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Mosquito larvicidal activity of transgenic *Anabaena* PCC 7120 expressing toxin genes from *Bacillus thuringiensis* subsp. *israelensis*

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Abstract

Genes encoding the mosquito larvicidal toxins Cry4Aa, Cry11Aa, Cyt1Aa and the regulatory P20 from *Bacillus thuringiensis* subsp. *israelensis* were introduced into the nitrogen-fixing, filamentous cyanobacterium *Anabaena* PCC 7120 for expression under control of two strong promoters P_{psbA} and P_{A1}. The clone pRVE4-ADRC displayed toxicity against fourth-instar larvae of *Aedes aegypti*, the highest ever achieved in cyanobacteria. It was about 2.5-fold more toxic than the respective clone without *cyt1Aa* [Wu et al., Appl. Environ. Microbiol. 63 (1997) 4971–4975]. Cyt1Aa synergized the combination of Crys by about five-fold. Consistently, the lethal times exerted by pRVE4-ADRC were also reduced (it killed exposed larvae more quickly). This clone may become a useful biological control agent which reduces the probability of resistance development in the target organisms [Wirth et al., Proc. Natl. Acad. Sci. USA 94 (1997) 10536–10540].

Keywords: Mosquitocidal *Anabaena*; δ-Endotoxin; *Bacillus thuringiensis* subsp. *israelensis*

1. Introduction

The entomopathogenic bacterium *Bacillus thuringiensis* subsp. *israelensis* has widely been used as an efficient and safe biological agent to control mosquito larvae and hence mosquito-borne diseases because it is highly specific and does not cause resistance in the targets [20]. Its larvicidal activity is contained in parasporal crystal composed of at least four major insecticidal crystal proteins (ICPs) (of 134, 128, 72, and 27 kDa) encoded by *cry4Aa*, *cry4Ba*, *cry11Aa* and *cyt1Aa*, respectively, all residing on pBtoxis [5]. Translation of an accessory protein from *p20*, co-transcribed with *cry11Aa* [11], raises the levels of Cyt1Aa, Cry11Aa, Cry4Aa, in *Escherichia coli* [18,31,38] and in an acrystalliferous strain of *B. thuringiensis* [8].

The ICPs differ in toxicity levels and against different mosquito species [10,23]. Cyt1Aa is the least toxic but most synergistic compared to any of the others and their combinations in vitro [10,32] and in transgenic *E. coli* [15], as well as to heterologous *Bacillus sphaericus* binary toxin [33]. *Culex quinquefasciatus* strains resistant to combinations of the three Crys retained their original sensitivities in the presence of Cyt1Aa; the latter is thus critical in suppressing resistance and may be useful in resistance management [32].

Indeed, no resistance toward *B. thuringiensis* subsp. *israelensis* has been detected in field populations of mosquitoes despite extensive use during 20 years [17]. To overcome the low efficacy and short residual activity in nature of current formulations, the ICP genes were cloned into alternative hosts that multiply in their habitats [20]. Cyanobacteria are attractive for this purpose [7,16]: they are ubiquitous, float in the upper water layer and resist adverse environmental conditions. They are consumed by mosquito larvae [27], can be cultured on a large scale [6], and are genetically manipulatable [12,13,34].

Some success has been achieved in expressing a single *cry* or *cyt* gene in unicellular cyanobacteria [2,9,21,25–27]

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and other Gram-negative bacteria [14,30,37], but larvicidal activities were limited. Much higher toxicities (LC_{50} of 9×10^4 cells ml^{-1} against third-instar *Aedes aegypti*) were achieved when *cry4Aa* and *cry11Aa*, with and without *p20*, were expressed under dual constitutive promoters P_{psbA} and P_{A1} in the filamentous species *Anabaena* PCC 7120 [34], and retained following irradiation by high doses of UV-B [19].

Here, *cyt1Aa* was cloned for expression from the same strong promoters P_{psbA} and P_{A1} in the same species of *Anabaena*, with and without *p20* and together with *cry4Aa* and *cry11Aa*. Toxicities of the recombinant clones and synergistic interactions between Cyt1Aa and Crys were determined.

2. Materials and methods

2.1. Bacterial strains and plasmids (Table 1)

Strain XL-Blue MRF′ of *E. coli* (Stratagene, La Jolla, CA, USA) was used for cloning genes from *B. thuringiensis* subsp. *israelensis* [15]. Plasmids pRM4-RC and pRM4-C [18] served for amplifying *cyt1Aa*, with and without *p20*, respectively. The shuttle vector pRL488p [34] was used for cloning genes from *B. thuringiensis* subsp. *israelensis* into *E. coli* and *Anabaena* PCC 7120 (Fig. 1). *E. coli* donor strain DH10B (kindly provided by Dr. X. Wu), carrying helper plasmid pRL623 (derivative of pRL528), conjugal plasmid pRL443 (derivative of RP4) [12,13] and incoming plasmid, was used for the biparental conjugation system.

2.2. Cloning for expression of B. thuringiensis subsp. israelensis genes into Anabaena PCC 7120

cyt1Aa and *p20-cyt1Aa*, each under P_{A1} and two tandem *lacO* operators, were amplified from pRM4-C and pRM4-RC [18] with Vent DNA polymerase (New England Biolabs) in a DNA MiniCycler (MJ Research, Watertown, MA, USA) for a 30-reaction cycle (40 s at 94°C, 30 s at 62°C, 1–2 min at 72°C each). The following two primers (both 26-mers) were used to obtain *p20+cyt1Aa*:

- 5′-TAGGCGTA**TCTAGA**GGCCCTTTCGTC-3′, with a *Xba*I restriction site (bold-faced);
- 5′-TCACCGTCATCACCGAAACGCGCGAG-3′, which annealed five bases downstream of the original unique *Xba*I site on pRM4-RC.

Two additional primers (both 27-mers) were used to obtain *cyt1Aa* alone with a *Kpn*I restriction site (bold-faced):

- 5′-AAAATAGG**GGTACC**ACGAGGCCCTTTC-3′;
- 5′-GTCTCC**GGTACC**TGCATGTGTCAGAGG-3′.

The *Xba*I-digested amplicon (with P_{A1}-*p20-cyt1Aa*) was ligated to *Xba*I-cleaved (a) pSBJ1 harboring the *cry4Aa-cry11Aa-p20* operon [34], to yield pSVE4-ADRC, and (b) pBluescript II SK+, to yield pBVE4-RC (Fig. 1). Both plasmids, pSVE4-ADRC and pBVE4-RC, were digested by *Kpn*I/*Sac*I, and the appropriate fragments (with P_{A1}-*cry4Aa-cry11Aa-p20*+P_{A1}-*p20-cyt1Aa* and P_{A1}-*p20-cytGAa*, respectively) were inserted into the *E. coli-Anabaena* shuttle vector pRL488p [34]. The final constructs (Fig. 1) were designated pRVE4-ADRC (21.6 kb, with *cry4Aa*, *cry11Aa*, *cyt1Aa* and two copies of *p20*) and pRVE4-RC (14.5 kb, with a single *p20* and *cyt1Aa*).

In addition, the *Kpn*I-digested amplicon (with P_{A1}-*cytGAa*) was ligated to *Kpn*I-cut pRL488p to produce (Fig. 1) pRVE4-C (14.1 kb). The three plasmids were introduced by electroporation into *E. coli* DH10B containing helper plasmid pRL623 and conjugal plasmid pRL443 [12]. Competent cells were prepared and plasmids were isolated by standard procedures.

Table 1
Plasmids used

Plasmid	Genes cloned from *B. thuringiensis* subsp. *israelensis*	Reference
A. In *E. coli*		
pRM4-C	*cyt1Aa*	[18]
pRM4-RC	*p20* and *cyt1Aa*	[18]
pBVE4-RC	*p20* and *cyt1Aa*	This study
pSBJ1	*cry4Aa*, *cry11Aa*, and *p20*	[34]
pVE4-ADRC	*cry4Aa*, *cry11Aa*, *p20* and *cyt1Aa*	[15]
pSVE4-ADRC	*cry4Aa*, *cry11Aa*, *p20* and *cyt1Aa*	This study
B. In *Anabaena* PCC 7120		
pSBJ2#11[a]	*cry4Aa*, *cry11Aa*, and *p20*	[34]
pRVE4-C	*cyt1Aa*	This study
pRVE4-RC	*p20* and *cyt1Aa*	This study
pRVE4-ADRC	*cry4Aa*, *cry11Aa*, *p20* and *cyt1Aa*	This study
C. Other	Function, description	Reference
pRL488p	Shuttle vector, derived from pRL488, with P_{psbA} from pRL435K	[34]
pRL623	Helper plasmid, derived from pRL528, with M. *Eco*47II, M. *Ava*I, M. *Ava*III	[12]
pRL443	Conjugal plasmid, derived from RP4	[12]

[a]Genes from the clone with plasmid #11 were chromosomally integrated [16].

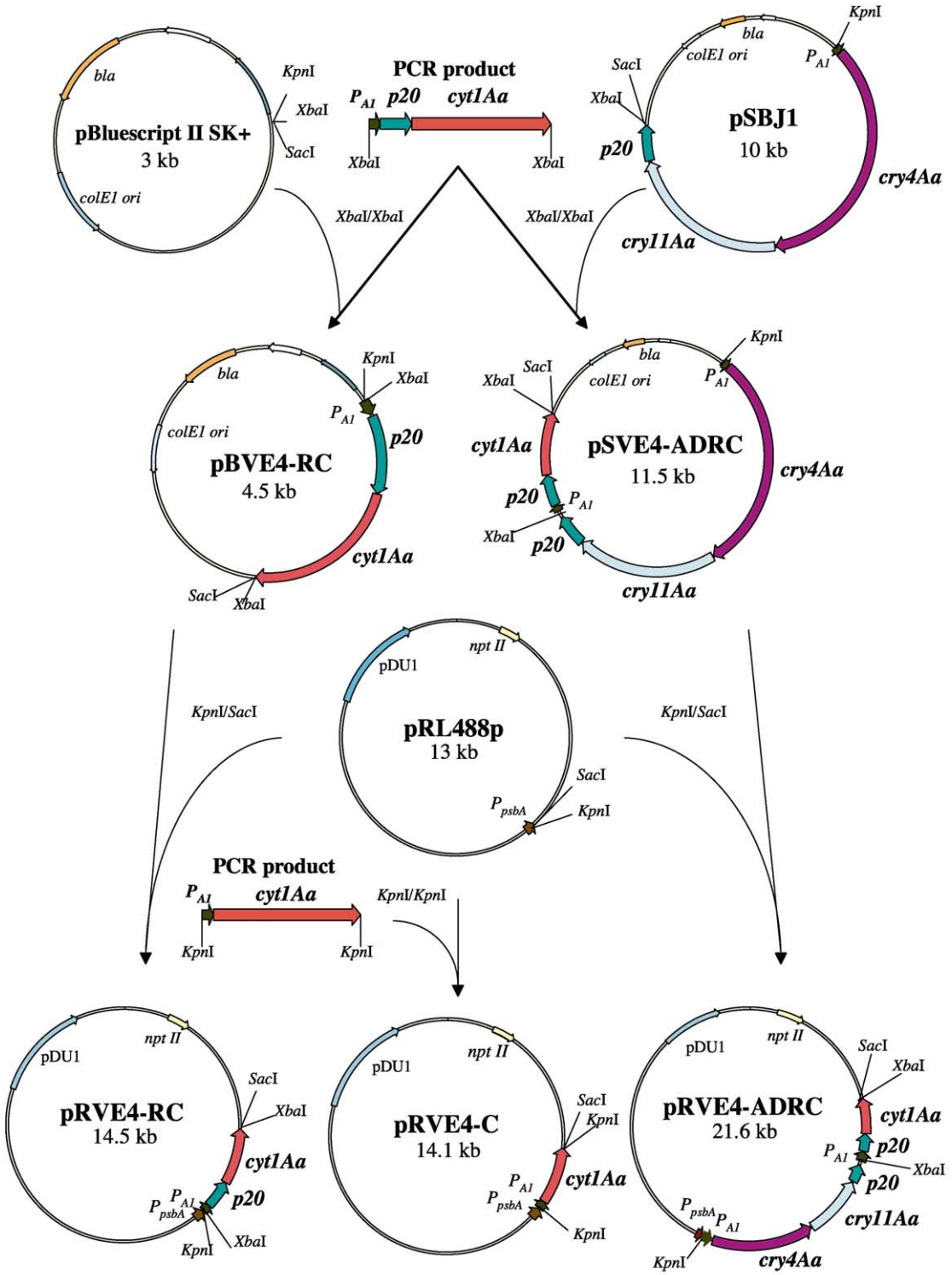

Fig. 1. Cloning of *cyt1Aa* with and without *p20* into the *E. coli-Anabaena* shuttle vector pRL488p and into the expression vector pSBJ1 harboring *cry4Aa*, *cry11Aa*, and *p20*. PCR products are 1648 and 1112 bp long for the P_{A1}-*p20*-*cyt1Aa* and P_{A1}-*cyt1Aa*, respectively.

2.3. *Conjugal introduction of* B. thuringiensis *subsp.* israelensis *genes into* Anabaena *PCC 7120*

The final three recombinant plasmids were introduced into *Anabaena* PCC 7120 by biparental mating [12,13]. *E. coli* DH10B harboring each of these plasmids was mixed (separately) with *Anabaena* PCC 7120, pre-grown in BG11 liquid medium at 28°C under cool white fluorescent light illumination, and the mixed suspensions were spread onto nitrocellulose membranes over BG11 agar plates. A day later, the membranes were transferred to selective plates containing neomycin (25 μg ml^{-1}). Colonies that

appeared after 10 days were inoculated in 1 ml BG11 with neomycin and grown for 2 weeks, when true ex-conjugants arose. These were purified by repeated streaking on agar plates with the antibiotic, each was diluted into BG11 with neomycin and cultivated under the same conditions. Growth was followed by determining chlorophyll concentrations in methanol extracts [13]. The existence of inserted genes was confirmed by polymerase chain reaction (PCR) amplification with appropriate primers [16].

2.4. Immunoblot analysis of toxic polypeptides

Cultures were concentrated 25–50-fold and disrupted by sonication (MSE Sonifier, five times 40 s each with 20-s intervals, 0°C). Samples with denaturing buffer were loaded on SDS–polyacrylamide (10–15%) gels after boiling for 10 min. Proteins (ca. 45 μg per lane; determined by Bio-Rad protein kit with bovine serum albumin as standard) were separated by electrophoresis and electro-transferred onto nitrocellulose filters. The blots were exposed to antisera directed against either whole *B. thuringiensis* subsp. *israelensis* crystal, Cyt1Aa or P20 (kindly provided, respectively, by Armelle Delecluse, Pasteur Institute, Sarjeet S. 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 visualizing using the chromogenic substrate for alkaline phosphatase fast-5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium tablets (Sigma), diluted in 10 ml water. *B. thuringiensis* subsp. *israelensis* (grown 4 days in LB broth at 30°C) and recombinant *E. coli* clones

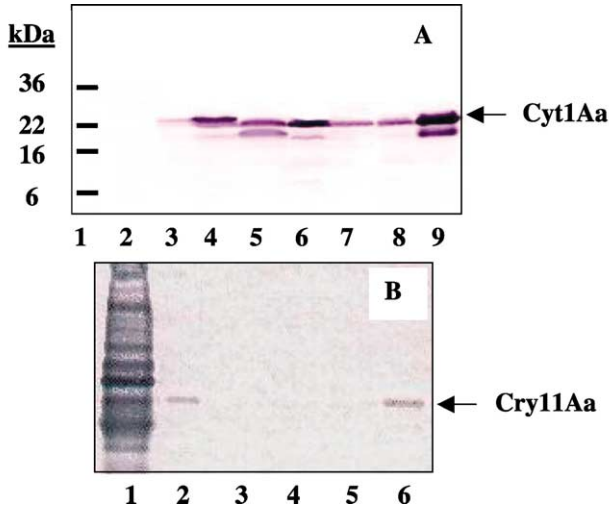

Fig. 2. Western blots of transgenic *Anabaena* and *E. coli* expressing CytGAa (A) and Cry11Aa (B) using anti-Cyt1Aa and antiserum against whole crystals, respectively. A: Lane 1, molecular size marker; lane 2, *Anabaena* PCC 7120 as a control; lane 3, pRVE4-C; lane 4, pRM4-C; lane 5, pRVE4-RC; lane 6, pRM4-RC; lane 7, pRVE4-ADRC; lane 8, pVE4-ADRC; lane 9, *B. thuringiensis* subsp. *israelensis*. B: Lane 1, *B. thuringiensis* subsp. *israelensis*; lane 2, pSBJ2; lane 3, *Anabaena* PCC 7120 as a control; lane 4, pRVE4-C; lane 5, pRVE4-RC; lane 6, pRVE4-ADRC.

(induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside at optical density 0.2 (ca. 2×10^8) for 4 h after growth at 37°C with 100 μg ml^{-1} ampicillin) were used as controls.

2.5. Bioassays for mosquito larvicidal activity

Cultures of the recombinant *Anabaena* were grown at 30°C in BG11 medium supplemented with 25 μg ml^{-1} neomycin into 250-ml flasks for 12 days, as previously described [16]. Cells were harvested by centrifugation 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. LC_{50} and LC_{90} (concentrations of cells that kill 50 and 90% of the exposed populations, respectively), as well as LT_{50} (time taken to kill 50% of exposed larvae), were determined by using probit analysis (EPA Probit analysis program) in at least six doses. All bioassays were performed at least three times in duplicate for each concentration.

3. Results and discussion

3.1. Expression in Anabaena PCC 7120

The clone with pRVE4-RC (Table 1) grew as well as its parental strain, while those with pRVE4-C and pRVE4-ADRC grew slightly more slowly than pSBJ2#11 [34]. The cells expressing *cyt1Aa* without *p20* from pRVE4-C enlarged and some of them later lysed (data not shown), indicating that they are sensitive to Cyt1Aa, as *E. coli* cells are [18]. Cells harboring pRVE4-C that survived may be resistant mutants.

Expression levels of Cyt1Aa in two of the three recombinant *Anabaena* clones (those with pRVE4-C and pRVE4-RC) were lower than in their *E. coli* counterparts with pRM4-C and pRM4-RC (lanes 3 and 5 in Fig. 2A to be compared with lanes 4 and 6, respectively). This difference is not observed between pRVE4-ADRC and pVE4-ADRC (compare lanes 7 and 8, respectively) perhaps because production of the additional two large proteins (Cry4Aa and Cry11Aa) exhausts the transcription/translation resources. In the transgenic *Anabaena*, the clone with pRVE4-RC expressed higher levels of Cyt1Aa than the clone with pRVE4-ADRC, and a faint signal could only be detected in the case of pRVE4-C (lanes 5, 7, and 3, respectively, in Fig. 2A). The higher level of CytGAa in the clone with pRVE4-RC than with pRVE4-C (both controlled by the tandem promoters P_{psbA}-P_{A1}) may be due to the presence of *p20* in the former (Fig. 1); preliminary data (some of the cells without *p20* enlarged and some lysed; pictures not shown) indicate that P20 protects *Anabaena* from the deleterious action of Cyt1Aa, as it does in *E. coli* [15,18]. P20 was, however, not detected with the specific anti-P20 (data not shown) in the trans-

Table 2
Toxicities of recombinant *Anabaena* PCC 7120 strains against fourth-instar *A. aegypti*, and synergism between Cyt1Aa and two Cry toxins

Strain	Larvicidal activity[a]	
	LC_{50} $\times 10^5$	LC_{90} $\times 10^5$
pSBJ2#11 (*cry4Aa*, *cry11Aa*, *p20*)	0.83 (0.65–1.05)	5.83 (3.88–10.53)
pRVE4-C[b] (*cyt1Aa*)	–	–
pRVE4-RC[b] (*p20*, *cyt1Aa*)	–	–
pRVE4-ADRC (*cry4Aa*, *cry11Aa*, *p20*, *cyt1Aa*)	0.35 (0.27–0.43)	1.80 (1.36–2.61)
Theoretical value[c]	1.66	11.66
Synergy factor[d]	4.74	6.48

[a]Values of LC_{50} and LC_{90} (in cells ml^{-1}) represent averages of three bioassays performed as described previously [15]. Numbers in parentheses are 95% confidence limits of probit.
[b]Clones pRVE4-RC and pRVE4-C did not exhibit any toxicity, even at a concentration of 6×10^8 cells ml^{-1}.
[c]Theoretical values of LC_{50} were calculated using the equation of [28] assuming a 1:1 ratio of cells with pSBJ2 and either pRVE4-RC or pRVE4-C, that the latter two are not toxic, and that all genes are expressed identically in all four clones.
[d]Synergy factors were obtained from the ratio between the theoretical (see c) and the observed LC values using Tabashnik's equation [28].

genic *Anabaena* with pRVE4-ADRC and pRVE4-RC, engineered to express *p20* from a single or tandem promoter, respectively (Fig. 1). The second copy of *p20* in the former clone is the third gene in an operon regulated by the same tandem promoters as in the clone carrying pSBJ2 [34], in which no expression of P20 could be detected either [35]. We cannot explain this negative result by a low quality anti-P20 because it did detect this accessory protein in *E. coli* with pVE4-ADRC or pRM4-RC [15].

The relatively low level of Cyt1Aa in the pRVE4-ADRC-containing clone, on the other hand, may be due to *cyt1Aa* being regulated under a single P_{A1} promoter (Fig. 1) or a limited capacity of the host to express three δ-endotoxins of *B. thuringiensis* subsp. *israelensis*. This relatively low level was, however, sufficient to synergize Crys (Table 2). No toxicity was found and a very low level of Cyt1Aa was detected by immunoblot (faint 25-kDa band) in *Synechocystis* PCC 6803 [9], the only work previously reporting expression of *cyt1Aa* in cyanobacteria. In this case, the gene was expressed under control of a single P_{psbA} that had been integrated into the chromosome. When two tandem promoters were employed, expression and toxicity levels of *cry4Ba* in another unicellular species *Synechococcus* PCC 7942 were higher [25]. Two tandem promoters were also more efficient to express *cry4Aa* in the filamentous cyanobacterium *Anabaena* PCC 7120 [34], as found here for *cyt1Aa* (in clone pRVE4-RC; Fig. 2A).

Large amounts of Cry11Aa were detected with antiserum against whole crystals in crude extracts of *Anabaena* with pRVE4-ADRC, in similar quantities as in the clone with pSBJ2 (expressing *cry4Aa*, *cry11Aa* and *p20* [35]) (Fig. 2B, lanes 6 and 2, respectively) and as in the *E. coli* clone with pVE4-ADRC [15]. Expression levels of *cry11Aa* in *Agmenellum quadruplicatum* PR-6 was high when fused in frame to the first six codons of *cpcB* under P_{psbB} with little or no apparent degradation, which may be due to protection by its crystal association [21], but toxicity of transgenic cells was very low. The high level found here in our *Anabaena* PCC 7120 (Fig. 2B) may be due to the tandem promoters employed or favorable codon usage.

A faint signal of Cry4Aa was detected by Western analysis in the crude extract and in the pellet of the clone with pRVE4-ADRC but not in the supernatant (data not shown), as found in the pellet of the one with pSBJ2 [35]. Expression of Cry4Aa from similar clones (pVE4-ADRC and pHE4-ADR, respectively) in *E. coli*, on the other hand, was high [15]. This observation is consistent with results of previous attempts to express Cry4Ba (homologous to Cry4Aa) in three unicellular cyanobacterial species, *A. quadruplicatum* PR-6 [2], *Synechocystis* PCC 6803 [9] and *Synechococcus* PCC 7942 [25,26], yielding exceedingly low protein levels and toxicities, while their *E. coli* counterparts with the same shuttle vectors displayed relatively high protein levels [2,9,25]. Moreover, very low expression levels of mosquito larvicidal proteins from *B. sphaericus* in *Synechococcus* and *Anabaena* have been found [24,36]. This series of observations has never

Table 3
LT_{50} of *A. aegypti* larvae (fourth-instar) by the transgenic *Anabaena* PCC 7120 strains expressing toxins from *B. thuringiensis* subsp. *israelensis*[a]

Chlorophyll concentration (ng ml^{-1})	LT_{50} (h)	
	pRVE4-ADRC	pSBJ2#11
100	3.35 (2.9–3.8)	9.39 (8.2–10.8)
80	3.34 (2.5–4.0)	7.0 (5.5–8.5)
60	4.20 (3.7–4.6)	11.02 (9.7–12.7)
40	5.13 (4.6–5.6)	21.32 (17.8–27.4)
20	14.42 (12.1–18.1)	79.95 (43.9–293.8)

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

been discussed or explained in the literature. It could be due to low solubility of Cry proteins, which varies according to the organism in which they are expressed and in what combination: (a) solubility of Cry4Aa is two-fold higher in *B. thuringiensis* subsp. *kurstaki* than in subsp. *israelensis* [3]; (b) solubility of δ-endotoxin in *B. thuringiensis* subsp. *aizawai* was reduced by absence of Cry1Ab, and restored upon reintroduction [4].

3.2. Toxicities of recombinant clones and synergism between toxins (Table 2)

The two clones expressing *cyt1Aa*, with or without *p20*, were not toxic at all (even at 6×10^8 cells ml^{-1}). Clone pRVE4-ADRC producing all four proteins displayed the highest toxicity against fourth-instar *A. aegypti* larvae (lowest LC_{50}, of 0.35×10^5 cells ml^{-1}), as did *E. coli* carrying the same combination [15], the highest toxicity ever reported for recombinant cyanobacteria with *B. thuringiensis* subsp. *israelensis* toxin genes [7]. The same construct lacking *cyt1Aa* (pSBJ2) was about 2.4-fold less toxic.

Synergism between Cyt1Aa and the two Crys in pRVE4-ADRC was defined by the synergy factor (SF; Table 2) obtained using Tabashnik's equation [28]. Values of SF in pRVE4-ADRC for LC_{50} and LC_{90} were 4.74 and 6.48, respectively, similar to those in *E. coli* harboring the same gene combination [15]. Synergy factors of about 15, 11 and 3 between Cyt1Aa and Cry4Aa or Cry4Ba or Cry11Aa, respectively, have previously been demonstrated in vitro against larvae of *A. aegypti*, whereas the SF between all four proteins was 5.6 [10]. Higher values of SF (between 16.6 and 70.5) were obtained in combinations of Cyt1Aa with the same Crys when tested against resistant strains of *C. quinquefasciatus*; these values were higher than for the susceptible strain, demonstrating the significance of Cyt1Aa in suppressing resistance [32].

The synergism is reflected by enhanced toxicity when the kinetics of larval mortality was determined (Table 3). Values of LT_{50} were obtained by using at least six doses (repeated at least thrice in duplicate), resembling results obtained with *E. coli* [15]. For example, at 20 ng chlorophyll ml^{-1}, pRVE4-ADRC killed 5.5 times more quickly than the respective clone without *cyt1Aa* (pSBJ2).

The clone with pRVE4-ADRC lost its high larvicidal activity after four subcultures in neomycin-free medium over a month, but regained toxicity following a challenge with neomycin (data not shown). It is therefore likely that *B. thuringiensis* subsp. *israelensis* genes expressed in this clone are plasmid-borne as are those in the clone (#7) with pSBJ2 [16], and that the plasmid is lost in most cells in the absence of antibiotic selection.

Toxicity endurance of the parental clone (#11) pSBJ2 where the plasmid had been integrated into the chromosome [16] is longer than that of *B. thuringiensis* subsp. *israelensis* itself (as Bactimos) either when mixed with silt or exposed to sunlight (to be published in Environ. Microbiol. vol. 5, 2003). Toxicity in outdoors experiments (affected by sunlight intensity and temperature) was extended 2–10-fold over that of Bactimos. Endurance of the clone with pRVE4-ADRC, expressing *cyt1Aa* as well, is anticipated to be at least as persistent as its parent.

3.3. Evaluation

Toxicities of the filamentous *Anabaena* PCC 7120 clones expressing two *cry* genes of *B. thuringiensis* subsp. *israelensis* [16,34], and with *cyt1Aa* (Tables 1 and 2), were higher than those reported in all unicellular cyanobacteria expressing these genes or *cry4Ba* (e.g., [2,9,21,25–27]). The advantage of multicellular over unicellular cyanobacterial species for mosquito control purposes is supported by comparisons between such species expressing the binary toxin of *B. sphaericus*: when these are expressed in filamentous species (*Anabaena* PCC 7120 and *Anabaena cylindrica*) [36], toxicities are much higher than in unicellular species [24,29].

Despite the very high toxicity of the *Anabaena* clone with pRVE4-ADRC described here, there is still room for improvement. For example, toxicity may be enhanced by expressing in addition the fourth major ICP, Cry4Ba, because it is also synergistic to the others [10,23]. Heterogeneous expression in prokaryotes can be affected by several factors such as mRNA stability (regulated by STAB-SD) [1] and transcription termination [22]. Addition of STAB-SD and an appropriate terminator may raise the expression levels of the cloned genes.

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References

[1] Agaisse, H. and Lereclus, D. (1996) STAB-SD: a Shine-Dalgarno sequence in the 5′ untranslated region is a determinant of mRNA stability. Mol. Microbiol. 20, 633–643.
[2] Angsuthanasombat, C. and Panyim, S. (1989) Biosysnthesis of 130-kilo-dalton mosquito larvicide in the cyanobacterium *Agmenellum quadruplicatum* PR-6. Appl. Environ. Microbiol. 55, 2428–2430.
[3] Angsuthanasombat, C., Crickmore, N. and Ellar, D.J. (1992) Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism in vivo. FEMS Microbiol. Lett. 94, 63–68.
[4] Aronson, A.I. (1995) The protoxin composition of *Bacillus thurin-*

giensis insecticidal inclusions affects solubility and toxicty. Appl. Environ. Microbiol. 61, 4057–4060.

[5] Berry, C., O'Neil, S., Ben-Dov, E., Jones, A.F., Murphy, L., Quail, M.A., Holden, M.T.G., Harris, D., Zaritsky, A. and Parkhill, J. (2002) Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 68, 5082–5095.

[6] Boussiba, S. (1993) Production of the nitrogen-fixing cyanobacterium *Anabaena siamensis* in a closed tubular reactor for rice farming. Microb. Releases 2, 35–39.

[7] Boussiba, S., Wu, X.-Q., Ben-Dov, E., Zarka, A. and Zaritsky, A. (2000) Nitrogen-fixing cyanobacteria as gene delivery system for expressing mosquitocidal toxins of *Bacillus thuringiensis* ssp. *israelensis*. J. Appl. Phycol. 12, 461–467.

[8] Chang, C., Yu, Y.-M., Dai, S.-M., Law, S.K. and Gill, S.S. (1993) High-level *cryIVD* and *cytA* gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. Appl. Environ. Microbiol. 59, 815–821.

[9] Chungjatupornchai, W. (1990) Expression of the mosquitocidal protein genes of *Bacillus thuringiensis* subsp. *israelensis* and the herbicide-resistance gene *bar* in *Synechocystis* PCC6803. Curr. Microbiol. 21, 283–288.

[10] Crickmore, N., Bone, E.J., Williams, J.A. and Ellar, D.J. (1995) Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. 131, 249–254.

[11] Dervyn, E., Poncet, S., Klier, A. and Rapoport, G. (1995) Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. 177, 2283–2291.

[12] Elhai, J., Vepritskiy, A., Muro-Pastor, A.M., Flores, E. and Wolk, C.P. (1997) Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. Am. Soc. Microbiol. 179, 1998–2005.

[13] Elhai, J. and Wolk, C.P. (1988) Conjugal transfer of DNA to cyanobacteria. Methods Enzymol. 167, 747–754.

[14] Khampang, P., Chungjatupornchai, W., Luxananil, P. and Panyim, S. (1999) Efficient expression of mosquito-larvicidal proteins in a gram-negative bacterium capable of recolonization in the guts of *Anopheles dirus* larva. Appl. Microbiol. Biotechnol. 51, 79–84.

[15] Khasdan, V., Ben-Dov, E., Manasherob, R., Boussiba, S. and Zaritsky, A. (2001) Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. Environ. Microbiol. 3, 798–806.

[16] Lluisma, A.O., Karmacharya, N., Zarka, A., Ben-Dov, E., Zaritsky, A. and Boussiba, S. (2001) Suitability of *Anabaena* PCC 7120 expressing mosquitocidal toxin genes from *Bacillus thuringiensis* subsp. *israelensis* for biotechnological application. Appl. Microbiol. Biotechnol. 57, 161–166.

[17] Ludwig, M. and Becker, N. (1997) Studies on possible resistance against *Bacillus thuringiensis israelensis* in *Aedes vexans* (Diptera, Culicinae) after 15 years of application. Zool. Beitr. 38, 167–174.

[18] Manasherob, R., Zaritsky, A., Ben-Dov, E., Saxena, D., Barak, Z. and Einav, M. (2001) Effect of accessory proteins P19 and P20 on cytolytic activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* in *Escherichia coli*. Curr. Microbiol. 43, 355–364.

[19] Manasherob, R., Ben-Dov, E., Wu, X., Boussiba, S. and Zaritsky, A. (2002) Protection from UV-B damage of mosquito larvicidal toxins from *Bacillus thuringiensis* subsp. *israelensis* expressed in *Anabaena* PCC 7120. Curr. Microbiol. 45, 217–220.

[20] Margalith, Y. and Ben-Dov, E. (2000) Biological control by *Bacillus thuringiensis* subsp. *israelensis*. In: Insect Pest Management: Techniques for Environmental Protection (Rechcigl, J.E. and Rechcigl, N.A., Eds.), pp. 243–301. CRC Press, Boca Raton, FL.

[21] Murphy, R.C. and Stevens, S.E.Jr. (1992) Cloning and expression of the *cryIVD* gene of *Bacillus thuringiensis* subsp. *israelensis* in the cyanobacterium *Agmenellum quadruplicatum* PR-6 and its resulting larvicidal activity. Appl. Environ. Microbiol. 58, 1650–1655.

[22] Nierlich, D.P. and Murakawa, G.J. (1996) The decay of bacterial messenger RNA. Prog. Nucleic Acid Res. Mol. Biol. 52, 153–216.

[23] Poncet, S., Delecluse, A., Kiler, A. and Rapoport, G. (1995) Evaluation of synergistic interactions among the CryIVA, CryIVB, and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. 66, 131–135.

[24] Sangthongpitag, K., Penfold, R.J., Delaney, S.F. and Rogers, P.L. (1997) Cloning and expression of the *Bacillus sphaericus* 2362 mosquitocidal genes in a non-toxic unicellular cyanobacterium, *Synechococcus* PCC 6301. Appl. Microbiol. Biotechnol. 47, 379–384.

[25] Soltes-Rak, E., Kushner, D.J., Williams, D.D. and Coleman, J.R. (1993) Effect of promoter modification on mosquitocidal *cryIVB* gene exoression in *Synechococcus* sp. strain PCC 7942. Appl. Environ. Microbiol. 59, 2404–2410.

[26] Soltes-Rak, E., Kushner, D.J., Williams, D.D. and Coleman, J.R. (1995) Factors regulating *cryIVB* expression in the cyanobacterium *Synechococcus* PCC 7942. Mol. Gen. Genet. 246, 301–308.

[27] Stevens Jr., S.E., Murphy, R.C., Lamoreaux, W.J. and Coons, L.B. (1994) A genetically engineered mosquitocidal cyanobacterium. J. Appl. Phycol. 6, 187–197.

[28] Tabashnik, B.E. (1992) Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 58, 3343–3346.

[29] Tandeau de Marsac, N., de la Torre, F. and Szulmajster, J. (1987) Expression of the larvicidal gene of *Bacillus sphaericus* 159M in the cyanobacterium *Anacystis nidulans*. Mol. Gen. Genet. 209, 396–398.

[30] Thanabalu, T., Hindley, J., Brenner, S., Oei, C. and Berry, C. (1992) Expression of the mosquitocidal toxins of *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* by recombinant *Caulobacter crescentus*, a vehicle for biological control of aquatic insect larvae. Appl. Environ. Microbiol. 58, 905–910.

[31] Visick, J.E. and Whiteley, H.R. (1991) Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* on production of the CytA protein by *Escherichia coli*. J. Bacteriol. 173, 1748–1756.

[32] Wirth, M.C., Georghiou, G.P. and Federici, B.A. (1997) CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. Proc. Natl. Acad. Sci. USA 94, 10536–10540.

[33] Wirth, M.C., Federici, B.A. and Walton, W.E. (2000) Cyt1A from *Bacillus thuringiensis* synergizes activity of *Bacillus sphaericus* against *Aedes aegypti* (Diptera: Culicidae). Appl. Environ. Microbiol. 66, 1093–1097.

[34] Wu, X., Vennison, S.J., Huirong, L., Ben-Dov, E., Zaritsky, A. and Boussiba, S. (1997) Mosquito larvicidal activity of transgenic *Anabaena* strain PCC 7120 expressing combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63, 4971–4975.

[35] Wu, X. (1999) Expression of *cry* Genes from *Bacillus thuringiensis* subsp. *israelensis* in Filamentous Nitrogen-fixing Cyanobacteria. Ph.D. Thesis, Ben-Gurion University of the Negev, Be'er-Sheva.

[36] Xu, X., Yan, G., Kong, R., Liu, X. and Hu, Y. (2000) Analysis of expression of the binary toxin genes from *Bacillus sphaericus* in *Anabaena* and potential in mosquito control. Curr. Microbiol. 41, 352–356.

[37] Yap, W.H., Thanabalu, T. and Porter, A.G. (1994) Expression of mosquitocidal toxin genes in a gas-vacuolated strain of *Ancylobacter aquaticus*. Appl. Environ. Microbiol. 60, 4199–4202.

[38] Yoshisue, H., Yoshida, K.-I., Sen, K., Sakai, H. and Komano, T. (1992) Effects of *Bacillus thuringiensis* var. *israelensis* 20-kDa protein on production of the Bti 130-kDa crystal protein in *Escherichia coli*. Biosci. Biotechnol. Biochem. 56, 1429–1433.