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Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*

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Summary

The genes *cyt1Aa* and *p20*, encoding, respectively, cytolytic and accessory proteins of *Bacillus thuringiensis* subsp. *israelensis*, were introduced into previously constructed clones expressing *cry4Aa* and *cry11Aa* in *Escherichia coli* (Ben-Dov *et al*., 1995). Fifteen clones with all possible combinations of the four genes were obtained and found to express the genes included. Two new combinations, pVE4-ADRC and pVE4-ARC, expressing *cyt1Aa*, *p20* and *cry4Aa*, with or without *cry11Aa*, respectively, were more toxic than their counterparts without *cyt1Aa*. They displayed the highest toxicity against *Aedes aegypti* larvae ever reached in transgenic bacteria. Five out of the six clones (except pVE4-DC) containing *cry4Aa* or *cry11Aa* (with or without *p20*) displayed varying levels of synergism with *cyt1Aa*: they are 1.5- to 34-fold more toxic than the respective clones without *cyt1Aa* against exposed larvae. Their lethal times also decreased (they kill larvae quicker), more so at higher cell concentrations. These clones are anticipated to dramatically reduce the likelihood of resistant development in the target organisms (Wirth *et al*., 1997).

Introduction

Bacillus thuringiensis subsp. *israelensis* (serovar H14) is a Gram-positive bacterium that forms parasporal crystal proteins (δ-endotoxins) during sporulation and has been accepted as the best biocontrol agent against larvae of mosquitoes and black flies, vectors of many human infectious diseases (Margalith and Ben-Dov, 2000). It is, however, short-lived under field conditions. One way to overcome this limitation is by using transgenic organisms expressing a combination of toxin genes (Ben-Dov *et al*., 1995; Wu *et al*., 1997; Boussiba *et al*., 2000).

All six genes, encoding the toxic proteins (of 134, 128, 78, 72, 27 and 29 kDa in size) *cry4Aa, cry4Ba, cry10Aa, cry11Aa, cyt1Aa* and *cyt2Ba*, reside on a 137 kb plasmid (pBtoxis) (Ben-Dov *et al*., 1996; 1999). The presence of a chaperone-like P20 [20 kDa protein encoded by the third gene of the *cry11Aa* operon (Dervyn *et al*., 1995)] raises the levels of Cyt1Aa, Cry11Aa and Cry4Aa in *E. coli* (Adams *et al*., 1989; Visick and Whiteley, 1991; Yoshisue *et al*., 1992; Manasherob *et al*., 2001) and in an acrystalliferous strain of *B. thuringiensis* (Chang *et al*., 1992; 1993; Wu and Federici, 1993). Transgenic bacteria are killed by expressing Cyt1Aa but are protected by coexpression with P20 (Douek *et al*., 1992; Wu and Federici, 1993; Manasherob *et al*., 2001).

The δ-endotoxin proteins differ, qualitatively and quantitatively, in their toxicity levels and against different species of mosquitoes (Poncet *et al*., 1995; Margalith and Ben-Dov, 2000). Cry4Aa, Cry4Ba and Cry11Aa are much more toxic than Cyt1Aa (Delecluse *et al*., 1991; Margalith and Ben-Dov, 2000), but this does not explain the high larvicidal activity of the crystal. All combinations of these four major proteins against three mosquito species display different synergy factors of between 2.5 and 15 (Crickmore *et al*., 1995; Poncet *et al*., 1995). Cyt1Aa is least toxic of the four but is most synergistic to any of the other three and their combinations (Wu and Chang, 1985; Tabashnik, 1992; Wu *et al*., 1994; Crickmore *et al*., 1995; Wirth *et al*., 1997; Yu *et al*., 2001). Cyt1Aa demonstrate synergism with heterologous mosquitocidal toxins as well. Its mixture with the binary toxin of *Bacillus sphaericus* was highly toxic against a strain of *Culex quinquefasciatus* resistant to the latter (Wirth *et al*., 2000a). This same mixture also extended the susceptibility range to *Aedes aegypti,* a species that is not normally susceptible to *B. sphaericus* (Wirth *et al*., 2000b). Partial susceptibility of resistant mosquito strains to the *B. sphaericus* binary toxin was achieved by coexpressing it with Cyt1Ab from *B. thuringiensis* subsp. *medellin* in *B. sphaericus* (Thiery *et al*., 1998) or with

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Cyt1Aa in the acrystalliferous strain of *B. thuringiensis* subsp. *israelensis* (Li *et al*., 2000).

Strains of *C. quinquefasciatus* resistant to single or multiple Cry4Aa, Cry4Ba and Cry11Aa toxins of *B. thuringiensis* subsp. *israelensis* retained their original sensitivity levels in the presence of moderate concentrations of Cyt1Aa (Georghiou and Wirth, 1997; Wirth *et al*., 1997). In addition, these resistant *C. quinquefasciatus* showed cross-resistance to Cry11Ba from *B. thuringiensis* subsp. *jegathesan*; Cyt1Aa combined with Cry11Ba can suppress most of the cross-resistance to Cry11Ba (Wirth *et al*., 1998). Cyt1Aa is toxic to the Cottonwood Leaf beetle, *Chrysomela scripta*, and suppressed high levels of resistance to Cry3Aa found in *B. thuringiensis* subsp. *tenebrionis* (Federici and Bauer, 1998). All the above findings suggest that the Cyt1Aa may play a critical role in suppressing resistance to the Cry toxins and may be useful in managing resistance to bacterial insecticides (Cheong and Gill, 1997; Georghiou and Wirth, 1997; Wirth and Georghiou, 1997; Wirth *et al*., 1997; Federici and Bauer, 1998; Wirth *et al*., 1998; 2000a; 2000b).

Here, *cyt1Aa* and *p20* of *B. thuringiensis* subsp. *israelensis* were added into previously constructed pHE4 clones bearing *cry4Aa* and *cry11Aa* (Ben-Dov *et al*., 1995) in *E. coli*. From all 15 possible combinations of the four genes, the clones pVE4-ADRC (expressing *cry4Aa, cry11Aa, p20* and *cyt1Aa*) and pVE4-ARC (*cry4Aa, p20* and *cyt1Aa*) were most highly toxic against larvae of *A. aegypti*. The expressed proteins apparently interact synergistically, thereby increasing mosquito larvicidal activity significantly and decreasing lethal time in standard bioassay compared with clones that did not harbour *cyt1Aa*.

Results and discussion

A full series of expression vectors

To complete the set of all 15 possible combinations obtainable from four genes (*cyt1Aa*, *p20*, *cry4Aa* and *cry11Aa*), the last six were constructed by adding *cyt1Aa* and *cyt1Aa* + *p20* (Fig. 1) to the previously described expression vectors (Ben-Dov *et al*., 1995). These, and an additional clone, pVRE4-DRC (in which *cyt1Aa* was added in tandem as the third gene of the operon), are displayed in Table 1. Expression of the cloned genes in all 16 recombinants was confirmed (Fig. 2), and toxicities and synergysm among the expressed proteins quantitated (Table 1).

Expression in the recombinants and their mosquito larvicidal activities

All four proteins were visualized by Western blots using

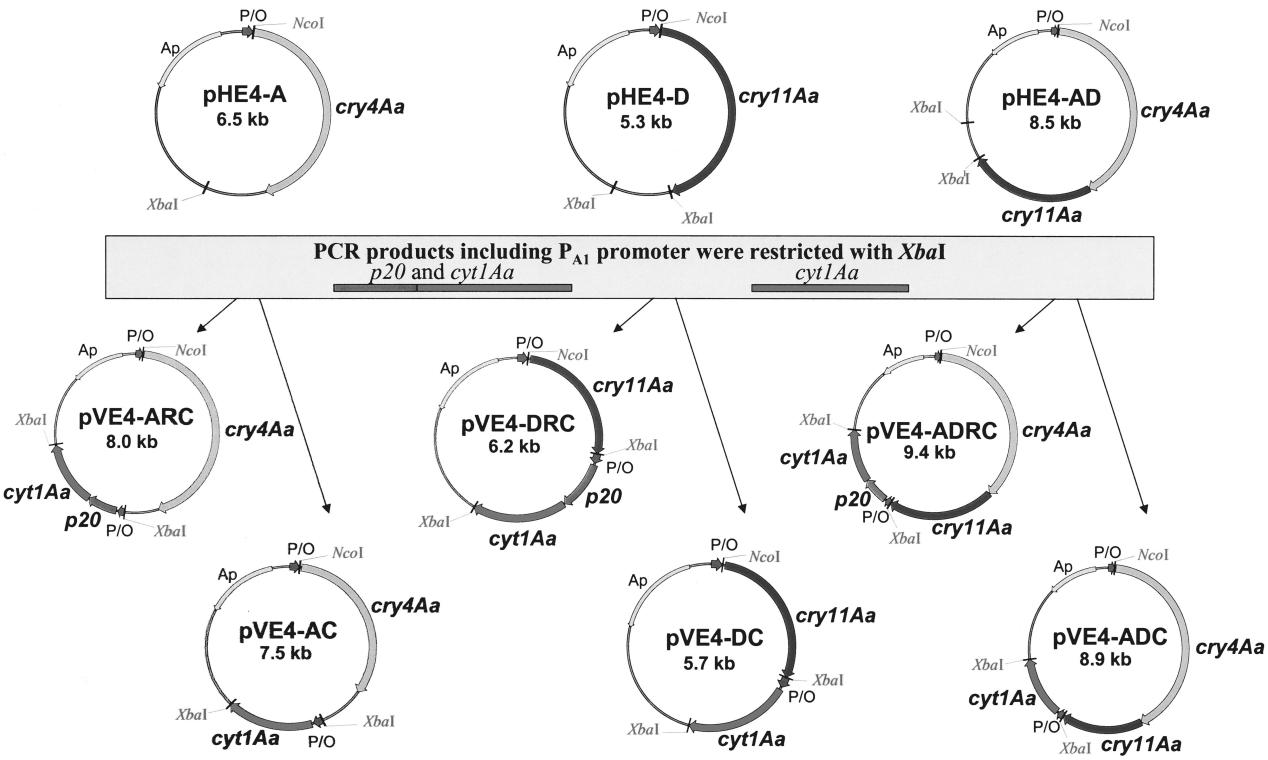

Fig. 1. Cloning of *cyt1Aa* with and without *p20* into the expression vectors pHE4-A, pHE4-D and pHE4-AD harbouring *cry4Aa* and *cry11Aa*. P/O indicate early T7 promoter P_{A1}, which utilizes the usual *E. coli* RNA polymerase and two tandem *lacO* operators.

Table 1. Mosquito larvicidal activities and synergy factors of transgenic *E. coli* strains carrying combinations of genes from *B. thuringiensis* subsp. *Israelensis* against fourth-instar *A. aegypti* larvae, *in cis and in trans* (as mixtures of pRM4-RC with pHE4-AD, -A and -D)[a].

Strain (reference)	Genes cloned from *B. thuringiensis* subsp. *israelensis*	Larvicidal activity $LC_{50} \times 10^6$	Larvicidal activity $LC_{90} \times 10^6$	Theoretical value of LC_{50}[b]	Synergy factor[c]
pHE4-ADR (Ben-Dov *et al.*, 1995)	*cry4Aa*, *cry11Aa* and *p20*	1.31 (1.01–1.66)	9.41 (6.58–15.62)		
pVE4-ADRC (This study)	*cry4Aa*, *cry11Aa*, *p20* and *cyt1Aa*	0.88 (0.80–0.96)	2.28 (2.00–2.68)	2.62	2.98
pHE4-AD (Ben-Dov *et al.*, 1995)	*cry4Aa* and *cry11Aa*	1.68 (1.33–2.04)	8.20 (6.59–10.87)		
pVE4-ADC (This study)	*cry4Aa*, *cry11Aa* and *cyt1Aa*	2.22 (1.87–2.62)	6.74 (5.44–8.86)	3.36	1.51
pHE4-AR (Ben-Dov *et al.*, 1995)	*cry4Aa* and *p20*	12.42 (9.49–17.42)	423.8 (210.3–1106.8)		
pVE4-ARC (This study)	*cry4Aa*, *p20* and *cyt1Aa*	0.96 (0.89–1.05)	2.21 (1.95–2.58)	24.82	25.87
pHE4-A (Ben-Dov *et al.*, 1995)	*cry4Aa*	5.06 (3.58–6.94)	82.28 (47.21–190.54)		
pVE4-AC (This study)	*cry4Aa* and *cyt1Aa*	1.52 (1.26–1.83)	6.00 (4.65–8.33)	10.12	6.66
pHE4-DR (Ben-Dov *et al.*, 1995)	*cry11Aa* and *p20*	~ 500	ND[d]		
pVE4-DRC (This study)	*cry11Aa*, *p20* and *cyt1Aa*	~ 200	ND	~ 1000	~ 5
pVRE4-DRC (This study)	*cry11Aa*, *p20* and *cyt1Aa*	29.03 (15.2–57.9)	224.6 (93.1–3876.0)	~ 1000	~ 34.45
pHE4-D (Ben-Dov *et al.*, 1995)	*cry11Aa*	~ 500	ND		
pVE4-DC (This study)	*cry11Aa* and *cyt1Aa*	ND	ND	–	–
pHE4-R (Ben-Dov *et al.*, 1995)	*p20*	ND	ND		
pRM4-C (Manasherob *et al.* 2001)	*cyt1Aa*	ND	ND		
pRM4-RC (Manasherob *et al.* 2001)	*p20* and *cyt1Aa*	ND	ND	–	–
pHE4-AD + pRM4-RC	*cry4Aa*, *cry11Aa* + *p20*, *cyt1Aa*	1.30 (1.13–1.47)	2.97 (2.56–3.59)	3.36	2.6
pHE4-A + pRM4-RC	*cry4Aa* + *p20*, *cyt1Aa*	1.66 (1.43–1.89)	4.51 (3.92–5.32)	10.12	6.1
pHE4-D + pRM4-RC	*cry11Aa* + *p20*, *cyt1Aa*	> 150	> 2000	> 1000	> 6.7

a. Genes in pHE4-, pRM4- and pVRE4-DRC are expressed under a single early T7 promoter (P_{A1}). In the pVE4-series, *cry4Aa* or *cry11Aa*, and *cyt1Aa* (with or without *p20*) are expressed under two P_{A1}, respectively. LC_{50} and LC_{90} values represent the average numbers (cells ml^{-1}) of three bioassays. Numbers in parentheses are 95% confidence limits, as determined by probit analysis.
b. Theoretical values of LC_{50} were calculated using Tabashnik's equation (Tabashnik, 1992) assuming that pRM4-RC, pHE4-R and pRM4-C are not toxic.
c. Synergy Factor contributed by expression of Cyt1Aa.
d. ND, no toxicity detected, even at a concentration of 5×10^8 cells ml^{-1}.

antibodies, either against whole δ-endotoxin of *B. thuringiensis* subsp. *israelensis* (Fig. 2A and B; to detect Cry4Aa, Cry11Aa and Cyt1Aa) or against P20 (Fig. 2C).

Toxicity of recombinant *Bacillus* spp. expressing δ-endotoxin genes from *B. thuringiensis* subsp. *israelensis* (Ward and Ellar, 1986; Ward *et al.*, 1986; Ward and Ellar, 1988; Chang *et al.*, 1993; Wu and Federici, 1993) is usually higher than of recombinant *E. coli* (Donovan *et al.*, 1988; Adams *et al.*, 1989; Visick and Whiteley, 1991; Chang *et al.*, 1992; Douek *et al.*, 1992) because of weak promotion, low stability and proteolytic cleavage of polypeptides and malformation of crystals (for an extensive review, see Margalith and Ben-Dov, 2000). Consistently, our *E. coli* clones expressing all seven combinations of *cry11Aa*, *cyt1Aa* and *p20* (excluding pVRE4-DRC) displayed very low toxicities, if at all (even at 5×10^8 cells ml^{-1}, Table 1), even though they do express the respective proteins (Fig. 2). The additional clone pVRE4-DRC expressing the same three genes as pVE4-DRC, which produces a lower level of Cyt1Aa but a higher level of Cry11Aa (compare lanes 10 and 9 in Fig. 2A respectively), is an order of magnitude more toxic (Table 1). These two clones were derived differently: in pVRE4-DRC, *cyt1Aa* was added as the third gene without an additional promoter to form a single operon, whereas in pVE4-DRC, *p20* and *cyt1Aa* were added with the promoter P_{A1} to the original clone pHE4-D (Ben-Dov *et al.*, 1995) expressing Cry11Aa from another P_{A1} (Fig. 1). The difference in toxicities between the two clones is discordant with results reported by Wu *et al.*

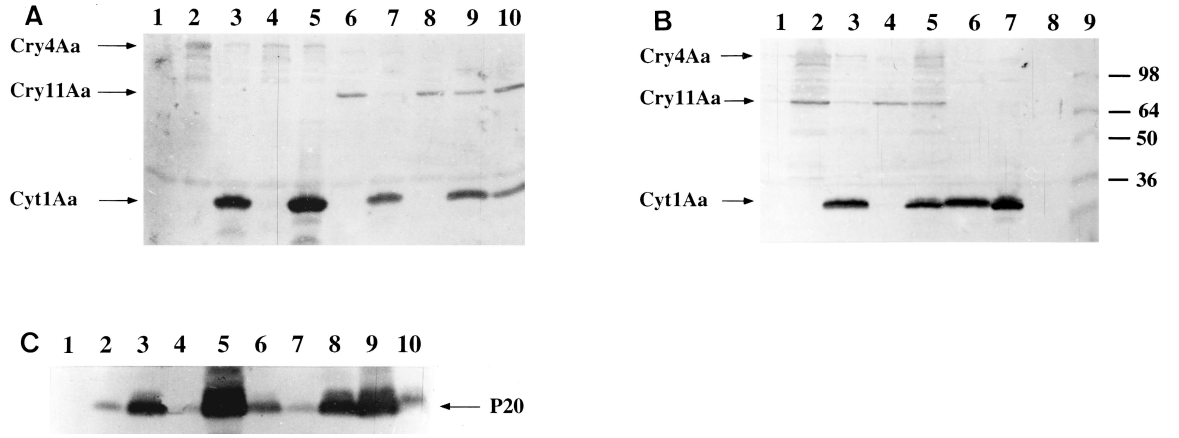

Fig. 2. Immunoblot (A, B, C) analyses of *E. coli* clones (of Table 1) expressing different combinations of *cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20* from *B. thuringiensis* subsp. *israelensis*.
A. Lane 1, pUHE-24 as a control; lane 2, pHE4-A; lane 3, pVE4-AC; lane 4, pHE4-AR; lane 5, pVE4-ARC; lane 6, 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.
C. Lane 1, pUHE-24 as a control; lane 2, pVRE4-DRC; lane 3, pVE4-DRC; lane 4, pHE4-DR; lane 5, pVE4-ARC; lane 6, pHE4-AR; lane 7, pHE4-ADR; lane 8, pVE4-ADRC; lane 9, pRM4-RC; lane 10, pHE4-R.

(1994), in which toxicity was the same when first instar *A. aegypti* larvae have been fed *in vitro* with various ratios of Cry11Aa and Cyt1Aa. This discrepancy may be explained by different susceptibilities of different instars to toxin composition. Lower sensitivities (three to eightfold) were also observed here for fourth instar larvae (Table 1) than previously for third instars (Table 2 in Ben-Dov *et al*., 1995) to four clones expressing *cry4Aa*, *cry11Aa* and *p20* (pHE4-A, -AR, -AD and -ADR).

Among the six new clones expressing *cyt1Aa* constructed in this study (Fig. 1), levels of Cry4Aa and Cry11Aa in the three clones with *p20* as well, pVE4-ARC, -DRC and -ADRC (Fig. 2A, lanes 5 and 9, and Fig. 2B, lane 5 respectively) were higher than in those without *p20* (pVE4-AC, -DC and -ADC; Fig. 2A, lanes 3 and 7; Fig. 2B, lane 3 respectively).

All eight clones expressing Cry4Aa (Fig. 2A and B) displayed various levels of toxicity (Table 1). Highest levels were achieved in pVE4-ADRC and pVE4-ARC producing Cry4Aa + Cyt1Aa + P20, with and without Cry11Aa, respectively (LC_{50} of less than 1×10^6 cells ml^{-1}; Table 1), consistent with the amounts of expressed proteins (lanes 5 in both Fig. 2A and B). The same constructs lacking *p20* (pVE4-ADC and pVE4-AC) were about twofold less toxic, most likely because cells expressing Cyt1Aa lose viability (Douek *et al*., 1992) unless coexpressed with P20 (Manasherob *et al*., 2001). Indeed, the four clones with *cyt1Aa* but not *p20* (pRM4-C, pVE4-AC, pVE4-ADC and pVE4-DC) quickly lose colony-forming abilities, at rates that seem to be negatively correlated with toxicities (data not shown). This series of bioassays demonstrates the significance of Cyt1Aa contribution to toxicity of *B. thuringiensis* subsp. *israelensis* against *A. aegypti* larvae, and the importance of P20 for best results in transgenic *E. coli*. To substantiate this conclusion, pRM4-RC expressing P20 and Cyt1Aa was mixed with the clones expressing Cry4Aa, Cry11Aa or both (pHE4-A, -D and -AD respectively) in a 1:1 ratio by cell number (Table 1). This test *in trans* indeed yielded increased toxicities (decreased LC_{50}s).

Three new clones with both *p20* and *cyt1Aa* (pVE4-ARC, -DRC and -ADRC) produced higher levels of P20 (Fig. 2C, lanes 5, 3 and 8 respectively) than previously constructed (Ben-Dov *et al*., 1995) P20-expressing clones pHE4-AR, -DR and -ADR (lanes 6, 4 and 7 respectively). This may be as a result of at least one of the following differences. First, *p20* is immediately downstream of the promoter (P_{A1}) here (Fig. 1), whereas it is the last gene there. Second, a stretch of 245 bp separates the stop codon of the preceding *cry* gene and the start codon of *p20* in pHE4-AR, -DR and -ADR. The same spacer stretch separates *p20* from P_{A1} in clone pHE4-R (containing *p20* alone) hence it also produces a low level of P20 (Fig. 2C, lane 10). In addition, the genes in pHE4-R, pHE4-DR and pVRE4-DRC were expressed (lanes 10, 4 and 2 respectively) using their own ribosomal binding sites (RBS) rather than the T5 RBS existing in the clones harbouring *cry4Aa* (Ben-Dov *et al*., 1995). The interpretation that the 245 bp spacer and missing T5-RBS cause reduced expression of *p20* is supported by the high level of P20 in the RBS-containing clones pRM4-RC (lane 9 in Fig. 2C; also see Manasherob *et al*., 2001), pVE4-DRC, -ARC and -ADRC (lanes 3, 5 and 8 respectively).

Presence of *p20* seems not to affect the expression

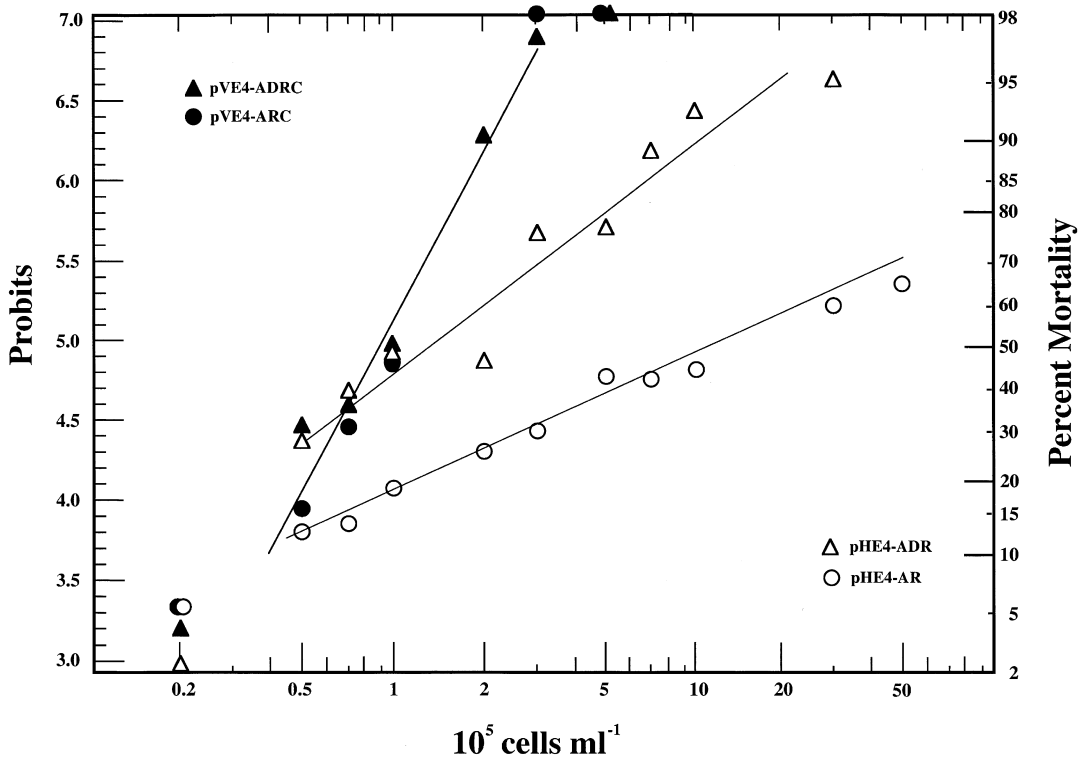

Fig. 3. Toxicity analyses by Probit of *E. coli* recombinants expressing *B. thuringiensis* subsp. *israelensis* Cry toxins with and without Cyt1Aa.

level of Cry11Aa [compare pHE4-DR (lane 8 in Fig. 2A) with pHE4-D (lane 6)]. It does, however, raise the level of Cyt1Aa [compare pRM4-RC (lane 7 in Fig. 2B) with pRM4-C (lane 6)]. This difference is distinct when the total protein loaded on the gel for blotting was diluted (see Fig. 8 in Manasherob *et al.*, 2001).

The dose–response curves (Fig. 3) of the most toxic clones (pVE4-ADRC and pVE4-ARC) were substantially steeper than of their counterparts without *cyt1Aa* (pHE4-ADR and pHE4-AR respectively). This observation is concordant with previous *in vitro* results comparing the dose–response curves of Cyt1Aa to those of the other Cry toxins (Crickmore *et al.*, 1995), as well as when Cyt1Aa has been added to *B. sphericus* toxins against *A. aegypti* larvae (Wirth *et al.*, 2000b). This phenomenon may indicate different mechanisms of pore formation between these toxins. It is consistent with the observation that LT_{50} values of Cyt1Aa-expressing clones were significantly shorter than of their counterparts without *cyt1Aa* (Table 2) and reflects this enhanced toxicity. For example, at 3×10^7 cells ml^{-1}, pVE4-ARC and pVE4-ADRC killed 32 and eight times quicker than the respective clones without *cyt1Aa* (pHE4-AR and pHE4-ADR) (Table 2). At the same cell concentration, the two *cyt1Aa*-expressing clones killed 100% of exposed larvae in 2 h, whereas mortalities with pHE4-AR and pHE4-ADR were about 60% and 90% only in 24 h respectively (data not shown). The differences between clones declined with decreased cell concentration used (Table 2). The same

Table 2. Time needed to kill 50% of exposed population (LT_{50}) of fourth-instar *A. aegypti* larvae by the transgenic *E. coli* strains expressing toxins from *B. thuringiensis* subsp. *israelensis*[a].

Concentration (cell ml^{-1})	LT_{50} (h)			
	pHE4-AR	pVE4-ARC	pHE4-ADR	pVE4-ADRC
3×10^7	20.25 (17.07–26.05)	0.63 (0.22–0.85)	6.78 (6.00–7.65)	0.86 (0.60–1.05)
1×10^7	27.97 (21.96–42.24)	1.91 (1.70–2.10)	6.58 (5.96–7.27)	2.41 (2.04–2.76)
7×10^6	28.77 (22.18–44.31)	2.58 (2.32–2.83)	8.60 (7.77–9.60)	2.86 (2.47–3.24)
5×10^6	34.66 (25.43–60.33)	3.34 (3.03–3.63)	9.74 (8.29–11.86)	3.02 (2.65–3.36)
3×10^6	34.46 (24.98–63.70)	5.48 (5.01–5.96)	15.71 (13.87–18.49)	6.16 (5.64–6.69)
1×10^6	53.43 (33.57–152.98)	23.71 (18.20–33.47)	25.05 (19.41–37.69)	21.52 (17.32–29.92)

a. Numbers in parentheses are 95% confidence limits, as determined by probit analysis.

was true when pVE4-DRC and pVRE4-DRC were compared with pVE4-DR (data not shown).

LC_{50} (0.9×10^6 cells ml^{-1}) of the most toxic clone, pVE4-ADRC, resembles that of the second most toxic, pVE4-ARC (1×10^6 cells ml^{-1}) (Table 1), although the latter kills 30% faster at high cell concentrations (Table 2). This seems to be caused by a higher level of Cyt1Aa expressed in this clone (Fig. 2A, lane 5) than in the former (Fig. 2B, lane 5). This is consistent with the observation (Crickmore *et al*., 1995), that the concentration of Cyt1Aa required to kill 98% of exposed *A. aegypti* larvae is lower than of Cry4Aa or of Cry11Aa.

Toxicities of the two clones expressing Cry4Aa without Cry11Aa or Cyt1Aa (pHE4-A and pHE4-AR) were least toxic among the eight best (Table 1). They were still much better than clones expressing Cry4Aa derived by others (Bourgouin *et al*., 1988; Ward and Ellar, 1988; Yoshida *et al*., 1989). It is noteworthy here, that *cry4Ba* had been efficiently expressed in *E. coli* and formed phase-bright insoluble inclusions that were highly toxic to *A. aegypti* larvae (Angsuthanasombat *et al*., 1987; Chungiatupornchai *et al*., 1988; Delecluse *et al*., 1988; Ward and Ellar, 1988). Addition of *cry4Ba* to our most toxic clones is thus anticipated to yield a still better mosquito control agent despite the lower synergism it displays with Cry11Aa (Poncet *et al*., 1995) or with Cyt1Aa (Crickmore *et al*., 1995). A construct expressing all four major toxins would result in an *E. coli* strain with toxicity approaching that of the original *B. thuringiensis* subsp. *israelensis*; LC_{90} of the latter (1.3×10^4 spores ml^{-1}) against *second instar* larvae of *A. aegypti* (Silapanuntakul *et al*., 1983) is only about 200-fold lower than of our best clones against *4th instars* (2.2×10^6 cells ml^{-1}; Table 1).

Synergism

The data presented here demonstrates that three out of the major δ-endotoxins of *B. thuringiensis* subsp. *israelensis* (Cyt1Aa, Cry4Aa and Cry11Aa) interact synergistically to kill fourth-instar *A. aegypti* larvae when expressed in *E. coli* (Table 1), as they do *in vitro* (Wu and Chang, 1985; Wu *et al*., 1994; Crickmore *et al*., 1995; Poncet *et al*., 1995; Wirth *et al*., 1997).

Of the seven constructs harbouring *cry4Aa* or *cry11Aa* and *cyt1Aa* (with or without *p20*), all but one (pVE4-DC) displayed varying synergy factors (SF values, defined in *Experimental procedures*, between 1.5 and 34; Table 1), demonstrating that Cyt1Aa synergizes the two Cry proteins. Highest SF values (26 and 34) were displayed by two clones (pVE4-ARC and pVRE4−DRC respectively) with combinations including either of the two Crys with Cyt1Aa + P20.

Two clones among those derived in this study were found most suitable for mosquito biocontrol: pVE4-ARC, with LC_{50} of about 10^6 cells ml^{-1} and SF of 26; and pVE4-ADRC, with LC_{50} of about 0.9×10^6 cells ml^{-1} and SF value of about 3. Expression in the latter of an additional toxin, Cry11Aa, is an exceedingly important asset to reduce the chance to develop resistance in the target organism, *A. aegypti*. Inclusion of *cyt1Aa* in both clones assures such an advantage (Georghiou and Wirth, 1997; Wirth and Georghiou, 1997; Wirth *et al*., 1997, 1998; 2000a; 2000b).

As with toxicity, mixing clone pRM4-RC (expressing Cyt1Aa and P20) with each of the three clones expressing Cry4Aa, Cry11Aa or both (pHE4-A, -D and -AD respectively) in a 1:1 ratio by cell number also resulted in synergy between Cyt1Aa and the Crys, albeit with lower SF values (of 2.6- to 6.7-fold) than in pVE4-ADRC, -ARC and −DRC (Table 1), when these proteins were supplied *in cis*. The discrepancy in two of the three comparisons is yet to be resolved.

The two Cry proteins (−4Aa and −11Aa) synergized each other as well: SF values of clones pHE4-ADR and pHE4-AD (with and without *p20*) were calculated from the data of Table 1 to range between 6 and 18.

Values of SF gleaned from the literature for purified toxins or lyophilized powders of *B. thuringiensis* strains expressing the same genes (Wu and Chang, 1985; Wu *et al*., 1994; Crickmore *et al*., 1995; Poncet *et al*., 1995; Wirth *et al*., 1997) are similar to those obtained here *in vivo* (Table 1). In most cases, combinations with *cyt1Aa* are most synergistic. For *in vivo* studies with species (such as *E. coli*) to which Cyt1Aa is toxic (Douek *et al*., 1992), cells expressing Cyt1Aa must express P20 as well to remain alive (Manasherob *et al*., 2001).

The combinations of *cry4Aa* + *cry11Aa*, with and without *p20*, have previously been cloned through *E. coli* (Ben-Dov *et al*., 1995) in the nitrogen-fixing filamentous cyanobacterium *Anabaena* PCC7120 to yield a high level of toxicity (Wu *et al*., 1997). Our next goal is to clone the four major δ-endotoxin genes into cyanobacterial strains as an approach to overcome the low efficacy and short half-life in nature of current formulations of *B. thuringiensis* subsp. *israelensis* (Margalith and Ben-Dov, 2000). Cyanobacteria, which proliferate near the water surface and resist different environmental conditions, may thus provide an alternative for prolonged delivery of *B. thuringiensis* subsp. *israelensis* δ-endotoxin to mosquito larvae that breed in the same habitat (Boussiba *et al*., 2000).

Experimental procedures

Bacterial strains and plasmids

Strain XL-Blue MRF′ of *E. coli* (Stratagene, La Jolla, CA) was used as a host. Plasmids pRM4-RC and pRM4-C

(Manasherob *et al*., 2001) served for isolating *cyt1Aa* with and without *p20* respectively. Plasmids pHE4-A, pHE4-AR, pHE4-D, pHE4-DR, pHE4-AD, pHE4-ADR and pHE4-R (Ben-Dov *et al*., 1995) served for cloning all 15 combinations of four *B. thuringiensis* subsp. *israelensis* genes (Table 1).

Recombinant DNA methods

DNA modification and restriction enzymes were used as recommended by the suppliers, and carried out as described by Sambrook and Russell (2001). Competent cells were prepared and plasmids isolated by standard procedures. Transformants of *E. coli* strains XL-Blue MRF′ were selected on Luria–Bertani (LB) plates containing ampicillin (100 μg ml^{-1}) and tetracycline (10 μg ml^{-1}). DNA was analysed by electrophoresis on horizontal 0.7% agarose slab gels and visualized with ethidium bromide.

Polymerase chain reaction

Amplification was carried out with the high fidelity Vent DNA polymerase (New England Biolabs) with 3′-to-5′ proofreading exonuclease activity, in a DNA MiniCycler (MJ Research, Watertown, MA) for a 30-reaction cycle each. Cycle parameters consisted of 40 s at 94°C, 30 s at 62°C and 1–2 min at 72°C.

The two primers employed to obtain *p20* with *cyt1Aa* and *cyt1Aa* alone were: (i) a 26-mer 5′-TAGGCGTA**TCTAGA**GGCCCTTTCGTC-3′, with a *Xba*I restriction site (boldfaced); and (ii) a 26-mer 5′-TCACCGTCATCACCGAAACGCGCGAG-3′, which annealed five bases downstream from the original unique site of *Xba*I on pRM4-RC and pRM4-C.

Cloning of cyt1Aa *with and without* p20

cyt1Aa and *p20-cyt1Aa*, each under early T7 promoter P_{A1}, which utilizes the usual *E. coli* RNA polymerase (Deuschle *et al*., 1986), were amplified from pRM4-C and pRM4-RC (Manasherob *et al.,* 2001). Respective amplicons were digested by *Xba*I and inserted into pHE4-A, pHE4-D and pHE4-AD (harbouring *cry4Aa*, *cry11Aa* and both, respectively, under P_{A1} (Ben-Dov *et al*., 1995) to yield clones pVE4-AC, pVE4-ARC, pVE4-DC, pVE4-DRC, pVE4-ADC and pVE4-ADRC (Fig. 1 and Table 1).

In addition to construct pVRE4-DRC (Table 1), *cyt1Aa* was removed from pRM4-C by *Hin*dIII–*Xba*I and inserted into the same sites of pHE4-DR downstream *cry11Aa and p20* under a single P_{A1}.

Protein analysis

Transgenic *E. coli* cells were grown at 37°C in LB broth supplemented with 100 μg ml^{-1} ampicillin and induced by Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM) when the culture reached an optical density at 660 nm of 0.2–0.3 ($\approx 2 \times 10^8$ cells ml^{-1}). Cells were harvested by centrifugation 4 h later, 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 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 sulphate polyacrylamide (10–15%) gel electrophoresis (SDS–PAGE) then stained with Coomassie blue.

For immunoblot analysis, proteins were electrotransferred from the gel onto nitrocellulose filters by 2051 Midget Multiblot Electrophoretic Transfer Unit apparatus (Hoefer Scientific Instruments, San-Francisco, CA). The blots were exposed to specific antiserum directed against whole *B. thuringiensis* subsp. *israelensis* crystal (kindly provided by Armelle Delecluse, Pasteur Institute) or P20 (kindly provided by David Ellar, University of Cambridge). Protein A-alkaline phosphatase conjugate was used as a primary antibody detector. Being chromogenic substrates for alkaline phosphatase, fast nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate tablets (Sigma Chemical Co) were used to visualize the antigen.

Bioassays for mosquito larvicidal activity

The recombinant *E. coli* were grown at 37°C in LB broth supplemented with 100 μg ml^{-1} ampicillin to optical density at 660 nm of 0.2–0.3 ($\approx 2 \times 10^8$ cells ml^{-1}) and induced by IPTG (0.5 mM). Cells were harvested by centrifugation after 4 h of induction, and resuspended in distilled water. Samples were added to 20 early fourth-instar *A. aegypti* larvae in disposable cups with 100 ml sterile tap water, and larvicidal activity was determined after 24 h at 28°C (Ben-Dov *et al*., 1995). Experimental LC_{50} and LC_{90} (concentrations of cells that kill 50% and 90% of the exposed populations respectively) were determined by using probit analysis (EPA Probit analysis programme) in at least six doses. When mixtures of two clones (after 4 h induction) were bioassays, 1:1 ratios of cell numbers were applied.

LT_{50} (time taken to kill 50% of exposed larvae) was determined by following the kinetics of larval mortality using probit analysis in at least six doses.

All bioassays were performed at least three times in duplicates for each concentration.

Synergism

Synergistic interactions between Cyt1Aa and Crys were evaluated by the ratios between the theoretical LC_{50} and the observed LC_{50}, the so-called synergy factor (SF). The theoretical value was obtained by adapting Tabashnik's equation (Tabashnik, 1992):

$$LC_{50}(\text{mrc}) = \left[\frac{r_a}{LC_{50}(a)} + \frac{r_b}{LC_{50}(b)}\right]^{-1}$$

where LC_{50}(mrc) is for the 'mixture of recombinant clones' expressing different toxins; r_a and r_b are the relative proportions, by cell numbers, of the two clones (here, always 1:1); LC_{50}(a) and LC_{50}(b) are the independent LC_{50} values for each recombinant clone expressing the respective toxin(s). The interactive effects between pairs of clones expressing different toxins were estimated using mixtures containing 1:1 ratios by cell number. When two proteins were expressed in one clone, their accumulation was assumed to be equal by weight.

To characterize the interaction among three toxins when Cry4Aa, Cry11Aa expressed in one clone and Cyt1Aa in another, or three of them expressed in same clone, the assumption was that Cry4Aa, Cry11Aa (r_{4A+11A}) to Cyt1Aa (r_{Cyt}) ratio is 1:1 in each case.

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