Inheritance Patterns, Dominance, Stability, and Allelism of Insecticide Resistance and Cross-Resistance in Two Colonies of *Culex quinquefasciatus* (Diptera: Culicidae) Selected With Cry Toxins From *Bacillus thuringiensis* subsp. *israelensis*

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ABSTRACT Mendelian crosses were used to analyze the patterns of inheritance of Cry-toxin resistance in two colonies of *Culex quinquefasciatus* Say larvae resistant to bacterial toxins produced by *Bacillus thuringiensis* subsp. *israelensis* de Barjac. Resistance levels exceeded 1000-fold at 95% lethal concentration of the Cry11Aa-resistant colony (Cq11A). F_1 offspring of reciprocal crosses to a susceptible colony revealed autosomal inheritance and offspring were intermediate in resistance to the susceptible and resistant parental lines. Dose-response tests on backcross offspring were consistent with polyfactorial inheritance of resistance toward Cry11Aa and Cry4Aa + Cry4Ba, whereas cross-resistance toward Cry11Ba best fit a monofactorial model. Resistance was 600-fold at 95% lethal concentration in the colony selected with Cry4A + Cry4B (Cq4AB). Inheritance of resistance in F_1 offspring was autosomal and intermediate to the susceptible and resistant parents. Inheritance of Cry4Aa + Cry4Ba and Cry11Ba resistance best fit a polyfactorial model in offspring of the Cq4AB backcross, whereas Cry11Aa-resistance inheritance fit a monofactorial model. Dominance values were calculated at different Cry-toxin concentrations for F_1 offspring of both resistant colonies; dominance generally decreased as treatment concentration increased. Resistance and cross-resistance remained stable in Cq11A and Cq4AB in the absence of insecticide pressure. Allelic complementation tests were complementary and suggested that Cq11A and Cq4AB evolved resistance to Cry toxins at common loci. The patterns of cross-resistance suggest cross-recognition of binding moieties by Cry11Aa, Cry4Aa + Cry4Ba, and Cry11Ba in these *Culex*, which may be partly responsible for the toxin synergy characteristic of *B. thuringiensis* subsp. *israelensis* de Barjac.

KEY WORDS *Bacillus thuringiensis*, Cry toxins, genetics, mosquitoes, resistance

The bacterium *Bacillus thuringiensis* Berliner is characterized by the production of crystalline proteinaceous inclusions during sporulation (Whiteley and Schnepf 1986). In some strains of *B. thuringiensis*, the inclusion proteins are toxic when eaten by sensitive insect species, and this trait has been exploited to produce insecticides to control pests of agricultural and medical importance (van Frankenhuyzen 1993). Strains such as *B. thuringiensis* subsp. *kurstaki* that are active against lepidopterans are used as insecticidal sprays on crops, and several of the insecticidal proteins of these have been genetically engineered into various crop plants to target susceptible pest species (Whalon and Wingerd 2003). *B. thuringiensis* subsp. *israelensis* de Barjac is primarily active against mosquitoes and black flies and is used to reduce larval population densities and disrupt the spread of various diseases, including West Nile virus, dengue fever, and malaria in human populations (Porter et al. 1993).

Insecticide resistance is considered a significant barrier to the long-term success of *B. thuringiensis*-based insect control strategies. Resistance to *B. thuringiensis* has evolved in field populations of *Plutella xylostella* (L.) in response to foliar sprays (Tabashnik et al. 1990, Shelton et al. 1993) and in greenhouse populations of *Trichoplusia ni* (Hübner) (Janmaat and Meyers 2003). Furthermore, other important insect pests have demonstrated the capacity to evolve resistance to *B. thuringiensis* under laboratory selection pressure (for a review, see Tabashnik 1994). In contrast, *B. thuringiensis* subsp. *israelensis* has been successfully used to control mosquito and black fly larvae for >20 yr, with no evidence of field control failure (Becker and Ludwig 1993, Becker 1997). Unlike the *B. thuringiensis* strains active against lepidopteran pests, *B. thuringiensis* subsp. *israelensis* synthesizes a diverse spectrum of four major toxins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, which interact synergistically with each other, and specifically with the latter cyto-

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lytic toxin, to greatly enhance toxicity (Crickmore et al. 1995, Poncet et al. 1995) and suppress insecticide resistance (Wirth et al. 1997). Despite this advantage, laboratory studies with *Culex quinquefasciatus* Say have shown that mosquitoes can evolve high levels of resistance to the various component toxins of *B. thuringiensis* subsp. *israelensis* in the absence of Cyt1Aa (Georghiou and Wirth 1997). Consequently, it would be shortsighted to assume that resistance will never occur.

Understanding the genetic basis of resistance to *B. thuringiensis* is key to developing predictive models and resistance-monitoring strategies, as well as managing resistance. On a fundamental level, knowledge of the mode of inheritance of *B. thuringiensis* resistance can provide tools to elucidate the mode of action of *B. thuringiensis* toxins and facilitate the design of engineered bacterial strains with enhanced host range and refractoriness to resistance. Genetic studies of *B. thuringiensis* resistance have been undertaken for major agricultural pest species, but no information is available for mosquitoes. In this study, we examined the inheritance of resistance and cross-resistance to Cry toxins from *B. thuringiensis* subsp. *israelensis* in two laboratory-selected colonies of *C. quinquefasciatus.* We used Mendelian crosses to evaluate maternal effect, sex linkage, and dominance. Backcrosses were used to estimate the number of loci involved in resistance and cross-resistance. The stability of resistance in the absence of selection was studied. Finally, allelic complementation tests examined whether the two resistant lines shared loci for resistance and cross-resistance.

Materials and Methods

Mosquito Colonies. Three laboratory colonies of *C. quinquefasciatus* were used for these studies. The Cq11A- and Cq4AB-resistant colonies were each established in 1990 and derived from a large synthetic population formed by pooling multiple field collections. Both colonies have been maintained under selection pressure with Cry11Aa or Cry4Aa + Cry4Ba, respectively, since that time (Georghiou and Wirth 1997). Resistance levels to Cry11Aa in Cq11A reached >913-fold by generation 28 and exceeded 7,000-fold 1 yr later (Wirth et al. 1998). Cq4AB resistance reached 122-fold in generation 28 (Georghiou and Wirth 1997) and 290-fold after an additional year of selection (Wirth et al. 1998). Subsequent selection has increased and stabilized the levels of resistance. Colony CqSyn was a synthetic population established from multiple field collections in 1995, was used in concurrent dose-response tests (bioassays) to estimate resistance ratios for the selected colonies, and served as the susceptible parental colony for the genetic crosses (Wirth et al. 2004).

Recombinant Bacterial Toxins. Crystalliferous strains of *B. thuringiensis* subsp. *israelensis* that synthesize Cry4Aa + Cry4Ba (Delécluse et al. 1993), Cry11Aa (Wu et al. 1994), or Cry11Ba (*B. thuringiensis* subsp. *jegathesan*) (Delécluse et al. 1995) were used to select the resistant colonies and/or for bioassay. The bacterial strains were grown on liquid media, as described previously (Park et al. 1998). Sporulated cells were washed in distilled water and centrifuged, and the resulting pellet was lyophilized. For mosquito selections and bioassays, stock suspensions of the crystal/spore powders were prepared by weight in deionized water and homogenized with glass beads to create fine particle suspensions. Stocks were prepared monthly, and 10-fold serial dilutions were prepared weekly. All stocks and dilutions were frozen at – 20°C when not in use.

Selection and Bioassay Procedures. Early fourth instars were used for bioassay tests and selections. Bioassays exposed groups of 20 larvae to different concentrations of crystal/spore suspension in 100 ml of deionized water in 8-ounce plastic cups. At least seven different concentrations, plus a water control, were replicated a minimum of five times on 5 different days. A minimum of 10 replicates was performed for backcross assays to increase the reliability of the data. Mortality was determined 24 h after treatment, and data were analyzed using a Probit program for the computer (Raymond et al. 1993). Resistance ratios (RR_{50}, RR_{95}) were determined from concurrent bioassay tests on CqSyn and the resistant colonies using the same bacterial stocks and dilutions, and were calculated by dividing the 50% lethal concentration (LC_{50}) or the 95% lethal concentration (LC_{95}) of the selected colony by the LC_{50} or LC_{95} of CqSyn. Dose-response values with overlapping fiducial limits were not considered significantly different.

The two resistant colonies were reared under weekly insecticide pressure with either Cry11Aa or Cry4Aa + Cry4Ba (Georghiou and Wirth 1997). Groups of 1,000 early fourth instars were placed in enamel metal pans containing 1 liter of deionized water and exposed to their respective recombinant bacterial powder suspension for 24 h. Survivors are removed to clean water, fed, and used to continue the colony. Initially, selection pressure ensured 70–90% mortality, and generations were maintained separately. To avoid population bottlenecks, 500–1,000 adults were used to establish any single generation. However, generations have been allowed to overlap recently. Resistance has reached very high levels and has been very stable; therefore, resistance has been maintained in Cq11A and Cq4AB using a selection concentration of 40 μg/ml of either Cry11Aa or Cr4Aa + Cry4Ba powders. This concentration is 1,000-fold higher than the original selection concentration and generally kills <10% of the larvae.

Genetic Crosses. Reciprocal mass crosses were carried out between CqSyn and the respective resistant colonies (Cq11A or Cq4AB). Virgin males and females were obtained by isolating pupae in scintillation vials. A minimum of 300 males and 300 females was used for each mass cross. The following crosses and backcrosses were made, with the female parent listed first: 1) CqSyn × Cq11A; 2) Cq11A × CqSyn; 3) CqSyn × Cq4AB; 4) Cq4AB × CqSyn; 5) [Cq11A × CqSyn]

Table 1. Dose-response values and resistance ratios for Cry11Aa, Cry11Ba, or Cry4Aa + Cry4Ba toward parental and F_1 offspring of reciprocal crosses between Cq11A and CqSyn

Toxin(s)	Colony	*N*	LC_{50} (fiducial limit) μg/ml	LC_{95} (fiducial limit) μg/ml	Resistance ratio		χ^2	Slope
					RR_{50}	RR_{95}		
Cry11Aa	CqSyn	1,100	1.11 (0.442–2.78)	84.6 (15.0–481)	1.0	1.0	92.3	0.87
	Cq11A	900	493 (251–1,394)	240,000 (39,493–443,000)	444	2,836	3.6	0.61
	S × R	1,200	84.9 (25.2–305)	70,388 (805–> 1×10^6)	76.4	832	22.4	0.56
	R × S	1,100	139 (30.4–680)	79,023 (536–> 1×10^6)	125	934	28.9	0.59
Cry11Ba	CqSyn	700	0.0390 (0.0341–0.0444)	0.216 (0.173–0.282)	1.0	1.0	57.1	1.2
	Cq11A	1,200	0.177 (0.106–0.297)	4.77 (1.86–12.4)	4.5	22.0	10.9	2.2
	S × R	900	0.232 (0.169–0.318)	2.57 (1.46–4.71)	5.9	11.9	12.9	1.6
	R × S	800	0.184 (0.156–0.217)	2.13 (1.61–3.02)	4.7	9.9	9.2	1.5
Cry4Aa + Cry4Ba	CqSyn	900	0.199 (0.115–0.344)	2.80 (1.01–7.83)	1.0	1.0	49.5	1.4
	Cq11A	1,000	6.98 (3.77–12.9)	731 (158–3,524)	35	261	35.5	0.81
	S × R	1,000	2.31 (1.95–2.74)	37.1 (27.6–52.9)	11.6	13.2	9.8	1.4
	R × S	900	2.41 (1.41–4.1)	74.0 (25.1–223)	12.1	26.4	32.9	1.1

F_1 × CqSyn; 6) [Cq4AB × CqSyn]F_1 × Cq4AB; 7) Cq11A × Cq4AB; and 8) Cq4AB × Cq11A.

The standard backcross method was used to estimate the number of alleles involved in resistance. We accepted the assumption that all parental lines are homogeneous for resistance or susceptibility based on several lines of evidence. First, the fiducial limits of the dose-response lines to Cry11Aa, Cry4Aa + Cry4Ba, and Cry11Ba between the resistant and susceptible colonies did not overlap. Second, the fiducial limits to the three toxin powders in the susceptible colony and any of the F_1 offspring of the reciprocal crosses did not overlap. Third, resistance was stable to the three different toxin powders in the absence of the insecticide for 18 generations. These data are consistent with a high proportion of homogeneity for *B. thuringiensis* resistance in both Cq11A and Cq4AB. Because the determination of monfactorial or polyfactorial inheritance must be inferred from dose-response lines on backcross offspring, the technique is prone to error. However, we increased the number of replicates in the bioassays from 5 to 10 or more for each cross and included 10 or more test concentrations to improve the power of this test (Tabashnik 1991). χ^2 analysis was performed on the dose-response lines of the offspring of the backcrosses and compared with a single locus model derived from the dose-response lines of the resistant and susceptible parents and assuming a 1:1 distribution for resistance in backcross offspring. χ^2 deviations were calculated for the mortality at each insecticide concentration. χ^2 deviation values for all concentrations in a dose-response line were totaled and tested for significance using *n*-2 and 95% probability, where *n* is the number of test concentrations in a dose-response line.

Dominance Calculations. The single concentration method of Hartl (1992), as described by Liu and Tabashnik (1997), was used to estimate the degree of dominance of the resistance or cross-resistance trait, $h = (w_{12} - w_{22}) / (w_{11} - w_{22})$, where *h* is the degree of dominance, w_{11} is the fitness of the homozygous resistant parent, w_{12} is the fitness of the heterozygous offspring, and w_{22} is the fitness of the homozygous susceptible parent. The fitness of the resistant homozygous parent at any treatment concentration was assumed to be 1. The fitness of the susceptible parent and the heterozygous F_1 was estimated from the survival rate of the larvae at a specific treatment concentration divided by the survival rate of the resistant parent at the same concentration. Using this formula, an *h* value of 0 indicates fully recessive inheritance; an *h* value of 1 indicates a fully dominant trait; and an *h* value of 0.5 represents a codominant trait. When $0 < h < 0.5$, the trait is partly recessive, whereas when $0.5 < h < 1$, the trait is partly dominant.

Stability of Resistance. Three thousand larvae from each resistant colony, Cq11A, Cq4AB, and offspring of the cross, Cq11A × Cq4AB, were allowed to develop without exposure to insecticide and used to establish generation 1 of each unselected line. Subsequent generations were separated. The unselected lines derived from the Cq11A and Cq4AB were reared for 19 generations without exposure to insecticide. Susceptibility to insecticides was evaluated at generations 3, 7, 10, and 19. The offspring of the cross Cq11A × Cq4AB were reared for 13 generations without selection pressure, and susceptibility was evaluated in generations 1, 8, and 13.

The proportion of survivors of exposure to the concentrations 0.2, 2, 20, and 200 μg/ml for each insecticide was used to calculate the change in frequency of resistant genotypes in the populations in the absence of insecticide exposure, as described by Tabashnik et al. (1994). The rate of change in the absence of insecticide exposure (R) can be calculated from the following: R = (log [final proportion surviving treatment] – log [initial proportion surviving]) / *n*, where *n* is the number of generations not exposed to insecticide. A negative R value indicates a decline in the proportion of larvae surviving exposure to insecticide.

Results

The Cry11A-selected colony had LC_{50} and LC_{95} values of 493 and 240,000 μg/ml and resistance ratios of 444 and 2,836 toward Cry11Aa, respectively (Table 1). LC_{50} and LC_{95} values were significantly different from those of the susceptible colony, CqSyn, because of the lack of overlap in their fiducial limits. The F_1 offspring from reciprocal crosses between Cq11A and

Table 2. Dose-response values and resistance ratios for Cry4Aa + Cry4Ba, Cry11Aa, or Cry11Ba toward parental and F_1 offspring of reciprocal crosses between strains Cq4AB and CqSyn

Toxin(s)	Colony	*N*	LC_{50} (fiducial limit) μg/ml	LC_{95} (fiducial limit) μg/ml	Resistance ratio		χ^2	Slope
					RR_{50}	RR_{95}		
Cry4Aa + Cry4Ba	CqSyn	1,200	0.335 (0.277–0.405)	9.29 (6.64–13.8)	1.0	1.0	5.8	1.1
	Cq4AB	1,200	39.4 (19.5–80.1)	6,304 (1,026–40,292)	117	678	49.6	0.74
	S × R	1,100	4.30 (2.61–7.07)	291 (105–833)	12.8	31.3	37.2	0.82
	R × S	1,200	1.75 (1.05–2.91)	173 (59.4–514)	5.2	18.6	32.3	0.89
Cry11Aa	CqSyn	1,100	5.63 (3.21–9.84)	565 (159–2,086)	1.0	1.0	36.5	0.82
	Cq4AB	800	878 (543–1,843)	39,964 (12,178–282,665)	156	71	3.7	0.99
	S × R	1,000	40.4 (32.7–50.5)	1,640 (1,031–2,899)	7.2	2.9	3.9	1.0
	R × S	900	42.3 (33.1–55.0)	3,585 (1,925–7,982)	7.6	6.3	10.9	0.85
Cry11Ba	CqSyn	900	0.0443 (0.0390–0.0503)	0.219 (0.178–0.284)	1.0	1.0	4.1	2.4
	Cq4AB	1,100	1.52 (0.964–2.39)	70.4 (32.2–160)	34.4	321	38.1	1.0
	S × R	1,200	0.273 (0.195–0.381)	7.90 (4.23–14.5)	6.2	36.0	54.9	1.1
	R × S	1,100	0.316 (0.248–0.402)	9.87 (6.19–16.0)	7.1	45.0	21.3	1.2

CqSyn showed LC_{50} and LC_{95} values generally intermediate to their resistant and susceptible parents, and these values were also significantly different from those of CqSyn. RR_{50} of the F_1 offspring were 76.4 and 125, whereas RR_{95} were 832 and 934. The lethal concentration values (LC_{50}, LC_{95}) for the F_1 offspring of the reciprocal crosses were not significantly different from each other.

Using Cry11Ba toxin powder, the Cq11A colony showed low, but significant resistance ratios of 4.5 and 22 at the LC_{50} and LC_{95}, respectively (Table 1). Lethal concentration values for Cq11A and the F_1 offspring of the reciprocal crosses were significantly different from those for CqSyn. The LC values of F_1 offspring were more similar to the Cq11A parent at test concentrations below the LC_{50}, but were intermediate to the respective parental lines at the higher treatment concentrations. RR_{50} were 5.9 and 4.7, and RR_{95} were 11.9 and 9.9. The F_1 offspring of the reciprocal crosses were not significantly different from one another in their susceptibility to Cry11Ba.

Tests with Cry4Aa + Cry4Ba against Cq11A revealed resistance ratios of 35 and 261 at the LC_{50} and LC_{95}, respectively (Table 1). F_1 offspring of reciprocal crosses were intermediate to the respective parents, with RR_{50} of 11.6 and 12.1, and RR_{95} of 13.2 and 26.4. The LC values of Cq11A and the F_1 offspring of the reciprocal crosses were significantly different from CqSyn. The LC_{50} and LC_{95} values for the F_1 offspring of the reciprocal crosses were not significantly different from each other.

The offspring of the backcross (Cq11A × CqSyn) F_1 × CqSyn showed significant deviation from the monofactorial model when assayed with Cry11Aa ($\chi^2 = 67.7$, df = 9, $P < 0.05$), particularly in the moderately low and moderately high treatment concentrations (data not shown). When tested with Cry4Aa + Cry4Ba toxin powder, the backcross offspring also deviated from the monofactorial model in the moderately low and moderately high treatment concentrations ($\chi^2 = 49.4$, df = 12, $P < 0.05$). However, tests with Cry11Ba toxin powder fit the monofactorial model ($\chi^2 = 4.1$, df = 8, $P > 0.05$).

The Cq4AB-selected colony showed high resistance toward Cry4Aa + Cry4Ba with LC_{50} and LC_{95} values of 39.4 and 6,304 μg/ml, and resistance ratios of 117 and 678 at the LC_{50} and LC_{95}, respectively (Table 2). The F_1 offspring of reciprocal crosses were intermediate to their respective parents with RR_{50} of 12.8 and 5.2, and RR_{95} of 31.3 and 18.6. Cq4AB and the F_1 offspring were significantly different in susceptibility from CqSyn. The F_1 offspring of the reciprocal crosses were not significantly different from each other.

Cross-resistance levels to Cry11Aa in Cq4AB were lower than the resistance levels observed with colony Cq11A, with resistance ratios of 156 and 71 at the LC_{50} and LC_{95}. The F_1 offspring of the reciprocal crosses showed low, but significant cross-resistance to Cry11Aa, and resistance ratios at the LC_{50} were 7.2 and 7.6, and at the LC_{95} were 2.9 and 6.3. Both Cq4AB and the F_1 offspring of the reciprocal crosses were significantly different from CqSyn in susceptibility to Cry11Aa. The F_1 offspring of the reciprocal crosses were not significantly different from each other.

Cross-resistance ratios for Cq4AB toward Cry11Ba were 34.4 and 321 at the LC_{50} and LC_{95}, and were considerably higher than observed for Cq11A. F_1 offspring of reciprocal crosses were intermediate in resistance to their respective parents with resistance ratios of 6.2 and 7.1 at the LC_{50}, and 36 and 45 at the LC_{95}. Both Cq4AB and the offspring of the reciprocal crosses were significantly different from CqSyn in susceptibility to Cry11Ba. The F_1 offspring of the reciprocal crosses were not significantly different in susceptibility from each other.

The dose-response data for offspring of the (Cq4AB × CqSyn)F_1 × Cq4AB backcross with Cry4Aa + Cry4BA ($\chi^2 = 28.6$, df = 11, $P < 0.05$) (Fig. 1A) and Cry11Ba powders ($\chi^2 = 47.5$, df = 11, $P < 0.05$) (data not shown) were not consistent with the monofactorial model because of significant deviations at multiple treatment concentrations. However, the (Cq4AB × CqSyn)F_1 × Cq4AB backcross offspring fit the monofactorial model when tested with Cry11Aa ($\chi^2 = 5.5$, df = 6, $P > 0.05$) (Fig. 1B).

Dominance estimations at the various treatment concentrations showed that resistance to Cry11Aa toxin in Cq11A ranged from semidominant to dominant, depending on the cross and the treatment concentration (Table 3). For example, *h* values for the

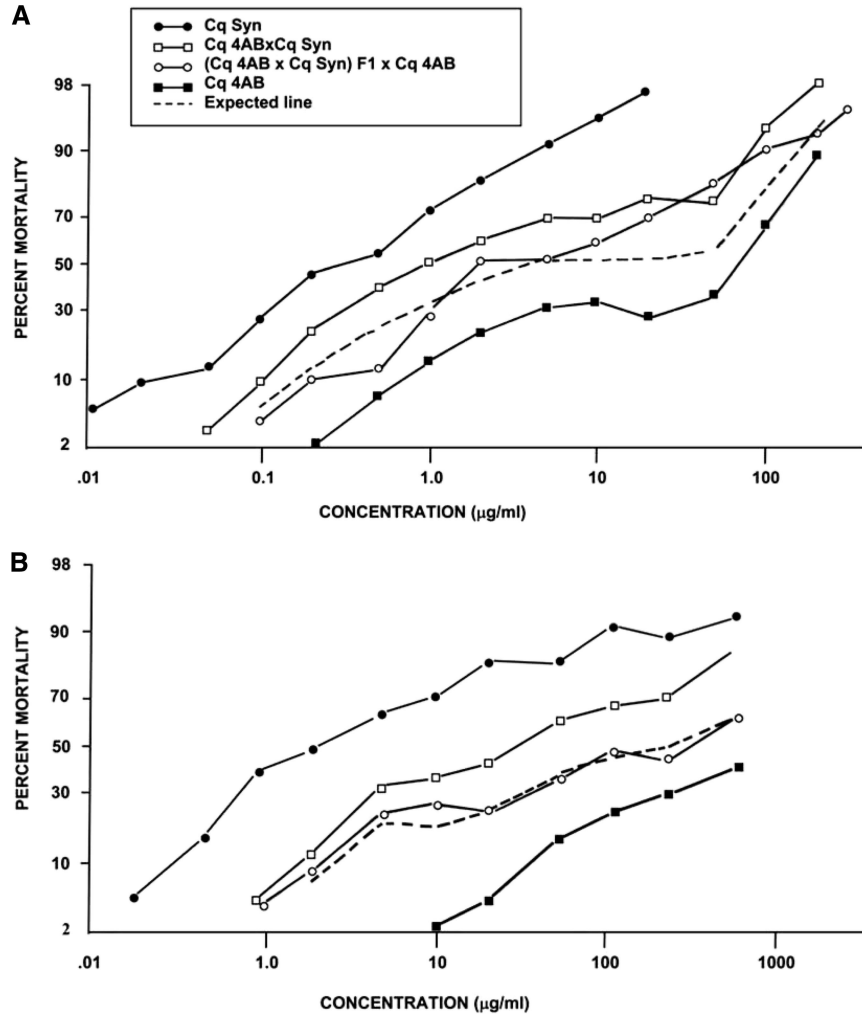

Fig. 1. Results of dose-response testing with *B. thuringiensis israelensis* toxins (A) Cry4Aa + Cry4Ba and (B) Cryl1Aa against the parental (Cq4AB and CqSyn), F_1, and backcross generations of colony Cq4AB. The dashed line is the line expected under a monofactorial model.

CqSyn × Cq11A cross were one (complete dominance) at 2.0 μg/ml, 0.70 (semidominant) at 20 μg/ml, and 0.89 (semidominant) at 200 μg/ml. Cross-resistance to Cry4Aa + Cry4Ba and to Cryl1Ba ranged from dominant ($h = 1$) to recessive ($h = 0$), and dominance declined as the treatment concentration increased.

Colony Cq4AB showed patterns of dominance different than those observed in colony Cq11A. Cross-resistance to Cryl1Aa ranged from dominant to semirecessive, and declined as treatment concentration increased. Resistance to Cry4Aa + Cry4Ba and cross-resistance to Cryl1Ba ranged from semidominant to semirecessive, and also declined with increasing treatment concentration.

In the absence of insecticide exposure, resistance remained stable in both selected colonies, particularly at the lower treatment concentrations. R values were calculated at 0.2, 2, 20, and 200 μg/ml for each colony/insecticide combination (Table 4). R values for colony Cq11A were generally positive for Cryl1Aa and <0.01. R values for Cq4AB were consistently negative, but less than −0.01, suggesting a very minor decline in resistance. Resistance was stable toward Cry4a + Cry4Ba in both colonies.

Offspring of the reciprocal crosses between Cq11A and Cq4AB were tested with the various toxin powders, and resistance levels were determined (Table 5). The offspring of the hybrid crosses were not significantly different from each other in their susceptibility, indicating autosomal inheritance with no maternal effects. Resistance and cross-resistance alleles were strongly complementary because the offspring of both crosses were as resistant, or more resistant, than their homozygous resistant parents. This was particularly notable at the LC_{95}, in which resistance ratios were an order of magnitude higher than those of either resistant parent. In the absence of selection for 13 gener-

Table 3. Estimation of dominance based on treatment concentration of *B. thuringiensis* toxins for F_1 offspring from reciprocal crosses between Cq11A, Cq4AB, and SynP

Toxin(s)	Concentration μg/ml	*h* value (crosses)			
		Cq11A × CqSyn	CqSyn × Cq11A	CqAB × CqSyn	CqSyn × CqAB
Cry11Aa	0.2	–	–	1	0.67
	2.0	0.83	1	0.81	0.88
	20	0.59	0.70	0.54	0.49
	200	0.84	0.89	0.28	0.14
Cry4Aa + Cry4Ba	0.02	–	–	0.89	1
	0.2	0	0	0.53	0.91
	2.0	1	0.38	0.37	0.60
	20	0.23	0.47	0.29	0.59
	200	0	0	0.20	0.30
Cry11Ba	0.02	1	1	0.68	0.76
	0.2	1	1	0.71	0.54
	2.0	0.88	1	0.55	0.52
	20	0	0	0.14	0.41

The *h* value indicates the degree of dominance of a phenotypic trait. An *h* value of 0 indicates fully recessive inheritance; an *h* value of 1 indicates a fully dominant trait; and an *h* value of 0.5 indicates a codominant trait. When $0 < h < 0.5$, the trait is partially recessive, whereas when $0.5 < h < 1$, the trait is partially dominant.

ations, mortality at selected concentrations fluctuated, but only extremely small declines were noted to either Cry11Aa or Cry4A + Cry4Ba. R values were generally negative, but very close to 0 (−0.0085 or less) (Table 6).

Discussion

Larvae of two colonies of *C. quinquefasciatus* reared in the laboratory under selection pressure since 1990 with the insecticidal toxins Cry11Aa or Cry4Aa + Cry4Ba evolved high levels of insecticide resistance and cross-resistance to a variety of mosquitocidal toxins, including Cry11Ba, to which they had not been exposed previously. F_1 offspring of reciprocal crosses in both colonies showed dose-response lines consistent with autosomal inheritance of the resistance alleles, and no maternal effects. These results are in agreement with previous reports of autosomal inheritance of resistance to *B. thuringiensis* in other insect species, including *Plodia interpunctella* (Hübner) (McGaughey 1985), *Plutella xylostella* (L.) (Tabashnik et al. 1992), *Heliothis virescens* (F.) (Gould et al. 1995), *Leptinotarsa decemlineata* (Say) (Rahardja and Whalon 1995), *Pectinophora gossypiela* (Saunders) (Tabashnik et al. 2002), *Ostrinia nubilalis* (Hübner) (Alves et al. 2006), and *Helicoverpa armigera* (Hübner) (Mahon et al. 2007).

Table 4. Stability of resistance in the absence of selection pressure in colonies Cq11A and Cq4AB

Toxins	Generation	Concentration μg/ml	Colony (% mortality)		R value	
			Cq11A	Cq4AB	Cq11A	Cq4AB
Cry11Aa	1	2	10	2		
		20	20	4		
		200	43	26		
	19	2	18	6	0.0005	−0.0010
		20	39	35	−0.0065	−0.0089
		200	27	41	0.0060	−0.0052
Cry4Aa + Cry4Ba	1	0.2	3	9		
		2	47	32		
		20	54	41		
		200	86	93		
	19	0.2	0	0	0.0027	0.0022
		2	10	21	0.0121	0.0034
		20	25	63	0.0112	−0.0107
		200	72	64	0.0150	0.0374

R value measures the rate of change in the absence of insecticide exposure. A negative R value indicates a decline in the proportion of individuals surviving exposure to insecticide. R values close to 0 indicate no change in the proportion of individuals surviving insecticide exposure.

The bioassay data with the various Cry proteins against offspring from the backcross experiments showed interesting similarities and differences between the phenotypic expression of resistance in the two colonies. Inheritance of Cry11Aa resistance was polyfactorial in Cq11A, whereas inheritance better fit a monofactorial model for colony Cq4AB. Cry4Aa + Cry4Ba resistance was a polyfactorial trait in both colonies. Cry11Ba cross-resistance inheritance patterns were consistent with the monofactorial model for Cq11A, but better fit a polyfactorial model for Cq4AB. These differences could result from experimental error as a result of the inherent problems with the classical Mendelian approach. However, the robustness of the data sets for all backcross offspring suggests these data reflect the distinct evolutionary path of each colony in response to selection pressure with different Cry toxins.

The phenotypic expression of dominance of resistance and cross-resistance also differed between the two selected colonies and was dependent on the treatment concentration and the specific protein(s) tested. Despite these differences, dominance generally decreased as the toxin concentration increased. Dominance of *B. thuringiensis* resistance has been reported to vary with treatment concentration, decreasing in response to increasing treatment concentration, in a number of insect species, including the following: *L. decemlineata* (Rahardja and Whalon 1995), *P. xylostella* (Liu and Tabashnik 1997, Sayyed et al. 2000, Liu et al. 2001), *P. gossypiella* (Tabashnik et al. 2002), and *O. nubilalis* (Alves et al. 2006). However, other expressions of dominance, such as incomplete dominance (Sims and Stone 1991, Huang et al. 1999), incomplete recessive (Hama et al. 1992, Kain et al. 2004), and fully recessive inheritance (Augustin et al. 2004, Tabashnik et al. 1992, Mahon et al. 2007) have been reported.

Although both resistant colonies followed the same general relationship between dominance and treatment concentration, distinctly different levels of dominance and overall resistance levels were characteristic. For example, Cry11Aa resistance in Cq11A F_1 offspring was codominant to partly dominant. Cross-resistance to Cry11Aa in colony Cq4AB F_1 offspring was partly recessive to partly dominant, and resistance ratios were an order of magnitude lower than observed in Cq11A. Differences were also observed in dominance for the F_1 offspring using Cry4Aa + Cry4Ba; dominance was generally lower in Cq11A F_1 offspring than Cq4AB F_1 offspring, although the levels of resistance attained after selection were very similar

Table 5. Dose-response assay values for Cry4Aa + Cry4Ba, Cry11Aa, or Cry11Ba against offspring of reciprocal crosses between Cq11A and Cq4AB

Toxin(s)	Cross	*N*	LC_{50} (fiducial limit) μg/ml	LC_{95} (fiducial limit) μg/ml	Resistance ratio		χ^2	Slope
					RR_{50}	RR_{95}		
Cry4Aa + Cry4Ba	Cq11A	1,000	6.98 (3.77–12.9)	731 (158–3,524)	35	261	35.5	0.81
	Cq4AB	1,200	39.4 (19.5–80.1)	6,304 (1,026–40,292)	117	678	49.6	0.74
	Cq11A × Cq4AB	800	110.9 (78.2–173)	11,669 (4,458–45,460)	331	1,256	8.7	0.81
	Cq4AB × Cq11A	1,000	107 (74.4–172)	16,696 (6,163–66,020)	537	5,962	12.1	0.75
Cry11Aa	Cq11A	900	493 (251–1,394)	240,000 (39,493–443,000)	444	2,837	3.6	0.61
	Cq4AB	800	878 (543–1,843)	39,964 (12,178–282,665)	156	71	3.7	0.99
	Cq11A × Cq4AB	800	2% mortality at 200 μg/ml					
	Cq4AB × Cq11A	800	21% mortality at 200 μg/ml					
Cry11Ba	Cq11A	1,200	0.177 (0.106–0.297)	4.77 (1.86–12.4)	4.5	22.0	10.9	2.2
	Cq4AB	1,100	1.52 (0.964–2.39)	70.4 (32.2–160)	34.4	321	38.1	1.0
	Cq11A × Cq4AB	1,000	18.2 (11.8–28.7)	2,555 (872–8,111)	466	11,828	19.9	0.74
	Cq4AB × Cq11A	900	15.5 (8.10–29.8)	795 (154–4,265)	397	3,680	39.5	1.0

in the two parental colonies. These differences could result from multiple causes, including the evolution of resistance at independent loci, allelic differences between the two colonies, or from differential expression of the same alleles (Strickberger 1976).

Resistance remained stable in both Cq11A and Cq4AB in the absence of exposure to insecticides. Overall, R values were very close to 0 and positive, indicating no decline in resistance toward Cry11Aa or Cry4Aa + Cry4Ba. Only extremely small negative R values were observed for Cq4AB using Cry11Aa, suggesting a very slight decline in resistance level. All the R values, including the latter values, are much lower than those reported for several lepidopteran species (Tabashnik 1994). Of those studied, *P. interpunctella* had the lowest reported rate of instability with an R value of −0.02. Our R values were an order of magnitude smaller. In most cases, instability of resistance was attributed to fitness costs linked to the resistance alleles (Tabashnik 1994). However, the more likely explanation for stability in our colonies is alleles for resistance came close to fixation at the various loci in response to prolonged selection pressure of a closed population (Strickberger 1976).

Cq11A and Cq4AB evolved similar, broad-spectrum resistance to multiple Cry toxins during their selection in the laboratory. One possible explanation is that both colonies evolved resistance at common loci. The complementation tests, consisting of the reciprocal crosses between Cq11A and Cq4AB, were used to test for allelism at loci for resistance and cross-resistance toward Cry11Aa, Cry4Aa + Cry4Ba, and Cry11Ba in the two colonies. When the resistant colonies were crossed to the susceptible colony, their heterozygous offspring showed resistance levels that were at least an order of magnitude lower than their respective resistant parent. Therefore, in the absence of an unusual interaction between independent loci, offspring of the cross between Cq11A and Cq4AB, which receive half their genes for resistance from each parent, would be expected to express very high levels of resistance if their resistance alleles were complementary, i.e., carried at the same loci in both resistant colonies. In the absence of resistance alleles at common loci, an independent additive genetic effect of multiple heterozygous loci would be expected. The latter would likely yield resistance levels equal to, or greater than, heterozygous resistant offspring, but less than homozygous resistant offspring. As seen in Table 5, offspring of the both crosses were as resistant, or much more resistant to the three Cry toxin powders than either homozygous resistant parent. These results indicate the strong likelihood that the two selected mosquito colonies have alleles for resistance at common loci. It does not indicate that the alleles are identical or that both colonies have all their loci for resistance in common. Both scenarios are unlikely, because Cq11A and Cq4AB showed different phenotypic levels of resistance and cross-resistance, and the backcross data indicated the two colonies differed in the number of loci involved in resistance to Cry11Aa and to Cry11Ba. In fact, in the absence of selection pressure, the Cq11A × Cq4AB line showed a slight increase in susceptibility at lower treatment concentration levels, which would be expected if some loci were not common to both colonies. Interestingly, resistance was more stable at the highest treatment concentration, suggesting that those alleles may be of more importance to the phenotypic expression of resistance.

Table 6. Stability of resistance in the absence of selection pressure in offspring of the cross Cq11A × Cq4AB

Toxin(s)	Concentration (μg/ml)	Generation (% mortality)			R value
		1	8	13	
Cry11Aa	2	0	2	4	−0.0014
	20	0	16	19	−0.0070
	200	2	24	24	−0.0085
Cry4Aa + Cry4Ba	0.2	0	0	0	0.000
	2	11	21	18	−0.0027
	20	30	60	43	−0.0015
	200	61	73	56	0.0040

R value measures the rate of change in the absence of insecticide exposure. A negative R value indicates a decline in the proportion of individuals surviving exposure to insecticide. R values close to 0 indicate no change in the proportion of individuals surviving insecticide exposure.

An alternative explanation for the high levels of resistance observed in the crosses between Cq11A and Cq4AB is overdominance or heterosis. Crossbreeding highly inbred strains can produce more vigorous hybrid offspring resulting from the increase in fitness associated with the dramatic reduction in homozygosity at multiple loci (Strickberger 1976). Because no increased fitness was noted in the hybrid offspring from crosses with the susceptible strain, this explanation seems less likely.

The cross-resistance that evolved between the colonies selected with Cry11Aa or Cry4Aa + Cry4Ba indicates that alleles selected by Cry4Aa + Cry4Ba confer resistance to Cry11Aa, whereas alleles selected by Cry11Aa confer resistance to Cry4Aa + Cry4Ba. Similarly, both colonies evolved cross-resistance to Cry11Ba in response to selection with Cry11Aa or Cry4Aa + Cry4Ba, despite the absence of prior exposure. Some similarity in evolutionary pathway for resistance would not be completely unexpected because the two selected colonies were derived from the same, albeit large, heterogeneous synthetic population (Georghiou and Wirth 1997). However, the extent of similarities suggests that the number of resistance alleles in the original source population was relatively constrained, because similar alleles at the same loci arose independently under two different selection regimes (Tabashnik et al. 1998). If these alleles represent variations in the binding affinities for these Cry proteins, as reported for many insects resistant to *B. thuringiensis*, then it is likely that Cry11Aa, Cry4Aa + Cry4Ba, and Cry11Ba share a binding site (or sites), or there is cross-recognition of the different binding sites by these toxins. Binding assays with *B. thuringiensis* subsp. *israelensis* Cry proteins using brush border membrane vesicles from *Aedes aegypti* L. suggested that Cry11Aa, Cry4Aa, and Cry4Ba toxins may share a common class of binding sites (de Barros Moreira Beltrão and Silva-Filha 2007). Shared receptors have also been reported for Cry1A-resistant *P. xylostella*, which has a single receptor that binds Cry1Aa, Cry1Ab, and Cry1Ac and Cry1 F toxins (Ballester et al. 1999, Granero et al. 1996). Binding studies are needed to determine whether this hypothesis is correct.

Cross-recognition of binding sites between Cry11Aa, and Cry4Aa + Cry4Ba might also explain the synergy of these three Cry toxin components of *B. thuringiensis* subsp. *israelensis.* Earlier research clearly demonstrated that the activity of various combinations of Cry proteins from *B. thuringiensis* subsp. *israelensis* was much greater than expected than if presented singly (Crickmore et al. 1995, Poncet et al. 1995). Cooperative receptor binding and/or the formation of hybrid pores were proposed as a possible explanation for these results (Poncet et al. 1995). Although there are now several studies suggesting this possibility for *B. thuringiensis* subsp. *israelensis*, more research is needed before this possible mechanism can be accepted.

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