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Cross-resistance spectra of *Culex quinquefasciatus* resistant to mosquitocidal toxins of *Bacillus thuringiensis* towards recombinant *Escherichia coli* expressing genes from *B. thuringiensis* ssp. *israelensis*

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Summary

Sixteen *Escherichia coli* clones were assayed against susceptible and *Bacillus thuringiensis*-resistant *Culex quinquefasciatus* larvae. The clones expressed different combinations of four genes from *Bacillus thuringiensis* ssp. *israelensis*; three genes encoded mosquitocidal toxins (Cry11Aa, Cry4Aa and Cyt1Aa) and the fourth encoded an accessory protein (P20). The cross-resistance spectra of the mosquitoes were similar to the profiles for recombinant *B. thuringiensis* strains expressing *B. thuringiensis* toxin genes, but with varied toxicity levels. The toxicity of the recombinants towards resistant mosquito larvae was improved when *p20* and *cyt1Aa* were expressed in combination with *cry4Aa* and/or *cry11Aa*. Recombinant pVE4-ADRC, expressing *cry4Aa*, *cry11Aa*, *p20* and *cyt1Aa*, was the most active against the resistant *Culex*, and resistance levels did not exceed fourfold. These results indicate that *B. thuringiensis* ssp. *israelensis* genes expressed in a heterologous host such as *E. coli* can be effective against susceptible and *B. thuringiensis*-resistant larvae and suppress resistance.

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Introduction

Bacillus thuringiensis ssp. *israelensis* de Barjac is a bacterium with toxicity against insects of the suborder Nematocera. The bacterium is an effective insecticide against mosquitoes, blackflies and Chironomid midges, and is used to control the larval stages of these pests (Margalith and Ben-Dov, 2000). Its insecticidal activity is due to expression of various toxic genes residing on a 128 kb plasmid known as pBtoxis (Ben-Dov *et al.*, 1999; Berry *et al.*, 2002). These genes encode key toxic proteins Cry4Aa (128 kDa), Cry4Ba (134 kDa), Cry11Aa (72 kDa) and Cyt1Aa (27 kDa), which accumulate during sporulation and assemble into a spherical parasporal crystal surrounded by a lamellar envelope (Ibarra and Federici, 1986). Additional genes on the plasmid affect the synthesis and assembly of these toxins. For example, the P20 accessory protein encoded within the *cry11Aa* operon increases the levels of expression of Cyt1Aa, Cry4Aa and Cry11Aa in acrystalliferous *B. thuringiensis* (Chang *et al.*, 1992; Wu and Federici, 1993) as well as in *Escherichia coli* (Adams *et al.*, 1989; Visick and Whiteley, 1991; Yoshisue *et al.*, 1992; Manasherob *et al.*, 2001).

The Cry proteins of *B. thuringiensis* ssp. *israelensis* are related to other Cry toxins in their amino acid sequence (Crickmore *et al.*, 1998; Schnepf *et al.*, 1998). Cyt1Aa is unrelated to Cry toxins, is cytolytic *in vitro* to a variety of cells in culture, and shows affinity for the unsaturated fatty acids in the lipid portion of cell membranes (Thomas and Ellar, 1983). The mechanism of action for Cry and Cyt toxins is also different. The Cry toxins appear to act by colloid-osmotic lysis to form cation-specific *trans*-membrane pores (Knowles and Ellar, 1987), whereas the mechanism of action of Cyt toxins is still unresolved. They

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may form trans-membrane pores (Promdonkoy and Ellar, 2003) or alternatively, cause a detergent-like disruption of the lipid fraction of the membrane (Butko, 2003; Manceva *et al*., 2005).

The mosquitocidal action of *B. thuringiensis* ssp. *israelensis* requires ingestion of the parasporal crystal by sensitive larval species, dissolution of the crystal, and subsequent processing of the protoxin proteins into active toxins by high pH and proteases in the larval midgut (Delécluse *et al*., 2000). The active toxins bind to specific receptors on the midgut microvillar membrane leading to toxicity. Purified parasporal crystals from *B. thuringiensis* ssp. *israelensis* show high activity towards mosquito larvae, with LC_{50} values in the range of 10–14 ng ml^{-1} against fourth-instar *Aedes aegypti*, *Anopheles stephensi* and *Culex pipiens* (Crickmore *et al*., 1995). The four major toxins of *B. thuringiensis* ssp. *israelensis*, however, differ in their toxicity levels and host range; for example, Cry4Ba is active primarily against *Anopheles* and *Aedes*, and poorly active against *Culex* (Crickmore *et al*., 1995; Poncet *et al*., 1995; Margalith and Ben-Dov, 2000).

The high toxicity of *B. thuringiensis* ssp. *israelensis* results from synergistic activity among its individual component toxins (Crickmore *et al*., 1995; Poncet *et al*., 1995). Synergy also plays an important role in the risk for insecticide resistance in mosquito populations. Resistance to *B. thuringiensis* ssp. *israelensis* has not been detected in treated field populations (Becker and Ludwig, 1993; Becker, 2000), whereas mosquitoes under long-term selection pressure in the laboratory with Cry toxins, but without the Cyt1Aa toxin, evolved substantial levels of resistance (Georghiou and Wirth, 1997). The results indicated that Cyt1Aa is a key protein delaying resistance. That hypothesis was tested in the laboratory by treating *Culex quinquefasciatus* with Cry11Aa and Cyt1Aa, either alone or in combination for more than 48 generations. Mosquitoes treated with a 3:1 mixture of Cry11Aa + Cyt1Aa evolved less than 10-fold resistance to the mixture and to Cry11Aa alone, while those treated only with Cry11Aa evolved > 1000-fold resistance, demonstrating that Cyt1Aa significantly delayed the evolution of resistance to Cry11Aa (Wirth *et al*., 2005). Furthermore, Cyt1Aa was able to overcome resistance after it evolved. For instance, the high level of Cry11Aa resistance in the Cry11Aa-treated colony was suppressed when it was exposed to a 3:1 ratio of Cry11Aa and Cyt1Aa (Wirth *et al*., 2005). High levels of resistance to another microbial strain, *Bacillus sphaericus* Neide, were also reduced when Cyt1Aa was fed with *B. sphaericus* (Wirth *et al*., 2000).

Despite the high toxicity and low resistance that characterize *B. thuringiensis* ssp. *israelensis*, these insecticides have shortcomings. The relatively short residual activity after it is applied to water surfaces for mosquito control requires weekly treatments, which are labour intensive and costly (Mulla, 1990). In contrast, *B. sphaericus*-based insecticides have longer residual activity against mosquitoes, particularly in water containing high levels of organic material where *B. thuringiensis* ssp. *israelensis* performs poorly, possibly because *B. sphaericus* spores can germinate and recycle in this environment (Lacey, 1990). Different strategies have been proposed to improve residual activity for *B. thuringiensis* ssp. *israelensis*. One approach is to express its mosquitocidal toxins in an alternative host such as cyanobacteria, which can replicate in mosquito-breeding sites and are a food source for larvae, in order to enhance the availability and prolong the efficacy of the toxins (Boussiba *et al*., 2000). The success of this strategy requires the new host to show high toxicity and little risk for resistance equivalent to *B. thuringiensis* ssp. *israelensis*.

To test the hypothesis that expressing *B. thuringiensis* ssp. *israelensis* toxin genes in alternative hosts would not affect the cross-resistance patterns of *B. thuringiensis*-resistant *C. quinquefasciatus*, we tested 16 *E. coli* clones that expressed all combinations of the four genes from *B. thuringiensis* ssp. *israelensis.* We report that the recombinant pVE4-ADRC expressing all four genes was active against both susceptible and resistant *C. quinquefasciatus* and that resistance to Cry toxins in the insecticide-resistant mosquitoes was substantially reduced. Therefore it is possible that these characteristics will be retained in other hosts such as *Anabaena*.

Results

Recombinants pHE4-R, pRM4-C and pRM4-RC expressing *p20*, *cyt1Aa* and *p20* + *cyt1Aa*, respectively, were not toxic at 200 μg ml^{-1} against the susceptible (CqSyn) or resistant mosquito colonies (data not shown). The dose–response values for the recombinant strains expressing *cry4Aa*, either alone or in combination with *p20*, with or without *cyt1Aa*, are reported in Table 1 and the immunoblots are shown in Fig. 1A. Recombinants pHE4-A and pHE4-AR showed low toxicity against the susceptible and resistant colonies and no Probit Analysis was possible. Those same clones showed moderate to low expression, respectively, in the immunoblots (Fig. 1A). Clone pVE4-AC showed lower levels of Cry4Aa but high levels of Cyt1Aa in the blots (Fig. 1A) and was more active than pHE4-A and pHE4-AR, with an LC_{50} of 1.50 μg ml^{-1} against CqSyn (the susceptible reference colony) and showed an LC_{50} of 18.6 μg ml^{-1} against Cq11A (selected for resistance to Cry11Aa) (Table 1). The resistance ratio for Cq11A was thus 12.4 at the LC_{50}. However, much lower and non-linear activity was shown against the resistant colonies Cq4AB, Cq4AB11A and Cq4AB11Acyt, which are resistant to Cry4Aa + Cry4Ba, Cry4Aa + Cry4Ba + Cry11Aa and Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa respectively. Against the Jeg and Cry11B colonies

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Table 1. Toxicity of various *E. coli* recombinant strains expressing Cry4Aa alone or in combination with p20, Cyt1Aa or p20 plus Cyt1Aa against susceptible *C. quinquefasciatus* or *C. quinquefasciatus* resistant to toxins from *B. thuringiensis* ssp. *israelensis* or *B. thuringiensis* ssp. *jegathesan*.

				Resistance ratio	
Recombinant strain	Mosquito line	LC_{50} (FL) (μg ml^{-1})	LC_{95} (FL) (μg ml^{-1})	LC_{50}	LC_{95}
pHE4-A	CqSyn	Plateau at 78.7% mortality between 5 and 200 μg ml^{-1}			
	Cq11A	Plateau at 54.5% mortality between 5 and 200 μg ml^{-1}			
	Cq4AB	Plateau at 27.0% mortality between 5 and 200 μg ml^{-1}			
	Cq4AB11A	Plateau at 37.0% mortality between 5 and 200 μg ml^{-1}			
	Cq4AB11Acyt	Plateau at 35.0% mortality between 5 and 200 μg ml^{-1}			
pHE4-AR	CqSyn	Plateau at 52.4% mortality between 5 and 200 μg ml^{-1}			
	Cq11A	Plateau at 28.0% mortality between 5 and 200 μg ml^{-1}			
	Cq4AB	Plateau at 10.0% mortality between 5 and 200 μg ml^{-1}			
	Cq4AB11A	Plateau at 25.0% mortality between 5 and 200 μg ml^{-1}			
	Cq4AB11Acyt	Plateau at 16.2% mortality between 5 and 200 μg ml^{-1}			
pHE4-AC	CqSyn	1.50 (1.18–1.65)	11.5 (8.79–16.3)	1.0	1.0
	Cq11A	18.6 (10.2–33.7)	538.7 (122–2504)	12.4	46.8
	Cq4AB	Plateau at 79.8% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB11A	Plateau at 65.8% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB11Acyt	Plateau at 46.5% mortality between 20 and 200 μg ml^{-1}			
	Jeg	15.7 (8.81–28.1)	341 (93.4–1272)	10.5	29.7
	Cry11B	106.6 (45.3–272)	15 344 (951–362 854)	71.0	1334
pVE4-ARC	CqSyn	0.928 (0.649–1.33)	6.08 (3.20–11.9)	1.0	1.0
	Cq11A	1.77 (0.603–5.19)	15.4 (2.17–109)	1.9	2.5
	Cq4AB	1.75 (1.50–2.03)	15.1 (11.7–20.4)	1.9	2.5
	Cq4AB11A	2.70 (1.33–5.48)	42.1 (11.4–156)	2.9	6.9
	Cq4AB11Acyt	1.96 (0.941–4.09)	23.0 (6.17–86.6)	2.1	3.8
	Jeg	1.28 (1.06–1.52)	12.3 (8.84–19.5)	1.4	2.0
	Cry11B	1.75 (1.48–2.05)	14.5 (11.2–20.2)	1.9	2.4

(resistant to *B. thuringiensis* ssp. *jegathesan* or its component toxin Cry11Ba respectively), LC_{50} values for pVE4-AC were 15.7 and 106.6 μg ml^{-1} and resistance ratios were 10.5 and 71.0 respectively. When *cry4Aa*, *p20* and *cyt1Aa* were expressed in a single recombinant, pVE4-ARC, expression of Cry4Aa and Cyt1Aa was higher than in pVE4-AC (Fig. 1A) and the dose–response curves against all the mosquito colonies were linear. Probit Analyses yielded LC_{50} values ranging from 0.928 μg ml^{-1} for CqSyn to 2.70 μg ml^{-1} for Cq4AB11A. Resistance ratios were generally low from 2.0 for Jeg to 2.4 for Cq4AB11A.

Recombinant *E. coli* expressing *cry11Aa* alone or in combination with *p20*, with or without *cyt1Aa*, consistently yielded linear dose–response curves, although toxicity values were generally lower than for the most active *cry4Aa* recombinant, pVE4-ARC. For example, the LC_{50} for pHE4-D was 6.70 μg ml^{-1} against CqSyn (Table 2). Resistant ratios ranged from 6.6 to 8.5 at the LC_{50} for pHE4-D, from 11.0 to 51.7 for pHE4-DR, and from 3.3 to

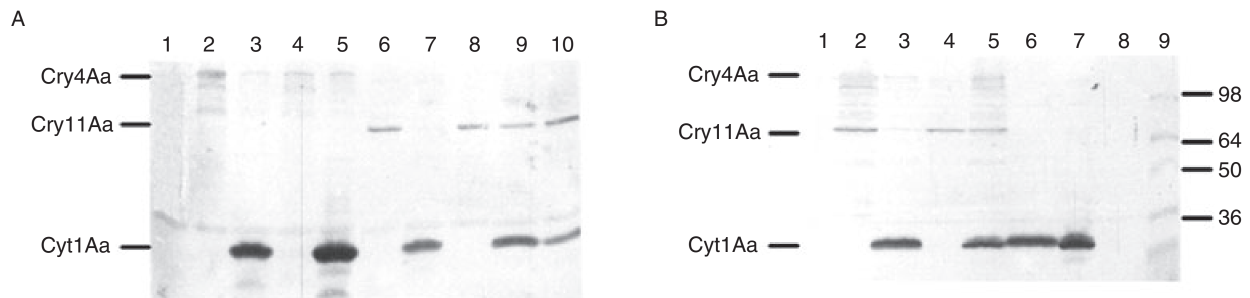

Fig. 1. Immunoblot analysis of *E. coli* clones expressing different combinations of *cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20* from *B. thuringiensis* ssp. *israelensis*.
A. Lane 1, pUHE-24 as a control; lane 2, pHE4-A; lane 3, pVE4-AC; lane 4, pVE4-AR; lane 5, pVE4-ARC; lane 6, pHE4-D; lane 7, pVE4-DC; lane 8, pHE4-DR; lane 9, pVE4-DRC; lane 10, pVRE4-DRC.
B. Lane 1, pUHE-24 as a control; lane 2, pHE4-AD; lane 3, pVE4-ADC; lane 4, pHE4-ADR; lane 5, pVE4-ADRC; lane 6, pRM4-C; lane 7, pRM4-RC; lane 8, pHE4-R; lane 9, molecular size marker. Modified from Khasdan and colleagues (2001).

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Table 2. Toxicity of various *E. coli* recombinant strains expressing Cry11Aa alone or in combination with p20, Cyt1Aa or p20 plus Cyt1Aa against susceptible *C. quinquefasciatus* or *C. quinquefasciatus* resistant to toxins from *B. thuringiensis* ssp. *israelensis* or *B. thuringiensis* ssp. *jegathesan.*

Recombinant strain	Mosquito line	LC_{50} (FL) (μg ml^{-1})	LC_{95} (FL) (μg ml^{-1})	Resistance ratio LC_{50}	Resistance ratio LC_{95}
pHE4 D	CqSyn	6.70 (5.85–7.67)	38.1 (30.0–51.3)	1.0	1.0
	Cq11A	45.4 (39.5–52.2)	258 (201–355)	6.8	6.8
	Cq4AB	44.2 (19.2–101)	367 (53.2–2603)	6.6	9.6
	Cq4AB11A	54.8 (32.0–94.3)	484 (139–1755)	8.2	12.7
	Cq4AB11Acyt	57.0 (39.7–78.0)	3 210 (1096–31 490)	8.5	84.2
pHE4-DR	CqSyn	6.46 (5.50–7.54)	60.4 (46.5–83.2)	1.0	1.0
	Cq11A	97.4 (82.3–118)	786 (525–1379)	15.1	13.0
	Cq4AB	126.7 (109–152)	678 (468–1173)	19.6	11.2
	Cq4AB11A	71.1 (62.1–81.8)	369.9 (283–526)	11.0	6.1
	Cq4AB11Acyt	334 (208–699)	20 040 (5763–159 376)	51.7	332
	Jeg	188 (147–262)	1 953 (1065–4953)	29.1	32.3
	Cry11B	84.1 (54.7–131)	587 (206–1888)	13.0	9.7
pVE4-DC	CqSyn	6.31 (4.14–9.57)	33.1 (15.7–71.7)	1.0	1.0
	Cq11A	53.9 (19.9–147)	1 212 (47.7–34 135)	8.6	36.6
	Cq4AB	20.9 (10.7–40.6)	202 (51.8–803)	3.3	6.1
	Cq4AB11A	88.6 (33.4–242)	2 490 (87.7–88 385)	14.1	75.2
	Cq4AB11Acyt	527 (297–1499)	23 600 (5534–385 145)	83.7	713
	Jeg	53.1 (40.4–73.2)	1 372 (700–3653)	8.4	41.5
	Cry11B	61.7 (114.5–90.0)	2 251 (865–13 390)	9.8	68.0
pVE4-DRC	CqSyn	9% mortality at 200 μg ml^{-1}			
	Cq11A	0% mortality at 200 μg ml^{-1}			
	Cq4AB	4% mortality at 200 μg ml^{-1}			
	Cq4AB11A	0% mortality at 200 μg ml^{-1}			
	Cq4AB11Acyt	4% mortality at 200 μg ml^{-1}			
pVRE4-DRC	CqSyn	4.72 (2.83–7.86)	22.8 (9.03–58.0)	1.0	1.0
	Cq11A	23.9 (8.90–64.2)	249.9 (30.5–2071)	5.1	11.0
	Cq4AB	17.3 (5.23–56.5)	1 306 (38.1–49 445)	3.7	57.3
	Cq4AB11A	34.8 (18.8–64.5)	447.9 (101–2053)	7.4	19.6
	Cq4AB11Acyt	59.1 (15.2–231)	2 735 (27.4–289 225)	12.5	120
	Jeg	54.3 (25.1–118)	1 190 (128–11 879)	11.5	52.2
	Cry11B	18.5 (9.83–34.7)	174 (50.1–619)	3.9	7.6

83.7 for pVE4-DC against the *B. thuringiensis*-resistant colonies. Immunoblots showed that Cry11Aa levels produced by pVE4-DC were low compared with pHE4-D and pHE4-DR (Fig. 1A). Recombinant pVE4-DRC was non-toxic despite the protein levels detected in the immunoblots. However, recombinant pVRE4-DRC, which expressed the same series of genes but in a different configuration and stoichiometry of produced proteins (Khasdan *et al.*, 2001), was much more active with an LC_{50} against CqSyn of 4.72 μg ml^{-1} and resistant ratios ranging from 3.7 to 12.5 at the LC_{50} for the various resistant colonies. In the immunoblots, pVRE4-DRC produced slightly lower levels of Cyt1Aa with higher levels of Cry11Aa than pVE4-DRC (compare lanes 10 and 9 in Fig. 1A) (Khasdan *et al.*, 2001).

When *cry4Aa* and *cry11Aa* were expressed in the same *E. coli* recombinant, with or without *p20* and *cyt1Aa*, toxicity was moderate against CqSyn (Table 3). However, against the *B. thuringiensis*-resistant colonies, recombinants pHE4-ADC and pHE4-ADR showed low activity (Table 3). When all four genes were expressed in recombinant pVE4-ADRC, toxicity was significantly higher, with an LC_{50} of 0.593 μg ml^{-1} against CqSyn, the highest toxicity observed among the recombinants. pVE4-ADRC was also very active against the *B. thuringiensis*-resistant colonies, with LC_{50} values ranging from 0.809 to 1.93 μg ml^{-1} and resistance ratios ranging from 1.4 to 3.3 at the LC_{50}. Recombinant pVE4-ADRC showed higher levels of Cry4Aa and Cry11Aa than pVE4-ADC or pHE4-ADR (Fig. 1B). In addition, higher levels of *p20* were reported for pVE4-ADRC compared with constructs pHE4-AR, pVE4-DRC and pHE4-ADR (Khasdan *et al.*, 2001).

Discussion

When genes for mosquito larvicidal proteins of *B. thuringiensis* ssp. *israelensis* were expressed individually in recombinant *E. coli*, suspensions using lyophilized powders of those strains showed low to moderate toxicity against larvae of susceptible *C. quinquefasciatus*. The recombinant pVE4-ADRC expressing simultaneously all

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Table 3. Toxicity of various *E. coli* recombinant strains expressing Cry11Aa plus Cry4Aa or in combination with p20, Cyt1Aa or p20 plus Cyt1Aa against susceptible *C. quinquefasciatus* or *C. quinquefasciatus* resistant to toxins from *B. thuringiensis* ssp. *israelensis* or *B. thuringiensis* ssp. *jegathesan.*

Recombinant strain	Mosquito line	LC_{50} (FL) (μg ml^{-1})	LC_{95} (FL) (μg ml^{-1})	Resistance ratio LC_{50}	Resistance ratio LC_{95}
pHE4-AD	CqSyn	1.51 (0.559–4.04)	342 (38.4–3111)	1.0	1.0
	Cq11A	Plateau at 35.4% mortality between 10 and 200 μg ml^{-1}			
	Cq4AB	Plateau at 19.8% mortality between 10 and 200 μg ml^{-1}			
	Cq4AB11A	Plateau at 31.8% mortality between 10 and 200 μg ml^{-1}			
	Cq4AB11Acyt	Plateau at 50.4% mortality between 10 and 200 μg ml^{-1}			
pHE4-ADC	CqSyn	4.24 (1.98–9.08)	51.6 (12.7–212)	1.0	1.0
	Cq11A	Plateau at 57.8% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB	Plateau at 50.0% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB11A	Plateau at 61.7% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB11Acyt	Plateau at 38.5% mortality between 20 and 200 μg ml^{-1}			
	Jeg	Plateau at 78% mortality between 50 and 200 μg ml^{-1}			
	Cry11B	Plateau at 63.3% mortality between 50 and 200 μg ml^{-1}			
pHE4-ADR	CqSyn	3.10 (1.15–8.30)	244 (28.9–2082)	1.0	1.0
	Cq11A	Plateau at 24.8% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB	Plateau at 15.0% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB11A	Plateau at 27.0% mortality between 20 and 200 μg ml^{-1}			
	Cq4AB11Acyt	Plateau at 57.0% mortality between 20 and 200 μg ml^{-1}			
pVE4-ADRC	CqSyn	0.593 (0.439–0.801)	1.80 (1.06–3.17)	1.0	1.0
	Cq11A	1.93 (1.13–3.35)	5.10 (1.78–15.3)	3.3	2.8
	Cq4AB	1.47 (0.938–2.30)	4.44 (1.91–10.4)	2.5	2.5
	Cq4AB11A	1.46 (0.0819–47.8)	5.44 (– –)	2.5	3.0
	Cq4AB11Acyt	1.52 (1.01–2.29)	7.39 (3.52–15.7)	2.6	4.1
	Jeg	0.809 (0.522–1.25)	3.10 (1.39–6.95)	1.4	1.7
	Cry11B	1.35 (1.23–1.47)	2.84 (2.43–3.58)	2.3	1.6

four genes, on the other hand, displayed an LC_{50} value (0.593 μg ml^{-1}; Table 3) one order of magnitude higher than the wild-type *B. thuringiensis* ssp. *israelensis*, with lethal concentration values of 0.02–0.03 μg ml^{-1} against susceptible *C. quinquefasciatus* (Wirth *et al*., 2004). This toxicity level for pVE4-ADRC is significant because the activities of endotoxin genes from *B. thuringiensis* expressed in *E. coli* are much lower than in the native host because of weak promotion and stability (Margalith and Ben-Dov, 2000). When tested against laboratory colonies of mosquitoes with high levels of resistance and cross-resistance to *B. thuringiensis* Cry toxins, the same recombinant was very active with resistance ratios of fourfold or less (Table 3). Furthermore, *E. coli* recombinants that expressed *cyt1Aa* in combination with *cry4Aa* or *cry11Aa* were very active, particularly if the regulatory gene *p20* was present (Tables 1 and 2). These results demonstrate that mosquito larvicidal genes from *B. thuringiensis* expressed in the heterologous host *E. coli*, produced highly active toxins that suppress Cry resistance in *Culex.*

The genes *p20* and *cyt1Aa* played important roles in the activity of the recombinant *E. coli* strains, especially against resistant mosquitoes. Strains containing both these genes were consistently more active with one exception, pVE4-DRC. The increase in toxicity is due to the higher rates of synthesis and stability of the Cry and Cyt proteins because of the post-transcriptional accessory role of *p20* (Visick and Whiteley, 1991; Yoshisue *et al*., 1992; Wu and Federici, 1993). Moreover, *p20* protects *E. coli* against the lethal effect of Cyt1Aa, which causes loss of the permeability barrier of the plasma membrane and nucleoid compaction by Cyt1Aa's perforation activity (Manasherob *et al*., 2001; 2003). For instance, in strain pVE4-DC, expression of *cyt1Aa* in the absence of *p20* resulted in rapid loss of colony-forming ability at rates that seem to be negatively correlated with toxicity (Khasdan *et al*., 2001).

Synergistic interactions among *B. thuringiensis* ssp. *israelensis* Cry and Cyt toxins are vital for high activity, and its ability to suppress Cry toxin resistance. Measurement of synergistic interactions was not feasible in this study. However, the increase in activity towards resistant *Culex* with recombinant strains containing Cyt1Aa and P20 is consistent with an earlier study using *B. thuringiensis* clones expressing individual Cry toxins mixed with Cyt1Aa (Wirth *et al*., 2000). The same *E. coli* recombinants were synergistic when mixed and tested against *Ae. aegypti* (Khasdan *et al*., 2001). Therefore, it is likely that synergy played a role in the observed toxicity against susceptible and resistant *C. quinquefasciatus*.

Wild-type *B. thuringiensis* ssp. *israelensis* contain *cry4Ba* in addition to the four genes in the current

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Table 4. Recombinant *E. coli* strains used for tests and the genes expressed.

Recombinant strain	Genes cloned from *B. thuringiensis* ssp. *israelensis*	Reference
1. pHE4-ADR	*cry4Aa*, *cry11Aa*, *p20*	Ben-Dov *et al.* (1995)
2. pVE4-ADRC	cry4Aa, cry11Aa, p20, cyt1Aa	Khasdan *et al.* (2001)
3. pHE4-AD	*cry4Aa*, *cry11Aa*	Ben-Dov *et al.* (1995)
4. pVE4-ADC	cry4Aa, cry11Aa, cyt1Aa	Khasdan *et al.* (2001)
5. pHE4-AR	*cry4Aa*, *p20*	Ben-Dov *et al.* (1995)
6. pVE4-ARC	*cry4Aa*, *p20*, *cyt1Aa*	Khasdan *et al.* (2001)
7. pHE4-A	*cry4Aa*	Ben-Dov *et al.* (1995)
8. pVE4-AC	*cry4Aa*, *cyt1Aa*	Khasdan *et al.* (2001)
9. pHE4-DR	*cry11Aa*, *p20*	Ben-Dov *et al.* (1995)
10. pVE4-DRC	*cry11Aa*, *p20*, *cyt1Aa*	Khasdan *et al.* (2001)
11. pVRE4-DRC	*cry11Aa*, *p20*, *cyt1Aa*	Khasdan *et al.* (2001)
12. pHE4-D	*cry11Aa*	Ben-Dov *et al.* (1995)
13. pVE4-DC	*cry11Aa*, *cyt1Aa*	Khasdan *et al.* (2001)
14. pHE4-R	*p20*	Ben-Dov *et al.* (1995)
15. pRMV-C	*cyt1Aa*	Manasherob *et al.* (2001)
16. pRM4-AC	*p20*, *cyt1Aa*	Manasherob *et al.* (2001)

recombinants. Although Cry4Ba is not active against *C. quinquefasciatus*, it does have toxicity towards other important mosquito species, particularly *Anopheles* and *Aedes* (Crickmore *et al*., 1995). Cry4Ba has been shown to interact synergistically with Cry4Aa and Cry11Aa against *Ae. aegypti* (Crickmore *et al*., 1995) and *C. pipiens* (Poncet *et al*., 1995). Thus its expression in combination with the genes we tested would probably enhance toxicity and the host range of the recombinant. The role of Cry4Ba in resistance refractoriness is not known, but its contributions to toxicity and synergy suggest that it could be important.

The Cry, Cyt and P20 proteins essential for toxicity and resistance suppression were present in our recombinants and caused high activity against resistant *Culex*, particularly pVE4-ADRC, with all four genes. The same components in *B. thuringiensis* ssp. *israelensis* have proved effective in mosquito control and in preventing resistance (Becker and Ludwig, 1993; Georghiou and Wirth, 1997). However, the assumption that engineered strains will also avoid resistance when used against field populations for prolonged periods, especially in the absence of *cry4Ba*, that is normally present in wild-type *B. thuringiensis* ssp. *israelensis*, remains to be tested.

A highly efficient transformation system for expressing foreign genes exists for strain PCC 7120 of the nitrogen-fixing cyanobacterium *Anabaena* (Wu *et al*., 1997), which is a natural food source for mosquito larvae (Merritt *et al*., 1992; Avissar *et al*., 1994). The combinations *cry4Aa* + *cry11Aa* + *p20*, with and without *cyt1Aa*, have been cloned through *E. coli* (Khasdan *et al*., 2001) into this strain and shown to have the highest toxicity ever achieved in cyanobacteria against larvae of *Ae. aegypti* (Wu *et al*., 1997; Khasdan *et al*., 2003). The toxicity in transgenic *Anabaena* PCC 7120 harbouring *cry4Aa* + *cry11Aa* + *p20* remained stable after 8 years in culture without antibiotic selection (Lluisma *et al*., 2001), illustrating the potential for recombinant strategies to produce novel, mosquito larvicidal biopesticides for future vector control. Substantial work remains before this tactic can seriously be considered a practical control strategy, particularly because of concerns regarding ecological effects caused by releasing live engineered bacteria, although it is very likely that transgenic *Anabaena* PCC 7120 would be out-competed in the field by indigenous, wild species (Antarikanonda, 1984). Regardless of the strategy that will ultimately be used to enhance availability and efficacy of *B. thuringiensis* ssp. *israelensis* insecticidal toxins, this report provides a proof of principle that heterologous expression of *B. thuringiensis* ssp. *israelensis* toxins might be useful in future mosquito control.

Experimental procedures

Recombinant strains, growth conditions and powder preparation

Recombinant *E. coli* strains (16) and the *B. thuringiensis* ssp. *israelensis* genes that they expressed, as described previously (Ben-Dov *et al*., 1995; Khasdan *et al*., 2001; Manasherob *et al*., 2001), are listed in Table 4. Each strain was inoculated from a 30 ml overnight batch culture grown in Luria–Bertani broth supplemented with 100 μg ml^{-1} ampicillin at 37°C into 600 ml batch cultures vigorously shaken in 3 l flasks with identical medium. Cells were induced to express the cloned genes with 0.5 mM of IPTG at OD_{660} of 0.2–0.3 (about 2×10^8 cells ml^{-1}), harvested by centrifugation after overnight incubation, washed in distilled water, frozen with liquid nitrogen, and lyophilized. Bioassays utilized lyophilized powder suspended in 50 ml deionized water after brief sonication and agitation using about 25 glass beads.

Cells for protein analysis were grown as described above except that cells were harvested by centrifugation after 4 h incubation and induction with IPTG. Cells were resuspended in distilled water at a 50-fold concentration, and disrupted by ultrasonic disintegration until complete lysis. Protein concentrations were determined by Bio-Rad protein kit; BSA was

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used as the standard. The aliquots were boiled (10 min) in sample treatment buffer (62.5 mM Tris-CL, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 0.1 M DTT). Total proteins (~45 µg per lane) were separated by sodium dodecyl sulfate polyacrylamide (10–15%) gel electrophoresis, then stained with Coomassie blue. Proteins were then electrotransferred from the gel onto nitrocellulose filters by 2051 Midget Multiblot Electrophoretic Transfer Unit apparatus (Hoefer Scientific Instruments, San Francisco, CA) for immunoblot analysis. The blots were exposed to specific antisera directed against whole *B. thuringiensis* ssp. *israelensis* crystal (provided by Armelle Delécluse, Pasteur Institute) or P20 (provided by David Ellar, University of Cambridge). Protein A–alkaline phosphatase conjugate was used as a primary antibody detector. Fast nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate tablets (Sigma Chemical) were used to visualize the antigen (Khasdan *et al*., 2001). Figure 1A and B was originally published in Khasdan and colleagues (2001) and is reproduced here with permission from the authors.

Mosquito colonies

Seven laboratory colonies of *C. quinquefasciatus* were used to measure the activity of the various *E. coli* recombinant strains. Four mosquito colonies that are resistant to single or multiple toxins from *B. thuringiensis* ssp. *israelensis* were used: Cry11Aa (colony Cq11A), Cry4Aa + Cry4Ba (colony Cq4AB), Cry4Aa + Cry4Ba + Cry11Aa (colony Cq4AB11A) and Cry4Aa + Cry4Ba + Cry11Aa + Cyt1Aa (colony Cq4AB11Acyt). These colonies were originally established from a large synthetic population consisting of 19 pooled field collections and have been maintained under laboratory selection pressure since 1990 (Georghiou and Wirth, 1997). Two colonies are resistant to the mosquitocidal bacterium *B. thuringiensis* ssp. *jegathesan*, colony Jeg, or to Cry11Ba of *B. thuringiensis* ssp. *jegathesan*, colony Cry11B. These colonies have been under laboratory selection pressure since 1995 (Wirth *et al*., 2004). A susceptible reference colony, CqSyn was used to establish baseline susceptibility values for the recombinant powders (Wirth *et al*., 2004).

Selection for resistance and bioassays

Resistant colonies are maintained under continuous selection pressure with the appropriate recombinant or wild-type *B. thuringiensis* strains. The selection procedure involves exposing groups of 1000 early fourth instars to suspensions of lyophilized powders of the bacterial strains in 1000 ml deionized water for 24 h. Survivors are removed to fresh water, fed and used to continue the colony. Generations are allowed to overlap. Recent measurement of resistance ratios, calculated by dividing the LC_{50} or LC_{95} values for the resistant colony by the concurrently determined LC value for CqSyn, are as follows: Cq11A (formerly known as Cq4D), resistance ratio at the LC_{95} (RR_{95}), 94 000; Cq4AB, RR_{95}, 264; Cq4AB11A, RR_{95}, 101; Cq4AB11Acyt (also known as Cq80), RR_{95}, 5.1 (Wirth *et al*., 2003). Resistant ratios for Jeg and Cy11B at the RR_{95} are 2.0 and 21.8 respectively (Wirth *et al*., 2004).

Escherichia coli recombinant strains were fed to groups of 20 early fourth instars at different concentrations of suspended, lyophilized powders in 100 ml deionized water in 250 ml plastic cups for 24 h. Initially, a range of nine different concentrations between 0.1 and 200 $\mu g\ ml^{-1}$ and an untreated control cup were tested. If little or no mortality was observed after 24 h, subsequent replications were limited to the highest concentration of 200 $\mu g\ ml^{-1}$ plus the control. All tests were replicated five times on five different days. The only exceptions were tests with pVE4-ADRC against colonies Jeg and Cry11B. Those tests were replicated three times before the powders were depleted. Recombinant strains that showed linear mortality in response to the test doses were analysed using a Probit analysis (Finney, 1971; Raymond *et al*., 1993).

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