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ORIGINAL PAPER

Larvicidal activities against agricultural pests of transgenic *Escherichia coli* expressing combinations of four genes from *Bacillus thuringiensis*

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Abstract The genes *cry1Ac* and *cry1Ca* from *Bacillus thuringiensis* subsps. *kurstaki* HD-73 and *aizawai* 4J4, respectively, encoding δ-endotoxins against lepidopteran larvae were isolated, cloned and expressed in *Escherichia coli*, with and without *cyt1Aa* (encoding cytolytic protein) and *p20* (accessory protein) from subsp. *israelensis*. Nine combinations of the genes under control of an early T7, P_{A1} inducible promoter, produced the encoding proteins. Toxicities were examined against larvae of three major agricultural pests: *Pectinophora gossypiella*, *Helicoverpa armigera* and *Spodoptera littoralis*. The clones expressing *cyt1Aa*, with or without *p20*, were not toxic. The clone expressing *cry1Ac* (pBt-1A) was the most toxic to *P. gossypiella* (LC_{50} of 0.27×10^8 cells g^{-1}). Clone pBt-1CA expressing *cry1Ca* and *cry1Ac* displayed the highest toxicity (LC_{50} of 0.12×10^8 cells ml^{-1}) against *S. littoralis*. Clone pBt-1CARCy expressing all four genes (*cry1Ca*, *cry1Ac*, *p20*, *cyt1Aa*) in tandem exhibited the highest toxicity to *H. armigera* (LC_{50} of 0.16×10^8 cells ml^{-1}). Cyt1Aa failed to raise the toxicity of these Cry toxins against *P. gossypiella* and *S. littoralis* but significantly enhanced toxicity against *H. armigera*. Two additional clones expressing either *cry1Ac* or *cry1Ca* under tandem promoters, P_{A1} and P_{psbA} (constitutive), displayed significantly higher toxicities (7.5- to 140-fold) than their counterparts with P_{A1} alone, reducing the LC_{50} values to below 10^7 cells ml^{-1}.

Keywords *Bacillus thuringiensis* · δ-Endotoxin · Transgenic *Escherichia coli* · Agriculture pests

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Introduction

Various subspecies of the entomopathogenic bacterium *Bacillus thuringiensis* are specific and environmentally safe microbial control agents against larvae of lepidopteran, dipteran, and coleopteran species (Margalith and Ben-Dov 2000). The larvicidal activity is included in a proteinaceous crystalline δ-endotoxins synthesized during sporulation (Schnepf et al. 1998). Extensive use of *B. thuringiensis* to control lepidopteran pests resulted in a number of field

populations with different levels of resistance to the insecticidal crystal proteins (ICPs) (Tabashnik 1994; Sayyed et al. 2000). Some laboratory-selected insects resistant to Cry1 toxins display cross-resistance to other ICPs; e.g., *Pectinophora gossypiella* (Tabashnik et al. 2000), *Plutella xylostella* (Tabashnik et al. 1994), *Heliothis virescence* (Gould et al. 1995) and *Spodoptera exigua* (Moar et al. 1995). Various strategies have been suggested to avoid selection for resistance in these pests, including refuges of susceptible plants along with transgenic insect-resistant plants (Shelton et al. 2000). Mixtures of Cry toxins can be attained: by inter-planting two cultivars, each with a different genetic basis for resistance (mosaic planting); by sequentially planting two cultivars; by gene pyramiding, whereas at least two Cry toxins are expressed in the same cultivar (Cao et al. 2002; Zhao et al. 2003), and by mixing with non-Cry insecticidal genes such as *gna* (snowdrop lectin) (Maqbool et al. 2001) and *CPT1* encoding a cowpea trypsin inhibitor (Zhao et al. 1999).

Contrary to lepidopteran pests, which have evolved resistance to subsps. *kurstaki* and *aizawai* of *B. thuringiensis*, resistance to subsp. *israelensis* has not been observed in mosquito species despite extensive use during several decades (Ludwig and Becker 1997; Margalith and Ben-Dov 2000). Mosquito larvicidal activity of *B. thuringiensis* subsp. *israelensis* resides in at least four major ICPs (of 134, 128, 72 and 27 kDa) encoded by *cry4Aa*, *cry4Ba*, *cry11Aa* and *cyt1Aa*, respectively, all mapped on pBtoxis (Berry et al. 2002). These δ-endotoxins differ in toxicity levels and specificity against different species of mosquitoes (Margalith and Ben-Dov 2000). Despite the low toxicity of Cyt1Aa against exposed larvae, it is highly synergistic with these Cry toxins and their combinations in vitro (Crickmore et al. 1995; Wirth et al. 1997, 2003), because of a different mode of action (Butko 2003). Cyt1Aa synergizes with Cry toxins of *B. thuringiensis* subsp. *israelensis* when expressed simultaneously in transgenic *Escherichia coli* and *Anabaena* PCC 7120 (Khasdan et al. 2001, 2003, respectively), and also synergizes with the heterologous mosquitocidal binary toxin of *Bacillus sphaericus* against *Aedes aegypti* and *Culex quinquefasciatus* (Wirth et al. 2000a, b, 2005). Moderate concentrations of Cyt1Aa suppress resistance of *C. quinquefasciatus* strains selected to single or multiple Cry4Aa, Cry4Ba and Cry11Aa toxins (Georghiou and Wirth 1997; Wirth et al. 1997, 2003). Moreover, cottonwood leaf beetles (*Chrysomela scripta*) resistant to Cry3Aa and *P. xylostella* resistant to Cry1Ac, are sensitive in the presence of Cyt1Aa (Federici and Bauer 1998; Sayyed et al. 2001).

These reports confirm our working hypothesis that Cyt1Aa can play a significant role in suppressing resistance to Cry toxins and hence be useful in managing resistance to bacterial insecticides. This hypothesis was also tested here, by expressing in *E. coli* of *cry1Ac* and *cry1Ca* from *B. thuringiensis* subsp. *kurstaki* HD-73 and subsp. *aizawai* 4J4, respectively, with and without *cyt1Aa* (encoding cytolytic protein) and *p20* (accessory) of subsp. *israelensis.* These two *cry* genes were selected here because their products bind differentially to specific receptors on various insects (Estela et al. 2004; Herrero et al. 2005; Ferré and Van Rie 2002; Gonzáles-Cabrera et al. 2006). Consistently, a colony of *H. virescens* and of *P. gossypiella* selected with Cry1Ac pro-toxin attained over 10,000-fold and 300-fold resistance to Cry1Ac respectively but was not cross resistant to Cry1Ca (Gould et al. 1995; Tabashnik et al. 2000).

Toxicities of the recombinant clones expressing different combinations of the four genes, and interactions between the products of the three toxins, were evaluated against larvae of susceptible strains of *P. gossypiella*, *Helicoverpa armigera* and *Spodoptera littoralis*.

Materials and methods

Bacterial strains and plasmids

Strain XL-Blue MRF´ of *E. coli* (Stratagene, La Jolla, CA, USA) was used throughout as a host. Previously constructed plasmid pBS SK/N, a derivative of pBluescript II SK$^+$ (Wu et al. 1997) harboring the early T7 promoter P_{A1} that utilizes the usual *E. coli* RNA polymerase (Deuschle et al. 1986), served for cloning six combinations of four genes: *cry1Ac*, *cry1Ca*, *cyt1Aa* and *p20* (Table 1, Fig. 1). The plasmids pRM4-C, pRM4-R and pRM4-RC are derivatives of pUHE-24S harboring the same P_{A1} promoter (Table 1; Manasherob et al. 2001) were used for bioassay and isolating *cyt1Aa* and *p20*, separately and together, respectively. Previously constructed plasmid pRVE4-RC (Khasdan et al. 2003), a derivative of the shuttle vector pRL488p that carries two tandem P_{psbA} and P_{A1} promoters (Wu et al. 1997) served for cloning separately (Table 1, Fig. 2) *cry1Ac* isolated from *B. thuringiensis* subsp. *kurstaki*-4D4 (original code HD-73) and *cry1Ca* from *B. thuringiensis* subsp. *aizawai*-4J4 (original code HD-11).

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 were selected on LB plates containing ampicillin (100 μg ml^{-1}) and tetracycline (10 μg ml^{-1}). DNA was analyzed by electrophoresis on horizontal 1% agarose slab gels and visualized with ethidium bromide.

Table 1 Plasmids expressing combinations of δ-endotoxin genes from *B. thuringiensis*

Plasmid	Description (genes cloned from *B. thuringiensis*)	Reference
pBt-1C	Derivative of pBS-SK/N[a] (*cry1Ca*)	This study
pBt-1A	Derivative of pBS-SK/N (*cry1Ac*)	This study
pBt-1CA	Derivative of pBS-SK/N (*cry1Ca* and *cry1Ac*)	This study
pBt-1ACy	Derivative of pBS-SK/N (*cry1Ac* and *cyt1Aa*)	This study
pBt-1ARCy	Derivative of pBS-SK/N (*cry1Ac, cyt1Aa* and *p20*)	This study
pBt-1CARCy	Derivative of pBS-SK/N (*cry1Ca*, *cry1Ac*, *cyt1Aa* and *p20*)	This study
pRBt-1A	Derivative of pRVE4-RC[b] (*cry1Ac*)	This study
pRBt-1C	Derivative of pRVE4-RC (*cry1Ca*)	This study
pRM4-C	Derivative of pUHE-24S (*cyt1Aa*)	Manasherob et al. (2001)
pRM4-R	Derivative of pUHE-24S (*p20*)	Manasherob et al. (2001)
pRM4-RC	Derivative of pUHE-24S (*p20* and *cyt1Aa*)	Manasherob et al. (2001)

[a] pBS-SK/N, derivative of pBluescript II SK$^+$ with 716 bp *Xba*I–*Xho*I fragment carrying P_{A1} promoter from pUHE-24S (Wu et al. 1997)

[b] pRVE4-RC, derivative of pRL-488p (*p20* and *cyt1Aa*) (Khasdan et al. 2003)

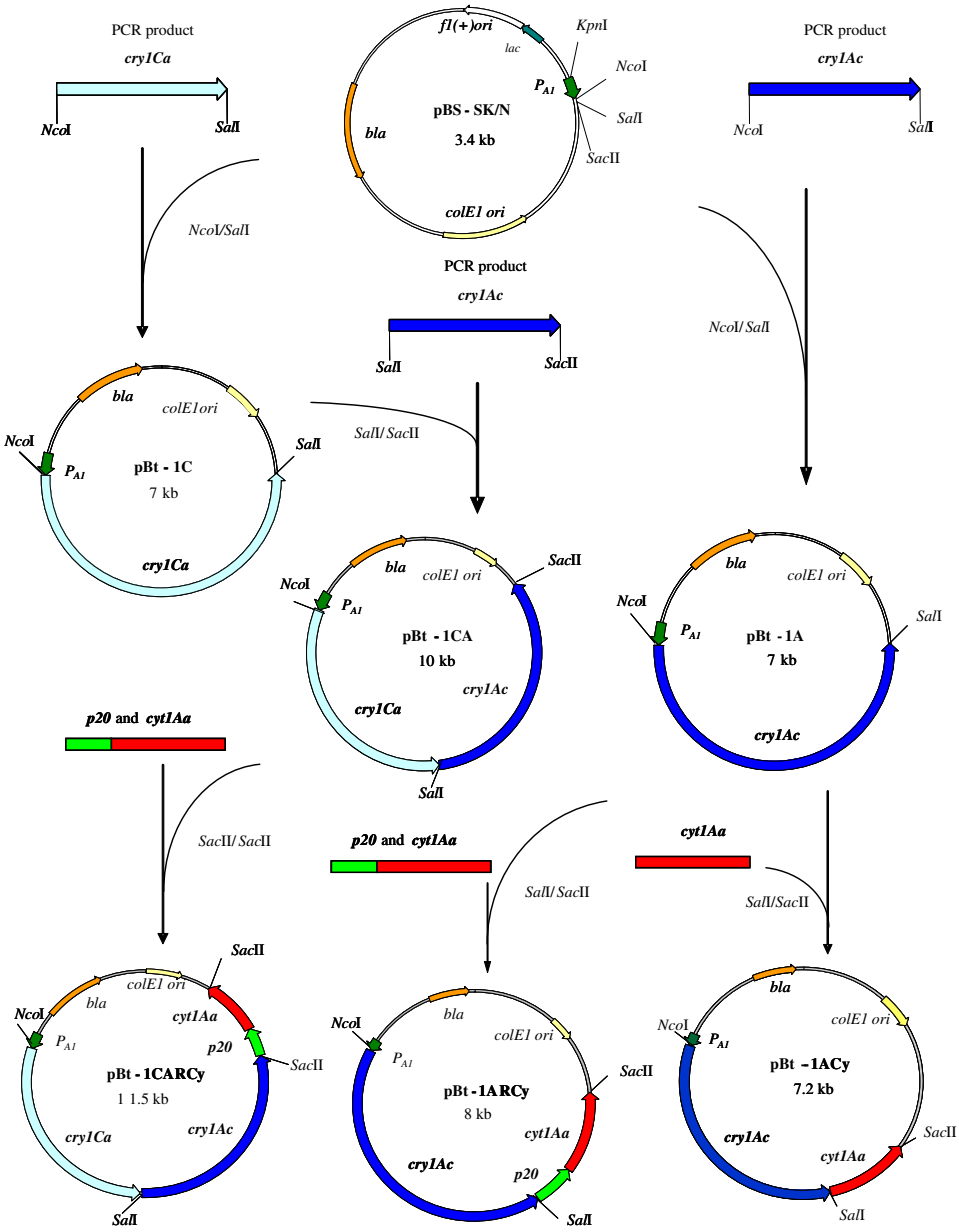

Fig. 1 Cloning of *cry1Ca*, *cry1Ac* with and without *cyt1Aa* and *p20* into the expression vector pBS SK/N that contains early T7 promoter P_{A1}

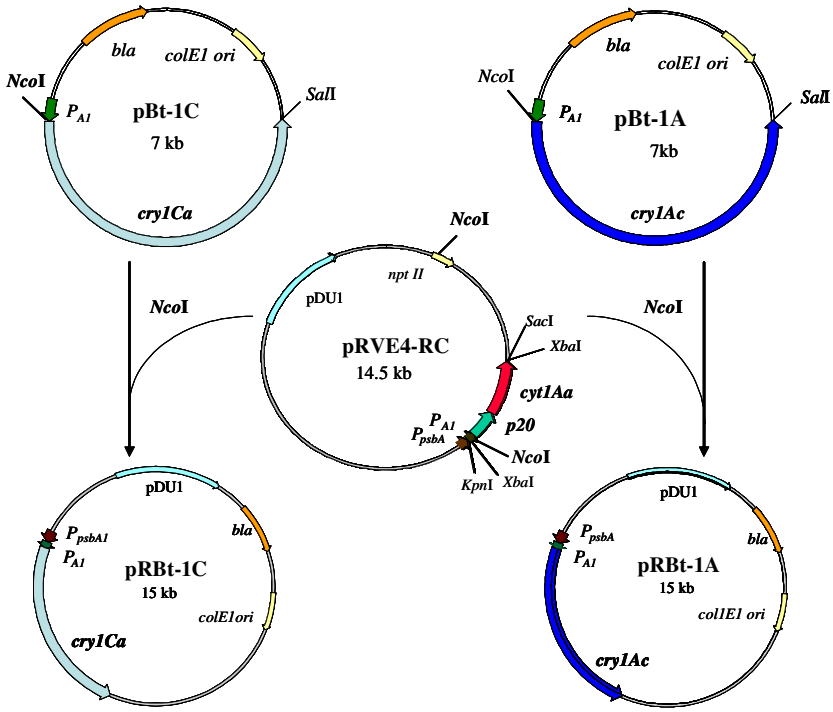

Fig. 2 Cloning of *cry1Ac* and *cry1Ca* into the *E. coli-Anabaena* shuttle vector pRVE4-RC

Polymerase chain reaction

Amplification was carried out with the high fidelity Vent DNA polymerase (New England Biolabs) or with FailSafe™ PCR PreMix Selection Kit (Epicentre) in a Biometra TGradient thermocycler (Biometra GmbH, Göttingen, Germany) set to a 30 reaction cycles for each. Cycle parameters consisted of 40 s at 94°C, 40 s at 55°C and 1–4 min at 72°C.

The following primers (Table 2) were employed to obtain: (a) *p20-cyt1Aa* (29-mer P20Cyt1A-*Sac*II-d or P20Cyt1A-*Sal*I-d, and a 28-mer P20Cyt1A-*Sac*II-r); (b) *cyt1Aa* alone (29-mer Cyt1A-*Sal*I-d and 28-mer Cyt1A-*Sac*II-r); (c) *cry1Ac* (31-mer Cry1A-*Nco*I-d and 34-mer Cry1A-*Sal*I-r or 36-mer Cry1A-*Sal*I-d, and 28-mer Cry1A-*Sal*I-r) and (d) *cry1Ca* (28-mer Cry1C-*Nco*I-d and 30-mer Cry1C-*Sal*I-r).

Cloning of *cry1Ac* and *cry1Ca* alone or in combinations with *cyt1Aa* and *p20*

The amplicons containing *cry1Ac* and *cry1Ca* were separately digested by *Nco*I/*Sal*I, and inserted each into pBS SK/N (Wu et al. 1997) under the P_{A1} promoter to yield clones pBt-1A and pBt-1C, respectively (Fig. 1). The *Sal*I/*Sac*II-digested fragment with *cry1Ac* was inserted into pBt-1C downstream from *cry1Ca* to obtain pBt-1CA with both genes under P_{A1} promoter (Fig. 1).

cyt1Aa and *p20-cyt1Aa* were amplified from pRM4-C and pRM4-RC (Manasherob et al. 2001), respectively. The *Sac*II-digested amplicon with *p20-cyt1Aa* was ligated to the *Sac*II-cleaved pBt-1CA harboring the *cry1Ca–cry1Ac* operon to yield clone pBt-1CARCy that expressed all four genes under P_{A1} promoter (Fig. 1). Similarly, *cyt1Aa* and

Table 2 List of primers designed and used in this investigation

Description/name	Sequence (from 5′ to 3′)[a]
Cry1C-*Nco*I-d	CGGAGGTATT**CCATGG**AGGAAAATAATC
Cry1C-*Sal*I-r	CTTTATTT**GTCGAC**GTTACATTTTATAACG
Cry1A-*Nco*I-d	GAGATGGAGGTAAC**CCATGG**ATAACAATCCG
Cry1A-*Sal*I-r	GTAATGCTGCTC**GTCGAC**AATTGATTTGAAAACG
Cry1A-*Sal*I-d	GAGTCATATGTTTTAAATT**GTCGAC**ATGAAAAACAG
Cry1A-*Sac*II-r	GTAATGCTGC**CCGCGG**ACAATTGATTTG
P20Cyt1A-*Sac*II-d	GGCGTATC**CCGCGG**CCCTTTCGTCTTCAC
P20Cyt1A-*Sal*I-d	GGCGTATCACGAGGCCCT**GTCGAC**TTCAC
P20Cyt1A-*Sac*II-r	GCTGCATGTGT**CCGCGG**TTTTCACCGTC
Cyt1A-*Sal*I-d	GGCGTATCACGAGGCCCT**GTCGAC**TTCAC
Cyt1A-*Sac*II-r	GCTGCATGTGT**CCGCGG**TTTTCACCGTC

d Means direct primer, *r* means reverse primer

[a] The restriction sites are bold-faced

p20-cyt1Aa amplicons were restricted with *Sal*I/*Sac*II and inserted into pBt-1A harboring *cry1Ac* to yield pBt-1ACy and pBt-1ARCy, respectively (Fig. 1).

*Nco*I-digested pBt-1C and pBt-1A were ligated (Fig. 2) to *Nco*I-cut pRVE4-RC, derivative of the *E. coli-Anabaena* shuttle vector pRL488p (Wu et al. 1997) under dual promoters P_{psbA} and P_{A1} to produce pRBt-1C, and pRBt-1A clones, respectively.

Protein analyses

Subspecies of *B. thuringiensis* were grown 5 days in LB broth at 30°C and used as control. Transgenic *E. coli* cells were grown at 37°C in LB supplemented with 100 μg ml^{-1} ampicillin and induced by Isopropyl-b-D-thiogalactopyranoside (IPTG; 0.5 mM) when the culture reached an optical density at 660 nm of 0.2–0.3 (≈2×10^8 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 with a Bio-Rad protein kit with BSA as 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 analyses, proteins were electrophoresed and electro-transferred onto nitrocellulose filters. The blots were exposed to specific antiserum directed against Cry1Ac, Cry1Ca, Cyt1Aa or P20 (kindly provided, respectively, by Alejandra Bravo, Universidad Nacional Autónoma de México; Avia Zilberstain, Tel-Aviv University; Sarjeet Gill, University of California; and David Ellar, University of Cambridge). Protein A-alkaline phosphatase conjugate was used as a primary antibody detector and bands were visualized using the chromogenic substrate for alkaline phosphatase-fast 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium tablets (Sigma) diluted in 10 ml water.

Insects

A colony of *P. gossypiella*, established at Gilat Research Center in 2001 with insects collected from cotton in Beit-Shean Valley (NE), and one of *H. armigera* were reared on an artificial diet (Manduca-Heliothis Premix, Stonefly, TX, USA) under standard controlled conditions: 27 ± 2°C, 60% RH and photoperiod of 14:10 h (L:D). A colony of *S. littoralis* was maintained at the Volcani Agricultural Center in Beit-Dagan on castor bean leaves at 25 ± 2°C, 60% RH with photoperiod of 14:10 h (L:D).

Bioassays for larvicidal activities

Cells of the recombinant *E. coli* were harvested by centrifugation after 4 h of induction and re-suspended in distilled water. For *P. gossypiella*, samples were thoroughly blended into the diet with a food processor to achieve the required concentrations (Tabashnik et al. 2000). For each treatment, ten plastic cups (50 ml) with 10 g of diet and ten eggs in each were incubated at 27 ± 2°C, 60% RH and photoperiod 14:10 h (L:D), and the number of survivors and their weight were recorded after 15 days.

For *H. armigera* and *S. littoralis* (1st-instar larvae), a disk (5-cm diameter) of fresh cotton leaf was immersed in a test solution for 10 s, allowed to dry at ambient temperature for 2 h, and placed in a Petri dish containing moistened filter paper. Two larvae were placed in each dish, and each treatment was repeated ten times. The dishes were incubated at 27 ± 2°C, 60% RH and photoperiod of 14:10 h (L:D). Mortality, larvae weight and leaf damage levels were determined after 5 days.

Values of LC_{50} and LC_{90} (concentrations of cells that kill 50 and 90% of the exposed populations, respectively) were determined by using EPA Probit analysis program with at least six doses. All bioassays were performed at least thrice for each concentration.

Results and discussion

A set of six different combinations of four genes from three subspecies of *B. thuringiensis* (*cry1Ac* from *kurstaki*, *cry1Ca* from *aizawai*, and *cyt1Aa* and *p20* from *israelensis*) was constructed for expression under an early T7, P_{A1} inducible promoter (Fig. 1, Table 1) using pBS SK/N (Wu et al. 1997). Two additional clones, pRBt-1A and pRBt-1C, expressing *cry1Ac* and *cry1Ca,* respectively, under tandem promoters, P_{A1} and the constitutive P_{psbA} (Fig. 2, Table 1), were obtained in the *E. coli-Anabaena* shuttle vector pRVE4-RC (Khasdan et al. 2003), derivative of pRL488p (Wu et al. 1997). Three clones (Table 1) with *cyt1Aa* and *p20*, alone or together (Manasherob et al. 2001), were used to amplify the genes and to measure toxicity (Table 3). Expression of the cloned genes in all 11 recombinants was confirmed (Figs. 3, 4), and their toxicities quantified (Table 3).

Our *E. coli* clones expressing *cyt1Aa* and *p20* alone (pRM4-C, pRM4-R) or together (pRM4-RC) consistently failed to display any toxicity towards exposed larvae of *P. gossypiella*, *H. armigera* and *S. littoralis*, even though the latter did express the respective proteins (Fig. 4b, c). Expression of *cyt1Aa* in an acrystalliferous strain of *B. thuringiensis* subsp. *kurstaki* is not toxic to susceptible and resistant strains of *P. xylostella* and *P. gossypiella* (Meyer et al. 2001), whereas it is toxic (when expressed in

Table 3 Larvicidal activities of the transgenic *E. coli* clones against three lepidopteran species

E. coli strains	*B. thuringiensis* genes	Larvicidal activity against[a]					
		P. gossypiella[b]		*H. armigera*[b]		*S. littoralis*[b]	
		LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
pBt-1A	*cry1Ac*	0.27 (0.04–5.3)	1.47 (0.2–8.2)	1.57 (0.6–3.9)	37.05 (11–56)	ND	ND
pBt-1ACy	*cry1Ac*, *cyt1Aa*	10.62 (3.62–27)	Not attained	7.56 (4.6–14)	Not attained	ND	ND
pBt-1ARCy	*cry1Ac*, *p20*, *cyt1Aa*	1.21 (0.6–3.9)	6.58 (3.9–16)	2.51 (1.2–6.4)	73.36 (32–116)	ND	ND
pBt-1C	*cry1Ca*	ND	ND	ND	ND	9.81 (4.1–17.1)	Not attained
pBt-1CA	*cry1Ca*, *cry1Ac*	0.4 (0.14–0.76)	4.8 (2–28)	ND	ND	0.12 (0.02–0.3)	Not attained
pBt-1CARCy	*cry1Ca*, *cry1Ac*, *p20*, *cyt1Aa*	1.35 (0.3–5)	20 (5–89)	0.16 (0.12–0.2)	0.79 (0.55–1.3)	27.93 (15–60)	Not attained
pRBt-1A	*cry1Ac*	0.04 (0.02–0.05)	0.13 (0.07–0.4)	0.09 (0.05–0.1)	0.64 (0.4–1.2)	ND	ND
pRBt-1C	*cry1Ca*	ND	ND	ND	ND	0.07 (0–0.4)	Not attained

Genes and gene combinations in the pBt-series are expressed under a single early T7 promoter (P_{A1}), and in the pRBt-series, under the dual P_{A1} and P_{psbA} promoters. *E. coli* strains without or with uninduced plasmids were not toxic to all three lepidopteran pests. Clones pRM4-R, pRM4-C and pRM4-RC that express *p20, cyt1Aa*, and *p20+cyt1Aa*, respectively, were not toxic to all three lepidopteran pests

[a] Values of LC_{50} and LC_{90} are averages of four bioassays. Numbers in parentheses are 95% confidence limits, as determined by probit analysis. ND, no toxicity detected, even at a concentration of 1×10^{10} cells ml^{-1} or g^{-1}

[b] Larvicidal activities are expressed in terms of 10^8 cells per either ml (for *H. armigera* and *S. littoralis*) or gram of the diet (for *P. gossypiella*)

an acrystalliferous variant of subsp. *israelensis*) to an insecticide-susceptible laboratory population of *P. xylostella* (Sayyed et al. 2001). Differences in crystal solubility that could affect relative toxicity have been observed with different *B. thuringiensis* hosts (Angsuthanasombat et al. 1992). Various endogenous proteases produced by subsps. *kurstaki*, *tenebrionis* and *israelensis* of *B. thuringiensis* could affect the specificity of the toxin as well (Reddy et al. 1998). In addition, different toxicity levels of acrystalliferous strains of *B. thuringiensis* expressing *cyt1Aa* may be explained by differences in the insect strains or species, the bioassay procedure, or the toxin itself (Sayyed et al. 2001). Toxicities of recombinant *Bacillus* spp. expressing δ-endotoxin genes from *B. thuringiensis* (Ward and Ellar 1986; Wu et al. 1994; Shao et al. 2001; Xue et al. 2005) are usually higher than those from recombinant *E. coli* (Douek et al. 1992; Khasdan et al. 2001; Kouskoura et al. 2001) because of weak promotion, low polypeptide stability, crystal malformation or deleterious effects on recombinants (Margalith and Ben-Dov 2000).

Interactions between Cry1Ac and Cry1Ca against lepidopteran species

The genes *cry1Ca* and *cry1Ac* were cloned separately and together to derive pBt-1C, pBt-1A and pBt-1CA, respectively (Fig. 1). As anticipated, pBt-1C displayed some

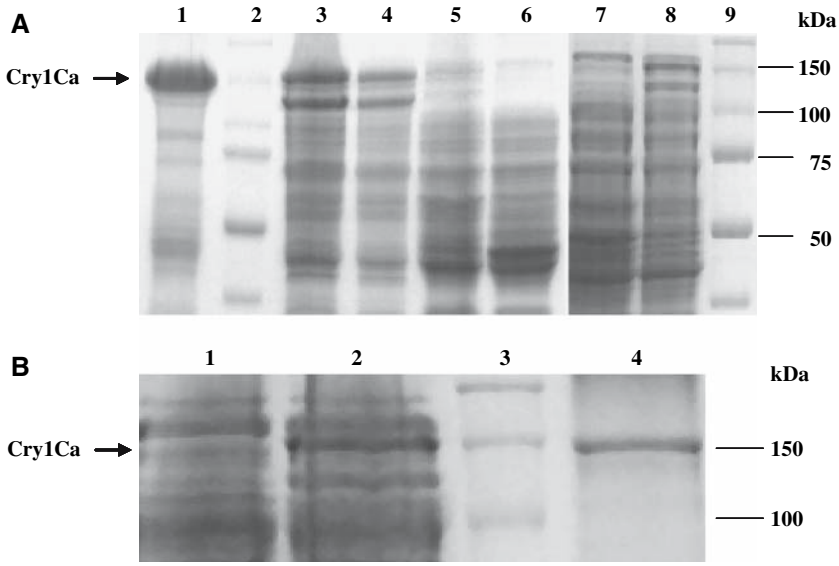

Fig. 3 SDS-PAGE: **a** *Lane 1 B. thuringiensis* subsp. *aizawai*, *lane 2* molecular size marker, *lane 3* pBt-1C, *lane 4* pBt-1CA (induced), *lane 5* pBt-1CA (uninduced), *lane 6 E. coli* as a control, *lane 7* pBt-1CARCy (uninduced), *lane 8* pBt-1CARCy (induced) and *lane 9* molecular size marker. **b** *Lane 1* pRBt-1C (uninduced), *lane 2* pRBt-1C (induced), *lane 3* molecular size marker and *lane 4 B. thuringiensis* subsp. *aizawai*

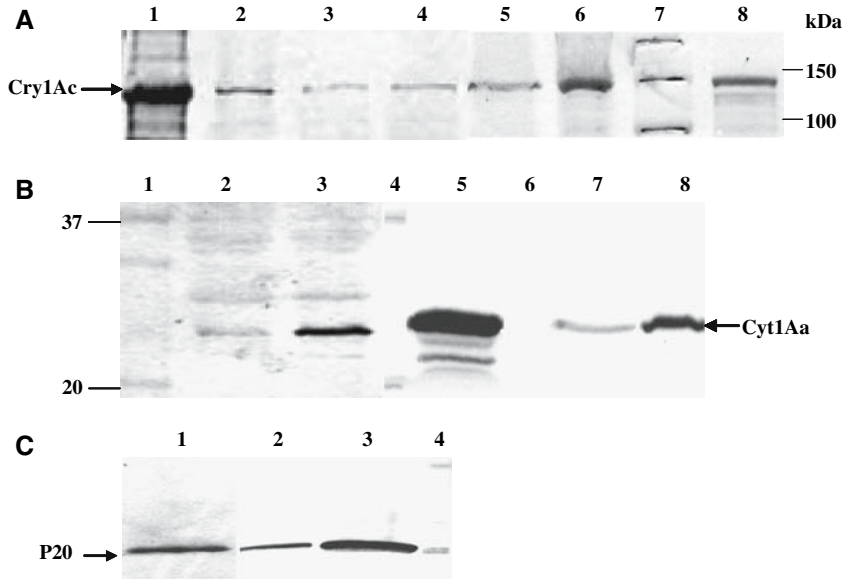

Fig. 4 Immunoblot analyses of *E. coli* clones (of Table 3) expressing different combinations of *cry1Ac*, *cyt1Aa* and *p20* from subsps. *kurstaki* and *israelensis* of *B. thuringiensis*. The antibodies used were against: **a** Cry1Ac. *Lane 1 B. thuringiensis* subsp. *kurstaki*, *lane 2* pBt-1A, *lane 3* pBt-1ACy, *lane 4* pBt-1ARCy, *lane 5* pBt-1CARCy, *lane 6* pBt-1CA, *lane 7* molecular size marker and *lane 8* pRBt-1A. **b** Cyt1Aa. *Lane 1* molecular size marker, *lane 2* pBt-1ACy, *lane 3* pBt-1ARCy, *lane 4* molecular size marker, *lane 5* pRM4-RC, *lane 6 E. coli* as a control, *lane 7* pBt-1CARCy (uninduced) and *lane 8* pBt-1CARCy (induced). **c** P20. *Lane 1* pBt-1ARCy, *lane 2* pBt-1CARCy, *lane 3* pRM4-RC, *lane 4* molecular size marker

toxicity against larvae of *S. littoralis* (LC_{50} of 9.8×10^8 cells ml^{-1}; Table 3) but none against *P. gossypiella* and *H. armigera*. In contrast, pBt-1A exhibited the highest level of toxicity against *P. gossypiella* (LC_{50} of 0.27×10^8 cells g^{-1}), a relatively high level against *H. armigera* (LC_{50} of 1.6×10^8 cells ml^{-1}), but none against *S. littoralis* (Table 3). However, clone pBt-1CA was not toxic to *H. armigera* and showed a similar level of toxicity (LC_{50} of about 0.4×10^8 cells g^{-1}) against *P. gossypiella* as that of pBt-1A; it was much more toxic against *S. littoralis* (LC_{50} of 0.12×10^8 cells ml^{-1}) than pBt-1C (LC_{50} of 9.8×10^8 cells ml^{-1}), whereas clone pBt-1A was not toxic at all (Table 3). The dramatic increased toxicity of pBt-1CA against *S. littoralis* and its slight decreased toxicity against *P. gossypiella* (depicted qualitatively in Table 4) clearly confirm that both polypeptides were produced in pBt-1CA. Moreover, while expression of *cry1Ac* was detected by anti-Cry1Ac (Fig. 4a), particularly in clone pBt-1CA (lane 6), expression of *cry1Ca* was not detected by anti-Cry1Ca, probably due to high cross-reactivity of this antiserum observed even with *E. coli* proteins. SDS-PAGE, on the other hand, which detected Cry1Ca in pBt-1C (Fig. 3, lane 3), is unable to confirm expression of *cry1Ca* in pBt-1CA (Fig. 3, lane 4) due to the size similarity between the two Cry proteins, but existence of Cry1Ca in pBt-1CA is concordant with the higher toxicity of this clone against *S. littoralis* than that of pBt-1A (Tables 3, 4).

The low toxicities of pBt-1CA against larvae of *P. gossypiella* and *H. armigera* may be explained by the interaction between Cry1Ca, which is not toxic to these pests, and Cry1Ac, which is toxic (Shao et al. 2001). The downstream position of *cry1Ac* in our operon (Fig. 1) may reduce its expression level and subsequently distort the appropriate stoichiometry. The differences in toxicity levels of pBt-1CA against larvae of all three pest species tested here may also result from the different ensemble of receptors in them,

Table 4 Larvicidal activities of the pBt-1A, pBt-1C and pBt-1CA clones (from Table 3)

E. coli strains	*Bt* genes	Relative larvicidal activity (and LC_{50}) against[a]		
		P. gossypiella	*H. armigera*	*S. littoralis*
pBt-1A	*cry1Ac*	+++ (0.27)	++ (1.57)	–
pBt-1C	*cry1Ca*	–	–	+ (9.81)
pBt-1CA	*cry1Ca*, *cry1Ac*	++ (0.4)	–	++++ (0.12)

[a] –, no toxicity detected, even at a concentration of 1×10^{10} cells ml^{-1} or g^{-1}. Numbers in parentheses are LC_{50} values, as determined by probit analysis and expressed in terms of 10^8 cells per either ml (for *H. armigera* and *S. littoralis*) or gram of the diet (for *P. gossypiella*)

and various modes of interactions of the toxins in the different environments that exist in the larval guts. Brush Border Membrane Vesicles (BBMV) of *S. littoralis* larvae have indeed been permeabilized to KCl by Cry1Ca but not by Cry1Ac, which is only marginally active, most likely due to its inability to perforate the membrane even if binding can take place (Escriche et al. 1998). Binding of Cry1Ca to its receptors in BBMV of *Sesamia nonagrioides* is enhanced by addition of Cry1Ac (Fig. 1C in Gonzáles-Cabrera et al. 2006), and both proteins bind to different receptors in *S. frugiperda* larvae (Rang et al. 2004). Both observations are consistent with the increased toxicity of the clone producing both toxins against *S. littoralis* (Tables 3, 4). It is noteworthy that the maximum osmotic swelling rate was much higher when the BBMV of *Manduca sexta* larvae were incubated with both Cry1Ac and Cry1Ca, an indication that the number of pores formed by each of the toxins is limited by the availability of their respective receptors (Fortier et al. 2007).

Moreover and on a different level, some combinations of co-expressed *cry* genes can dramatically affect the solubility and composition of an inclusion body and consequently on toxicity properties (Aronson 1995). For example, highest toxicities were observed against larvae of both, *S. exigua* and *H. armigera*, in the combination of Cry1Aa and Cry1Ca (produced in acrystalliferous *B. thuringiensis* 4Q7) at a ratio of 1:1 (Xue et al. 2005).

Does Cyt1Aa raise toxicity of Cry1 toxins?

Three clones with *cyt1Aa* (pBt-1ACy, -1ARCy and -1CARCy) revealed various levels of toxicity against larvae of three susceptible lepidopteran species (Table 3). The clone expressing all four genes (*cry1Ca*, *cry1Ac*, *p20*, *cyt1Aa*) in tandem (pBt-1CARCy; Figs. 1, 3a, 4) exhibited highest toxicity to *H. armigera* (LC_{50} of 0.16×10^8 cells ml^{-1}; Table 3). However, the same clone displayed low toxicity against *S. littoralis* (LC_{50} of 27.9×10^8 cells ml^{-1}) and against *P. gossypiella* (LC_{50} of 1.3×10^8 cells ml^{-1}).

The clone with pBt-1ACy (producing Cry1Ac and Cyt1Aa) was not toxic to *S. littoralis* and showed low toxicity against *P. gossypiella* and *H. armigera*, approximately 39- and 5-fold less than pBt-1A, respectively (Table 3). It was also about nine- and threefold less toxic then pBt-1ARCy (expressing *p20* in addition to *cry1Ac* and *cyt1Aa*) against the same pests, respectively (Table 3). These results are consistent with the low amounts of Cry1Ac and Cyt1Aa produced by pBt-1ACy (lane 3 in Fig. 4a and lane 2 in Fig. 4b, respectively), most likely because *E. coli* cells expressing *cyt1Aa* without *p20* lost their viability (Douek et al. 1992; Khasdan et al. 2001; Manasherob et al. 2001). The clone with pBt-1ARCy exhibited a comparable level of toxicity against *P. gossypiella* and *H. armigera* than pBt-1A (Table 3). Low toxicity of pBt-1ARCy is probably of the inappropriate stoichiometry among the produced proteins. For example, two clones (pVRE4-DRC and pVE4-DRC) that express the same three genes (*cry11Aa*, *p20* and *cyt1Aa*) under different expression systems produced different amounts of the proteins and displayed altered toxicities against larvae of *A. aegypti* (Khasdan et al. 2001).

Exceedingly low toxicity of purified Cry1Ca (Liao et al. 2002) and lack of toxicity of pBt-1C against *H. armigera* larvae (Table 3) are likely related to lack of an appropriate receptor (Estela et al. 2004; Herrero et al. 2005). Nevertheless, Cry1Ca produced in pBt-1CARCy increased the toxicity of pBt-1ARCy by 16-fold (from LC_{50} of 2.51×10^8 to 0.16×10^8 cells ml^{-1}), suggesting an interaction with Cyt1Aa. This is confirmed by the fact that pBt-1CA (expressing *cry1Ca* and *cry1Ac*) was also not toxic to *H. armigera* (Table 3). This raised toxicity is probably due to the detergent-like mode of Cyt1Aa action (Butko 2003; Manceva et al. 2005). High affinity to unsaturated phospholipids of Cyt1Aa, allows penetration of Cry1Ca into the membrane of midgut epithelial cells. Similar effects have been described with a mixture of Cyt1Aa and the binary toxin of *B. sphaericus*: (a) sensitizing larvae of *A. aegypti*, which are normally not susceptible to *B. sphaericus* (Wirth et al. 2000b) and (b) restoring toxicity against a line of *C. quinquefasciatus* that had been selected for resistance against the binary toxin (Wirth et al. 2000a). Insertion of Cry11Aa into the membrane of midgut epithelial cells was enhanced by binding to membrane-embedded Cyt1Aa, just like the interaction of Cry11Aa with its natural receptor (Pérez et al. 2005). Cyt1Aa thus enhances Cry11Aa toxicity and suppresses resistance after Cry-receptor mutations. The most common mechanisms of lepidopteran resistance to Cry1 toxins are through mutations that affect receptor amount or affinity (Ferré and Van Rie 2002); Cyt1Aa may thus promote Cry1-toxin(s) binding to target membranes and enhances the toxicity of the latter.

Higher yields of Cry1Ca (Rang et al. 1996) and Cry1Ac (Shao et al. 2001) in acrystalliferous *B. thuringiensis* have been obtained upon co-expression of the respective genes with *p20*. Toxicity of the latter recombinant (expressing *cry1Ac* with *p20*) against *H. armigera* larvae was correspondingly enhanced (Shao et al. 2001), concordant with the high toxicity of pBt-1CARCy expressing all four genes (Table 3), suggesting that P20 acts as a chaperone in vivo.

A mixture of Cyt1Aa and Cry1Ac (1:1 wt/wt) is highly synergistic and toxic against susceptible and Cry1Ac-resistant populations of *P. xylostella*, reducing the resistance ratio from 1,165 to just 5 (Sayyed et al. 2001). Purified Cyt1Aa is however not improved toxicity against the same pest in a mixture with *B. thuringiensis* subsp. *kurstaki*, crystals of which include Cry1Aa, Cry1Ab, and Cry2A in

addition to Cry1Ac (Meyer et al. 2001). This observation suggests that either the three additional toxins or an inappropriate stoichiometry suppresses the interaction of Cry1Ac with Cyt1Aa. Two other pests, *P. gossypiella*, (both susceptible and resistant strains; Meyer et al. 2001) and *Trichoplusia ni* (Rincón-Castro et al. 1999), are not sensitive to the same mixture of Cyt1Aa and Cry1Ac.

Expression of *cry1Ac* and *cry1Ca* under tandem P_{psbA} and P_{A1} promoters

The genes *cry1Ac* and *cry1Ca* were cloned separately into the previously constructed plasmid pRVE4-RC (Khasdan et al. 2003), derivative of the *E. coli-Anabaena* shuttle vector pRL488p (Wu et al. 1997), under tandem P_{psbA} and P_{A1} promoters (Fig. 2, Table 1). The additional promoter, chloroplast P_{psbA} is approximately 18 times more effective than a late *T7* promoter for hyper-expression of foreign protein-based polymers in *E. coli* (Brixey et al. 1997). This tandem promoter combination (P_{psbA} and P_{A1}) efficiently expresses toxin genes from *B. thuringiensis* subsp. *israelensis* in *Anabaena* PCC 7120 (Wu et al. 1997; Khasdan et al. 2003).

Clones pRBt-1A and pRBt-1C expressing in *E. coli cry1Ac* and *cry1Ca* (lane 8 in Fig. 4a and lane 2 in Fig. 3b, respectively), under dual (P_{psbA} and P_{A1}) promoters exhibited toxicities significantly higher than their pBt-1A and pBt-1C counterparts, expressing the same genes under a single P_{A1} promoter (Table 3). The toxicities of pRBt-1A against *P. gossypiella* (LC_{50} of 0.04×10^8 cells g^{-1}) and *H. armigera* larvae (LC_{50} of 0.09×10^8 cells ml^{-1}) were 7.5 and 18-fold higher, respectively, than those of pBt-1A. The toxicity of pRBt-1C against *S. littoralis* (LC_{50} of 0.07×10^8 cells ml^{-1}) was 140-fold-times higher than that of its counterpart (pBt-1C) (Table 3).

Expression in *Synechococcus* PCC 7942 of *cry4Ba* by two tandem promoters (its own and *lacP*) increased its mosquito larvicidal activity (Soltes-Rak et al. 1993). Toxicity against *A. aegypti* larvae of transgenic *Anabaena* PCC 7120 expressing *cry4Aa* with the same two tandem promoters (P_{psbA} and P_{A1}) is substantially higher than with P_{psbA} alone (Wu et al. 1997).

In the search for new toxin combinations, synergy and subsequent toxicity between pairs is usually examined (Rincón-Castro et al. 1999). In this report was demonstrated that it is difficult to predict which combination is most effective for control of particular pest(s), thus each combination must be tested separately. The clone pBt-1CARCy that expresses three toxin genes and another one for a helper protein (*cry1Ca*, *cry1Ac*, *cyt1Aa* and *p20*, respectively) has useful biological control potency, by reducing the likelihood of future resistance evolution in target organisms such as *H. armigera*.

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