein Bin from *L. sphaericus*, the Cyt1Aa protein from *B. thuringiensis* subsp. *israelensis*, and the Cry11Ba protein from *B. thuringiensis* subsp. *jegathesan* (Park et al. 2003), hereafter referred to as Bin11BCyA. Technical powder of *L. sphaericus* was obtained from Valent Biosciences (Libertyville, IL). The individual mosquitocidal active proteins were also produced. Cyt1Aa was produced in an acrySTALLIFEROUS strain of *B. thuringiensis* (Wu and Federici 1993), and Cry11Ba was produced in a strain of *B. thuringiensis* subsp. *thuringiensis* (H1; Délecluse et al. 1995). All materials were in the form of lyophilized, crystal/spore powders. The strains were grown on liquid media as described previously (Park et al. 2005). Sporulated cells were washed in distilled water, pelleted, lyophilized, and stored in the refrigerator.

Mosquitoes. Four colonies of *Cx. quinquefasciatus* were used in this study. The parental colony, RecSyn, was established from field-collected larvae combined with larvae from three other synthetic populations previously established in the laboratory (Wirth et al. 2010). After 3 mo of interbreeding in the synthetic parental colony, a second colony, named Bin11BCyA, was derived from RecSyn by selection with the recombinant bacterial strain Bin11BCyA. In post hoc tests, the susceptible reference colony CqSyn (Wirth et al. 2004a), a contributor to the RecSyn synthetic colony, was used to evaluate the interactions among the various components of the recombinant, as the RecSyn colony was no longer in culture. In addition, the laboratory Bin-resistant reference colony, BsR (Wirth et al. 2000b), was included in some testing to quantify any effects of toxin interaction on high-level Bin resistance.

Selection and Bioassay Procedures. Toxin suspensions and mixtures of toxin powders were prepared on the basis of the weight of the toxin/spore powders. A 1:1 ratio was used for the two-toxin mixtures. Scanned gels of the recombinant Bin11BCyA powder indicated that the ratios of the components were ~2.6:1.2:1.0, respectively (data not shown), thus a 3:1:1 ratio for the three-toxin mixture was used. Bioassays involved feeding groups of 20 early fourth instars with various concentrations of suspended crystal/spore powder in 250-ml plastic cups in 100 ml deionized water. Six or more concentrations plus a water control were used for each replication. Stock suspensions were prepared monthly and stored at -20°C when not in use, and dilutions were prepared weekly on the day of the assay. All bioassays included concurrent assays using the same materials and the same suspensions on the RecSyn colony to ensure that suspensions exhibited no loss of activity owing to storage. In total, five replications on five different days were performed. Mortality was determined after 48 h. Control mortality was rare, never exceeded 2%, and was corrected by using Abbott's formula. Colonies were assayed every third generation, except for Generation 15, which was skipped owing to high mortality and instability in the colony, described in greater detail below. Data were analyzed using a probit analysis program for the PC (Raymond et al. 1993). Synergy factors (SF_{50} and SF_{95}) were calculated according to Tabashnik (1992), in which values greater than 1.0 indicate synergy. Because the dose-response line for BsR treated with the mixture of Bin + Cry11Ba + Cyt1Aa was nonlinear, the LC_{50} and LC_{95} were read directly from the data as plotted on log-probit graph paper and used solely to estimate the presence or absence of synergy. Data for BsR, tested with Cyt1Aa and Bin + Cyt1Aa (1:1), were taken from Wirth et al. (2000c).

Selections involved exposing groups of 1,000 early fourth instars to suspensions of crystal/spore powder in enamel metal pans containing 1 liter of deionized water. Survivors were recovered into clean water and fed after 48 h. Generations were maintained separately for 20 generations, after which generations were allowed to overlap. Selection was briefly suspended for Generation 16 for logistical reasons and unselected

pupae of that generation were added to the cage to maintain high population density. However, selection was resumed immediately thereafter.

Results

Baseline dose–response values for powders of the recombinant strain Bin11BCytA against the synthetic population, RecSyn, were measured prior to the initiation of selection pressure. The LC_{50} was 0.00434 $\mu\text{g/ml}$, and the LC_{95} was 0.0254 $\mu\text{g/ml}$ after 48 h (data not shown). Large-scale fermentation was then used to produce significant quantities of lyophilized powder for the subsequent selections and bioassays throughout the project. That fermentation was slightly lower in activity than observed in the first tests. In Generation 3, the larger fermentation tested against RecSyn showed LC_{50} and LC_{95} values of 0.0142 and 0.134 $\mu\text{g/ml}$, respectively (Table 1). Concurrent assays on colonies RecSyn and Bin11BCytA already showed significant divergence in susceptibility in Generation 3. Bin11BCytA showed values of 0.127 and 2.26 $\mu\text{g/ml}$ and resistance ratios of 8.9 and 16.9 at the LC_{50} and LC_{95} , respectively. Susceptibility continued to decline under selection pressure, and resistance levels were 36.7-, 165-, and 999-fold at the LC_{50} in Generations 6, 9, and 12, respectively.

Generations 12 and 13 were selected with 6 and 8 $\mu\text{g/ml}$, respectively, and mortality was 77–78%. In Generation 14, the selected colony was treated with 8 $\mu\text{g/ml}$, and an unexpectedly high average mortality, exceeding 90%, was observed. Some selected pans yielded 100% mortality. That concentration, and higher concentrations, had been successfully used in the previous three selected generations, i.e., 11, 12, and 13. Consequently, despite treating 11,000 larvae, only 894 larvae survived after 48 h. In Generation 15, 10,000 larvae were selected at progressively lower concentrations

ranging from 6 $\mu\text{g/ml}$ down to 1 $\mu\text{g/ml}$. However, as overall survival was too low to guarantee continuing the colony, 5,000 more larvae were selected at 1 $\mu\text{g/ml}$ to rescue the colony and provide sufficient numbers of adults. Owing to the precarious state of the colony, scheduled bioassays at Generation 15 were omitted in the interests of stabilizing the colony and Generation 16 was unselected to guarantee that the colony would recover vigor. In Generation 17, a survivable concentration was identified as 0.8 $\mu\text{g/ml}$, reflecting mortality rates averaging 85%. During this period, the lyophilized powder from the large fermentation was rechecked by sodium dodecyl sulphate gel electrophoresis and was confirmed to be the recombinant strain Bin11BCytA and correctly expressing the desired proteins (data not shown). Bioassays were performed in Generation 19, and a large change in LC_{50} and LC_{95} was revealed, with values of 0.326 and 1.59 $\mu\text{g/ml}$, respectively, for the selected line, whereas the LC values for the unselected parental colony, RecSyn, were not significantly changed. Resistance ratios had declined from 999-fold at the LC_{50} in Generation 12 to 18.8-fold in Generation 19.

After Generation 20, generations were no longer maintained separately and ongoing generations overlapped, although selection continued. In approximate Generation 25, resistance levels were 3.4 at the LC_{50} , and although some selection pressure increases were tolerated, resistance fluctuated between 5.3- and 7.3-fold at the LC_{50} and, thereafter, up to an estimated Generation 66.

In Generation 25, susceptibility to the individual component toxins expressed by the recombinant strain, Bin, Cry11Ba, and Cyt1Aa, was evaluated by bioassay. High-level resistance to the Bin toxins of *L. sphaeiricus* was detected. LC_{50} and LC_{95} values were not measurable, and the population displayed average mortality of 40.8% to concentrations ranging from 50 to 200 $\mu\text{g/ml}$,

Table 1. Dose–response values for RecSyn and Bin11BCytA strains of *Cx. quinquefasciatus* fourth instars assayed during selection with the recombinant bacterium *B. thuringiensis* subsp. *israelensis* BinCry11BCytA

Colony	Generation	LC_{50} $\mu\text{g/ml}$	Fiducial limits	LC_{95} $\mu\text{g/ml}$	Fiducial limits	Resistance ratio	
						LC_{50}	LC_{95}
RecSyn	3	0.0142	(0.0106–0.0191)	0.134	(0.0797–0.232)	1.0	1.0
Bin11BCytA	3	0.127	(0.105–0.154)	2.26	(1.52–3.74)	8.9	16.9
RecSyn	6	0.0107	(0.00603–0.0187)	0.209	(0.0806–0.550)	1.0	1.0
Bin11BCytA	6	0.391	(0.319–0.482)	15.8	(10.4–25.9)	36.7	75.5
RecSyn	9	0.0201	(0.0126–0.0320)	0.271	(0.116–0.639)	1.0	1.0
Bin11BCytA	9	3.31	(2.91–3.77)	16.9	(13.6–22.0)	165	62.5
RecSyn	12	0.0941	(0.00635–0.0139)	0.138	(0.0683–0.289)	1.0	1.0
Bin11BCytA	12	9.40	(6.17–14.4)	116.9	(48.0–298)	999	848
RecSyn	19	0.0173	(0.0112–0.0265)	0.0937	(0.0429–0.213)	1.0	1.0
Bin11BCytA	19	0.326	(0.286–0.375)	1.59	(1.23–2.19)	18.8	16.9
RecSyn	25	0.0416	(0.0361–0.0476)	0.235	(0.187–0.313)	1.0	1.0
Bin11BCytA	25	0.143	(0.127–0.161)	0.583	(0.476–0.751)	3.4	2.5
RecSyn	~30	0.0729	(0.0520–0.102)	0.556	(0.296–1.07)	1.0	1.0
Bin11BCytA	~30	0.383	(0.208–0.701)	2.38	(0.781–7.35)	5.3	4.3
RecSyn	~35	0.0467	(0.0414–0.0529)	0.166	(0.133–0.223)	1.0	1.0
Bin11BCytA	~35	0.247	(0.214–0.285)	1.79	(1.41–2.41)	5.3	10.8
RecSyn	~45	0.110	(0.0664–0.182)	0.441	(0.161–1.37)	1.0	1.0
Bin11BCytA	~45	0.805	(0.445–1.45)	5.36	(1.78–16.6)	7.3	12.1
RecSyn	~66	0.151	(0.131–0.175)	1.11	(0.863–1.50)	1.0	1.0
Bin11BCytA	~66	0.819	(0.687–0.962)	8.50	(6.37–12.1)	5.4	7.7

and an estimated resistance level of >900-fold (Table 2). Susceptibility to *L. sphaeरिकus* was retested in approximate Generation 66, and susceptibility had declined further, to an average mortality of 37.4% between concentrations 0.1 and 200 µg/ml. In contrast, in Generation 25, the selected colony evolved only low-level resistance to the Cry11Ba, and no resistance was detected to Cyt1Aa. Bioassays with Cry11Ba revealed LC₅₀ and LC₉₅ values of 0.743 and 14.1 µg/ml, and low-to-moderate levels of resistance with ratios of 2.3 and 8.3, respectively. No significant resistance to Cyt1Aa was detected, and the LC₅₀ and LC₉₅ were 45.4 and 392 µg/ml, at the LC₅₀ and the LC₉₅, respectively.

Because specific interactions (i.e., synergy) had not previously been examined between all the various components of the recombinant bacterium, single, pairwise, and the mixture of the three components were assayed against a second susceptible reference colony, CqSyn, as the RecSyn colony had been terminated. A subset of those materials was also screened against the Bin-resistant laboratory colony, BsR (Wirth et al. 2000b). Bin + Cyt1Aa (1:1), Bin + Cry11Ba (1:1), Cry11Ba + Cyt1Aa (1:1), and Bin + Cry11Ba + Cyt1Aa (3:1:1) all interacted synergistically against the CqSyn colony (Table 3). The highest level of synergy was detected between Bin + Cry11Ba, with SF values of 16.4 and 7.1 at the LC₅₀ and LC₉₅, respectively. All other SF values ranged from 1.3 to 7.1. The mixture of Bin + Cry11Ba + Cyt1Aa showed SF values of 1.3 at the LC₉₅, whereas the SF value at the LC₅₀ was significantly higher at 6.7. Against the Bin-resistant colony, BsR, Bin + Cry11Ba revealed 3.0-fold synergy at both the LC₅₀ and LC₉₅, and Bin resistance was suppressed from >1000 to 23.2 and 3.3-fold at the LC₅₀ and the LC₉₅, respectively. Previous data showed that a 1:1 ratio of Bin + Cyt1Aa was synergistic, with SF values of 88 and 69 at the LC₅₀ and the LC₉₅, respectively, and resistance to Bin was reduced from >10,000 to 14.4 at the LC₉₅ (Wirth et al. 2000a). There was a notable decline in the activity in the mid-range of the dose-response data for the three-toxin mixture (Fig. 1) that resulted in a nonlinear dose response in BsR, whereas high concentrations were quite active. In contrast, the same mixture produced a linear dose-response line when tested against CqSyn. Synergy values for the

Bin + Cry11Ba + Cyt1Aa mixture were derived from the estimated LC₅₀ and LC₉₅ from the dose-response data line, found to be positive, and were between 3- and 4-fold at the LC₅₀ and LC₉₅, respectively.

Discussion

Under long-term selection pressure with a recombinant bacterial strain synthesizing the proteins for Bin, Cry11Ba, and Cyt1Aa, fourth instars of *Cx. quinquefasciatus* evolved moderate resistance after three generations of treatment. Resistance levels increased steadily up to Generation 12, where the highest measured resistance was 999-fold at the LC₅₀. However, high resistance was not sustained under continued selection pressure, and in Generation 15, the colony was unable to tolerate the same level of selection pressure, and resistance to Bin11BCyA declined dramatically. Despite ongoing selection pressure over ~66 generations, the colony failed to evolve higher resistance, and resistance ratios remained between 3- and 7-fold.

The result of selection using Bin11BCyA is in marked contrast to a previous selection using a different recombinant strain, Bti/Bs (Wirth et al. 2010). That recombinant strain consisted of *B. thuringiensis* subsp. *israelensis* engineered to express the Bin toxin of *L. sphaeरिकus* in addition to its native toxin complex (Park et al. 2005). Selection over 35 generations led to a maximum resistance level of 5-fold in Generation 21, but resistance declined to around 2-fold thereafter (Wirth et al. 2010). Because this latter recombinant was superior by this measure to the Bin11BCyA recombinant, the differences in their toxin composition and the interactions of those components may provide important insights into key traits necessary to optimal design of future recombinant bacterial strains.

Toxin interactions have been generally shown to influence the evolution of resistance in two ways. First, toxin synergy among the various components directly delays the rate and reduces the level of resistance that evolves in susceptible larvae. This effect was first observed in selection studies with *B. thuringiensis* subsp. *israelensis* and its component toxins, in which resistance levels were lower, and resistance onset was delayed, as the number of toxins increased and, most importantly, when Cyt1Aa was present (Georghiou and

Table 2. Resistance of *Cx. quinquefasciatus* fourth instars to individual toxins in response to selection pressure with the recombinant bacterium *B. thuringiensis* subsp. *israelensis* BinCry11BCyA

Toxin	Colony	Generation	LC ₅₀ µg/ml	Fiducial limits	LC ₉₅ µg/ml	Fiducial limits	Resistance ratio	
							LC ₅₀	LC ₉₅
BinCry11BCyA	RecSyn	25	0.0416	(0.0361–0.0476)	0.235	(0.187–0.313)	1.0	1.0
	Bin11BCyA	25	0.143	(0.127–0.161)	0.583	(0.476–0.751)	3.4	2.5
	RecSyn	25	0.222	(0.140–0.351)	1.89	(0.748–4.91)	1.0	1.0
	Bin11BCyA	25	Plateau of 40.8% mortality between 50–200 µg/ml				>900	
<i>L. sphaeरिकus</i>	RecSyn	~66	0.0780	(0.0674–0.0896)	0.427	(0.333–0.593)	1.0	1.0
	Bin11BCyA	~66	Plateau of 37.4% mortality between 0.1–200 µg/ml					
	RecSyn	25	0.324	(0.282–0.371)	1.70	(1.35–2.29)	1.0	1.0
	Bin11BCyA	25	0.743	(0.216–2.56)	14.1	(1.3–153)	2.3	8.3
Cry11B	RecSyn	25	31.8	(27.3–37.1)	273	(205–389)	1.0	1.0
	Bin11BCyA	25	45.4	(38.4–53.6)	392	(279–622)	1.4	1.4
Cyt1A	RecSyn	25						
	Bin11BCyA	25						

Table 3. Analysis of toxin interactions among *L. sphaericus* (strain 2362), Cry11Ba, and Cyt1Aa against a susceptible colon, CqSyn, and an *L. sphaericus*-resistant colony, BsR, of *Cx. quinquefasciatus*

Toxin	Colony	LC ₅₀	(Fiducial limit)	LC ₉₅ µg/ml	(Fiducial limits)	Synergism factor	
						LC ₅₀	LC ₉₅
BinCry11BCytA (Recombinant)	CqSyn	0.00166	(0.00134–0.00230)	0.0305	(0.0206–0.0510)	Na	Na
	BsR	0.0178	(0.00979–0.0322)	0.145	(0.0461–0.463)	Na	Na
Ls2362 (Bin)	CqSyn	0.0250	(0.00695–0.0903)	0.437	(0.0184–10.7)	Na	Na
	BsR	Average mortality of 6% at 0.5 µg/ml				Na	Na
Cry11Ba	CqSyn	0.160	(0.139–0.183)	0.847	(0.665–1.16)	Na	Na
	BsR	0.0409	(0.0349–0.0478)	0.398	(0.304–0.552)	Na	Na
Cyt1Aa	CqSyn	6.25	(2.96–13.1)	50.4	(13.9–184)	Na	Na
	BsR	32.5	(28.3–37.6)	222	(172–304)	Na ^a	Na ^a
Bin + Cyt1A (1:1)	CqSyn	0.0159	(0.0131–0.0194)	0.387	(0.258–0.645)	3.0	2.2
	BsR	0.735	(0.632–0.853)	6.49	(5.06–8.73)	88	69 ^a
Bin + Cry11Ba (1:1)	CqSyn	0.0119	(0.00876–0.0161)	0.0813	(0.0463–0.149)	16.4	7.1
	BsR	0.0276	(0.0188–0.0405)	0.266	(0.125–0.581)	3.0	3.0
Cry11Ba + Cyt1A	CqSyn	0.0797	(0.0688–0.0920)	0.543	(0.424–0.736)	3.9	3.1
Bin + Cry11B + Cyt1A (Mixture)	CqSyn	0.0326	(0.0273–0.0386)	0.384	(0.288–0.549)	6.7	1.3
	BsR ^b	0.066 ^b		0.470 ^b		3.4	4.2

^a Data taken from Wirth et al. 2000.
^b Nonlinear response in dose–response testing, estimated from graph for SF calculation only.

Fig. 1. Bioassay results using a Bin + Cry11Ba + Cyt1Aa mixture or a Bin + Cry11Ba + Cyt1Aa recombinant bacterium against fourth instars of an *L. sphaericus*-resistant *Cx. quinquefasciatus* colony, BsR.

Wirth 1997). This effect was also observed when larvae were selected with a simple mixture of Cry11Aa + Cyt1Aa versus Cry11Aa alone (Wirth et al. 2005b). The combination of Cyt1Aa + Bin shows a similar outcome; resistance evolution to the combination was lower than observed for selection with Bin alone (Wirth et al. 2005a). Second, synergistic interactions among the various toxins, particularly the Cyt1Aa toxin, mask the phenotypic expression of resistance to the other toxins in the mixture. This was clearly evident in

the *B. thuringiensis* subsp. *israelensis* selection studies (Wirth and Georgiou 1997; Wirth et al. 1998, 2004a) and was also observed in selections using *L. sphaericus* in the presence or absence of Cyt1Aa. Resistance to *L. sphaericus* evolves under selection using Bin alone or with Cyt1Aa, but that resistance is suppressed if Cyt1Aa is present (Wirth et al. 2005a). Mosquitoes with extremely high Bin resistance (>10,000-fold) express only 15-fold resistance in the presence of a 3:1 ratio of Bin and Cyt1A, thus suppressing 99% of Bin

resistance present (Wirth et al. 2000). Thus, high Bin resistance is suppressed or masked in the presence of Cyt1Aa; however, it is not eliminated.

The three specific components of our recombinant, Bin, Cry11Ba, and Cyt1Aa, were anticipated to interact and influence the evolution of resistance in the two ways described above. Bin interactions with Cry11Ba and Cyt1Aa were anticipated to suppress expression of Bin resistance, whereas the interactions between Cry11Ba and Cyt1Aa were anticipated to delay resistance to the Cry component in a manner similar to that observed for Cry11Aa. Comparable responses were detected in this study, as we observed high *L. sphaericus* resistance and very low Cry11Ba resistance in the selected colony when those materials were tested individually.

However, the phenomenon of high resistance to the recombinant itself that was observed between Generations 3 and 12 was not detected in other earlier studies. Thus, the inconsistency may lie in the interactions among the three components. Our post hoc measurements of synergy for binary interactions generally agreed with earlier reports. We observed a high level of synergy between Bin and Cry11Ba that was similar to those of Bin with other Cry toxins (Wirth et al. 2004b). However, the interaction of Cry11Ba and Bin against our Bin-resistant colony, although synergistic, was considerably lower than reported for other Cry toxins (Wirth et al. 2004b). Consequently, Cry11Ba may be poorly effective at suppressing high levels of Bin resistance, and this may be a contributing factor to the evolution of resistance to the recombinant. Another potential factor is the three-way interaction between Bin, Cry11Ba, and Cyt1Aa. Against susceptible larvae, the three-toxin mixture showed almost 7-fold synergy at the LC₅₀, but notably, synergy was only 1.3-fold at the LC₉₅, a synergy level that is generally considered to be insignificant, as it was less than 1.5-fold. Against BsR, the three-toxin mixture was also synergistic, but the actual dose-response line from the data set showed a pronounced decline in mortality in the mid-range concentrations, after which mortality rose rapidly at the higher concentrations. Although the three-toxin mixture was not linear against Bin-resistant BsR, the same mixture was linear when tested concurrently against CqSyn. Interestingly, and perhaps importantly, the recombinant BinCry11BCytA was linear against both the susceptible and Bin-resistant mosquito lines. The purpose of the artificial mixture was to approximate the proportions of these three proteins as expressed in the recombinant to measure synergy. The data show that the recombinant yielded higher overall activity than the mixture, which may be the consequence of the stability of the engineering and expression in the recombinant. These differences also suggest that the mixture, while useful for approximating possible synergistic interactions, may be an unreliable indicator of the source of the evolution of resistance observed in the early generations of selected larvae. We can speculate that the poor activity at moderate concentrations may have provided the opportunity for the population to evolve resistance in the early stages of selection, whereas the effectiveness of the

higher concentrations overcame that earlier advantage, after selection concentrations were increased.

The answer to why resistance evolved in the early stages of selection and was subsequently reversed is not totally clear. We have identified two possible aspects in the design of the recombinant that may have played some role in the early resistance; the poor synergy of Bin + Cry11Ba against high Bin resistance and the nonlinear dose-response line for the mixture Bin + Cry11B + Cyt1Aa. However, this question requires further research to better understand why this particular design resulted in such a usual pattern of evolution and what toxin combinations are ideal for avoiding resistance.

Based on the results from other selection studies, both ours and other groups using mosquitocidal toxins, the preponderance of evidence supports the hypothesis that synergy is beneficial to reducing the rate and magnitude of the evolution of resistance in *Cx. quinquefasciatus* as well as in suppressing the expression of resistance to component toxins. However, the unexpected early evolution of resistance and its reversal that we observed under selection with Bin11BCytA indicates that the precise interactions among the components and their robustness over the time must be investigated in depth before any potential application is considered. It may be relevant to note here that the failure to evolve any significant resistance under laboratory selection has only been achieved to date using wild-type *B. thuringiensis* subsp. *israelensis* against *Cx. quinquefasciatus* (Georghiou 1990, Georghiou and Wirth 1997, Saleh et al. 2003), or *Aedes aegypti* (Goldman et al. 1986, Paris et al. 2011), wild-type *B. thuringiensis* subsp. *jegathesan* (Wirth et al. 2004a), and with the combination of *B. thuringiensis* subsp. *israelensis* and *L. sphaericus*, either as a mixture (Zahiri and Mulla 2003) or as a recombinant strain that expressed both *B. thuringiensis* subsp. *israelensis* and *L. sphaericus* toxic proteins (Wirth et al. 2010). Although the data are primarily limited to *Cx. quinquefasciatus*, these results should be carefully considered in the development and use of future microbial pesticides.

Acknowledgments

This project was supported in part by grants from the National Institute of Health (AI45817), the U.S. Department of Agriculture (S-1029), The University of California Mosquito Control Program, and the Agricultural Experiment Station at the University of California – Riverside. Preparation of the figure by Stephanie Russell is gratefully acknowledged.

References Cited

- Ahmed, I., A. Yokota, A. Yamazoe, and T. Fujiwara. 2007. Proposal of *Lysinibacillus boronitolerans* gen. nov., *Lysinibacillus fusiformis* comb. nov., and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. Int. J. Syst. Evol. Microbiol. 57: 117–1125.
- Becker, N., and M. Ludwig. 1993. Investigations on possible resistance in *Aedes vexans* after a 10-year application of *Bacillus thuringiensis israelensis*. J. Amer. Mosq. Control Assoc. 9: 221–224.

- Berry, C. 2012. The bacterium, *Lysinibacillus sphaericus*, as an insect pathogen. *J. Invertebr. Pathol.* 109: 1–10.
- Berry, C., J. Hindley, A. F. Ehrhardt, T. Grounds, I. de Souza, and E. W. Davidson. 1993. Genetic determinants of host ranges of *Bacillus sphaericus* larvicidal toxins. *J. Bacteriol.* 175: 510–518.
- Crickmore, N., E. J. Bone, J. A. Williams, and D. J. Ellar. 1995. Contributions of the individual components of the δ -endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiol. Lett.* 131: 249–254.
- Délécluse, A., M.-L. Rosso, and A. Ragni. 1995. Cloning and expression of a novel toxin gene from *Bacillus thuringiensis* subsp. *jegathesan* encoding a highly mosquitocidal protein. *Appl. Environ. Microbiol.* 61: 4230–4235.
- Georgiou, G. P. 1990. Resistance potential to biopesticides and consideration of countermeasures, pp. 402–442. In J. E. Casida (ed.), *Pesticides and alternatives*. Elsevier Sci. Pub. New York, NY.
- Georgiou, G. P., and M. C. Wirth. 1997. Influence of single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). *Appl. Environ. Microbiol.* 63: 1095–1101.
- Goldberg, L. J., and J. Margalit. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univittatus*, *Aedes aegypti*, and *Culex pipiens*. *Mosq. News* 37: 355–358.
- Goldman, I. F., J. Arnold, and B. C. Carlton. 1986. Selection for resistance to *Bacillus thuringiensis* subsp. *israelensis* in field and laboratory populations of the mosquito *Aedes aegypti*. *J. Invert. Pathol.* 47: 317–324.
- Kellen, W. R., and C. M. Meyers. 1964. *Bacillus sphaericus* Neide as a pathogen of mosquitoes. *Proc. Calif. Mosq. Control Assoc.* 32: 37.
- Mulla, M. S. 1990. Activity, field efficacy, and use of *Bacillus thuringiensis israelensis* against mosquitoes, pp. 134–160. In H. de Barjac, and D. J. Sutherland (eds.), *Bacterial control of mosquitoes and blackflies: Biochemistry, genetics, and applications of Bacillus thuringiensis israelensis and Bacillus sphaericus*. Rutgers University Press. New Brunswick.
- Paris, M., G. Tetreau, F. Laurent, M. Llu, L. Deprés, and J. P. David. 2003. Persistence of *Bacillus thuringiensis israelensis* (Bti) in the environment induces resistance to multiple Bti toxins in mosquitoes. *Pest Manage. Sci.* 67: 122–128.
- Park, H.-W., D. K. Bideshi, and B. A. Federici. 2003. Recombinant strain of *Bacillus thuringiensis* producing Cyt1A, Cry11B, and the *Bacillus sphaericus* binary toxin. *Appl. Environ. Microbiol.* 69: 1331–1334.
- Park, H.-W., D. K. Bideshi, M. C. Wirth, J. J. Johnson, W. E. Walton, and B. A. Federici. 2005. Recombinant bacteria with markedly improved efficacy against *Culex* vectors of West Nile virus. *Am. J. Trop. Med. Hyg.* 72: 732–738.
- Poncet, S., A. Délécluse, A. Klier, and G. Rappoport. 1995. Evaluation of synergistic interactions among CryIVA, CryIVB, and CryIVD toxic components of *Bacillus thuringiensis* subsp. *israelensis*. *J. Invert. Pathol.* 66: 131–135.
- Porter, A. G., E. W. Davidson, and J.-W. Liu. 1993. Mosquitocidal toxins of *Bacilli* and their genetic manipulation for effective biological control of mosquitoes. *Microbiol. Rev.* 57: 838–861.
- Rao, D. R., T. R. Mani, R. Rajendran, A. S. Joseph, A. Gajana, and R. Reuben. 1995. Development of a high level of resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. *J. Amer. Mosq. Control Assoc.* 11: 1–5.
- Raymond, M., G. Prato, and D. Ratsira. 1993. Probability Analysis of Mortality Assays Showing Quantile Response, Version 3.3. Praxeme, Saint Georges D'Arques, France.
- Rodcharoen, J., and M. S. Mulla. 1994. Resistance development in *Culex quinquefasciatus* (Diptera: Culicidae) to *Bacillus sphaericus*. *J. Econ. Entomol.* 87: 1133–1140.
- Saleh, M. S., F. A. El-Meniawi, N. L. Kelada, and H. M. Zahran. 2003. Resistance development in mosquito larvae *Culex pipiens* to the bacterial agent *Bacillus thuringiensis* var. *israelensis*. *J. Appl. Entomol.* 127: 29–32.
- Silva-Filha, M.-H., L. Regis, C. Nielsen-LeRoux, and J.-F. Charles. 1995. Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). *J. Econ. Entomol.* 88: 525–530.
- Sinègre, C., M. Babinot, J.-M. Quermel, and B. Gaven. 1994. First field occurrence of *Culex pipiens* resistance to *Bacillus sphaericus* in southern France, p. 17. In *Proceedings, 8th European Meeting of Society for Vector Ecology*, 5–8 September 1994, Barcelona, Spain. Society for Vector Ecology, Santa Ana, CA.
- Su, T., and M. S. Mulla. 2004. Documentation of high-level *Bacillus sphaericus* 2362 resistance in field population of *Culex quinquefasciatus* breeding in polluted water in Thailand. *J. Am. Mosq. Control Assoc.* 20: 405–411.
- Tabashnik, B. E. 1992. Evaluation of synergism among *Bacillus thuringiensis* toxins. *Appl. Environ. Microbiol.* 58: 3343–3346.
- WHO. 1999. The World Health Report. World Health Organization, Geneva.
- Wirth, M. C., A. Délécluse, B. A. Federici, and W. E. Walton. 1998. Variable cross-resistance to Cry11B from *Bacillus thuringiensis* subsp. *jegathesan* in *Culex quinquefasciatus* (Diptera: Culicidae) resistant to single or multiple toxins of *Bacillus thuringiensis* subsp. *israelensis*. *Appl. Environ. Microbiol.* 64: 4174–4179.
- Wirth, M. C., A. Délécluse, and W. E. Walton. 2004a. Laboratory selection for resistance to *Bacillus thuringiensis* subsp. *jegathesan* or a component toxin, Cry11B, in *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol.* 41: 435–441.
- Wirth, M. C. and G. P. Georgiou. 1997. Cross-resistance among CryIV toxins of *Bacillus thuringiensis* subsp. *israelensis* in *Culex quinquefasciatus* (Diptera: Culicidae). *J. Econ. Entomol.* 90: 1471–1477.
- Wirth, M. C., B. A. Federici, and W. E. Walton. 2000a. Cyt1A from *Bacillus thuringiensis* synergizes activity of *Bacillus sphaericus* against *Aedes aegypti* (Diptera: Culicidae). *Appl. Environ. Microbiol.* 60: 1094–1097.
- Wirth, M. C., G. P. Georgiou, J. I. Malik, and G. H. Abro. 2000b. Laboratory selection for resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from California, USA. *J. Med. Entomol.* 37: 534–540.
- Wirth, M. C., J. A. Giannino, B. A. Federici, and W. E. Walton. 2004b. Synergy between toxins of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*. *J. Med. Entomol.* 41: 935–991.
- Wirth, M. C., J. J. Giannino, B. A. Federici, and W. E. Walton. 2005a. Evolution of resistance toward *Bacillus sphaericus* or a mixture of *B. sphaericus* and Cyt1A from *Bacillus thuringiensis*, in the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae). *J. Invert. Pathol.* 88: 154–162.
- Wirth, M. C., H.-W. Park, W. E. Walton, and B. A. Federici. 2005b. Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. *Appl. Environ. Microbiol.* 71: 185–189.
- Wirth, M. C., W. E. Walton, and B. A. Federici. 1997. CytA enables endotoxins of *Bacillus thuringiensis* to overcome high

- levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. *Proc. Natl. Acad. Sci. USA* 94: 10536–10540.
- Wirth, M. C., W. E. Walton, and B. A. Federici. 2000c.** Cyt1A from *Bacillus thuringiensis* restores toxicity of *Bacillus sphaericus* against resistant *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol.* 37: 401–407.
- Wirth, M. C., W. E. Walton, and B. A. Federici. 2010.** Evolution of resistance to the *Bacillus sphaericus* Bin toxin is phenotypically masked by combination with the mosquitocidal proteins of *Bacillus thuringiensis* subsp. *israelensis*. *Environ. Microbiol.* 12: 1154–1160.
- Wu, D., and B. A. Federici. 1993.** A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *J. Bacteriol.* 175: 5276–5280.
- Yuan, Z., Y. Zhang, Q. Cai, and E.-Y. Liu. 2000.** High level field resistance to *Bacillus sphaericus* C3-41 in *Culex quinquefasciatus* from southern China. *Biocontrol. Sci. Technol.* 10: 41–49.
- Zahiri, N. S., and M. S. Mulla. 2003.** Susceptibility profile of *Culex quinquefasciatus* (Diptera: Culicidae) to *Bacillus sphaericus* on selection with rotation and mixture of *B. sphaericus* and *B. thuringiensis israelensis*. *J. Med. Entomol.* 40: 672–677.

Received 4 March 2015; accepted 17 July 2015.