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Suitability of *Anabaena* PCC7120 expressing mosquitocidal toxin genes from *Bacillus thuringiensis* subsp. *israelensis* for biotechnological application

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Abstract We present evidence that *Anabaena* PCC7120 (*A.*7120) strains expressing mosquitocidal toxin genes from *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) have a strong potential for biotechnological application. Characterization of two 4-year-old recombinant *A.*7120 clones constructed previously in our laboratory [clone 7 and clone 11, each carrying three *Bti* genes (*cry4Aa*, *cry11Aa*, and *p20*)] revealed three facts. First, the *Bti* genes were stable in *A.*7120 even in the absence of antibiotic selection when the genes were integrated in the chromosome (in clone 11); and the genes were also stable as plasmid-borne constructs (in clone 7), provided the cultures were maintained under continued selection. Second, clone 7 (kept under selection) and clone 11 (either kept or not kept under selection) continued to be mosquitocidal through 4 years of culture. Third, growth of the recombinant clones was comparable to the wild type under optimal growth conditions, indicating that growth was not compromised by the expression of toxin genes. These results clear the way for the development of mass production techniques for *A.*7120 strains expressing *Bti* toxin genes.

Introduction

Cyanobacterial strains expressing toxin genes from *Bacillus* spp [i.e., *B. thuringiensis* subsp. *israelensis* (*Bti*) and *B. sphaericus*] are currently being developed into effective but environment-friendly biopesticides against mosquitoes and blackflies (for reviews, see Boussiba et al. 2000; Margalith and Ben-Dov 2000). As part of this effort, *Bti* δ-endotoxin genes [*cryIVA* (*cry4Aa*), *cryIVD cry11Aa*)] and a regulatory gene (*p20*) were introduced into the filamentous cyanobacterium *Anabaena* PCC7120 (*A.*7120) in our laboratory (Wu et al. 1997). Two recombinant clones, clone 7 and clone 11, each carrying the three genes, exhibited high larvicidal activities [$LC_{50}<1\times10^5$ cells ml^{-1} or ≈30 ng chlorophyll a (chl-a) ml^{-1}] against *Aedes aegypti* larvae (Wu et al. 1997), the highest reported for cyanobacteria expressing *Bti* δ-endotoxin genes (Boussiba et al. 2000).

The feasibility of using recombinant cyanobacteria for mosquito control remains to be demonstrated. Of particular interest is whether recombinant *A.*7120 strains are suitable for long-term culture and mass production. Because the toxin genes could be lost or undergo mutation resulting in loss of toxicity, it is also necessary to show that the clones continue to carry the genes and to be mosquitocidal over a long period of time, especially if the genes are borne by plasmid vectors. Whether the expression of the toxin genes compromises the strains' growth is also a major concern. Because the use of antibiotics for selection in large-scale cultures is environmentally unsound, strains that continue to be larvicidal in the absence of selection would be desirable if not essential.

So far, little work has been done to assess the biotechnological potential of recombinant cyanobacteria. To evaluate the suitability of recombinant *A.*7120 strains expressing *Bti* toxin genes for large-scale and long-term production, we further characterized the two mosquitocidal *A.*7120 clones 4 years after they were constructed.

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Materials and methods

Strains and plasmids

The wild-type *A.*7120 and the recombinant clones, clone 7 and clone 11 (each carrying *cry4Aa*, *cry11Aa*, and *p20* genes from *Bti*),

used in this study were as previously described (Wu et al. 1997). The plasmid pSBJ2, containing the three *Bti* genes transferred to *A.*7120 (Wu et al. 1997), was used as the control for the PCR and restriction analyses. *Escherichia coli* XL1Blue-MRF′ (Stratagene, USA) was used for plasmid-rescue experiments.

Culture conditions

Two batch-culture systems were used: (1) flask cultures maintained in an incubator-rotary shaker [150 ml medium, at 30 °C, with continuous illumination (50 μmol photon m^{-2} s^{-1}) and a shaking speed of 160 rpm], and (2) glass-column cultures [500 ml medium, at 30 °C, with continuous illumination (85 μmol photon m^{-2} s^{-1})]. The cultures were continuously aerated and mixed by bubbling air containing 1.5% CO_2 and, in both systems, the medium used was BG11 (Wu et al. 1997), supplemented, when necessary, with neomycin (25 μg ml^{-1}).

To determine the effect of absence of antibiotic selection on gene stability as well as toxicity, flask cultures of clone 11 were continuously subcultured, either with or without neomycin since 1996, when the clones were constructed. Flask-cultures of clone 7 were also continuously maintained under neomycin selection since 1996; and neomycin-free cultures were started in 1999.

PCR analysis

Portions of the *Bti* genes were amplified by PCR [30 cycles of denaturation at 94 °C for 1 min (3 min for the first cycle), annealing at 57 °C (for *cry4Aa*, *cry11Aa*) or 52 °C (for *p20*) for 1 min, and extension at 72 °C for 1 min (4 min for the last cycle)], using a MJ Research thermal cycler (MJ Research, USA) or a Stratagene Robocycler (Stratagene, USA) and using the following primers: Un4(d) and Un4(r) for *cry4Aa*, EE-11A(d) and EE-11(r) for *cry11Aa* (for sequences, see Ben-Dov et al. 1997), and EB-*p20*(d) (GTGTTTTATAAAATATTCACAACAG) and EB-*p20*(r) (CGTT-CCGATTAAGTTAAATAAGTC) for *p20*.

Recovery of plasmids from *A.*7120 clones

Total DNA was extracted from wild-type and recombinant *A.*7120 clones using the protocol described by Williams (1988) and introduced into *E. coli* XL1-Blue MRF′ cells by electroporation. Transformants growing on LB plates supplemented with ampicillin (50 μg ml^{-1}) and kanamycin (25 μg ml^{-1}) were screened by PCR for presence of the *Bti* genes and were assayed for toxicity to *A. aegypti* larvae.

Toxicity of *A.*7120 and *E. coli* to *A. aegypti* larvae

Cells of *A.*7120 or *E. coli* were harvested by centrifugation (expression of the *Bti* genes in the latter was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM, 1 h before harvesting), washed with distilled water, and fed to *A. aegypti* larvae (3rd instar stage) at various doses as described previously (Wu et al. 1997). The number of dead larvae was counted after 24 h and the LC_{50} was calculated by probit analysis (Daum 1970).

Measurement of growth

The increase in algal biomass was monitored by measuring chl-a and protein. The cells were extracted with methanol for chl-a determination (Mackinney 1941) and the pellet was used for protein extraction and quantification using the procedure of Lowry et al. (1951).

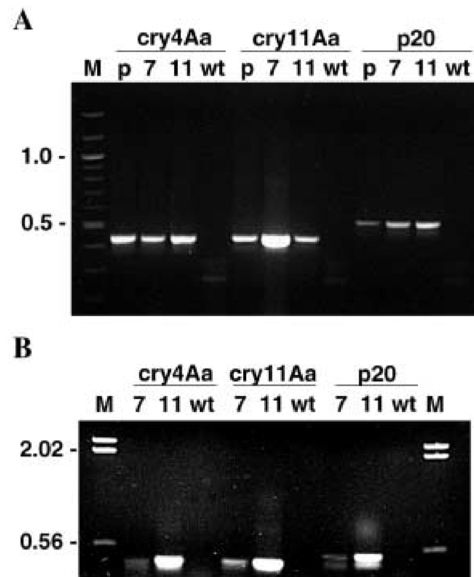

Fig. 1A,B Presence of the *Bti* genes in the wild-type and recombinant clones of *Anabaena* PCC7120 (*A.*7120) as revealed by PCR. Total DNA was extracted from the wild type (*wt*, grown in antibiotic-free medium) and from clone 7 (*7*) and clone 11 (*11*) which were continually subcultured with (**A**) or without (**B**) neomycin selection (see Materials and methods). This DNA was used as template for amplification of fragments of *cry4Aa* (expected size, 439 bp), *cry11Aa* (445 bp), and *p20* (542 bp). The plasmid pSBJ2 (*p*) was used as the control template. *M* Marker. Sizes are given in kilobase pairs (kb)

Results

Long-term stability of the *Bti* toxin genes in the recombinant *A.*7120 clones

The *Bti* genes *cry4Aa*, *cry11Aa*, and *p20* were apparently stable in *A.*7120. Two recombinant clones, clone 7 and clone 11 (each carrying the three *Bti* genes), continually subcultured in the presence of neomycin for 4 years, still contained the three genes, as confirmed by PCR (Fig. 1A) and by their toxicity to mosquito larvae (see below). Interestingly, clone 11 continually subcultured in antibiotic-free conditions for the same period continued to retain the genes (Fig. 1B) and to be strongly mosquitocidal. In contrast, clone 7 cultures maintained without antibiotic selection for 1 year were apparently losing the plasmid, as indicated by poor PCR amplification of the genes (Fig. 1B); and these cultures were not mosquitocidal (see below).

To test the hypothesis that the *Bti* genes had integrated into the chromosome in clone 11 but had remained as part of an autonomously replicating plasmid in clone 7 (thus accounting for the stable presence of the genes in the former but not in the latter in the absence of antibiotic selection), we attempted to recover plasmids from the two clones via cloning in *E. coli* (see Materials and methods). A plasmid was recovered from clone 7 (continuously subcultured in the presence of neomycin) and was cut with *Kpn*I and *Pst*I, the same enzymes used

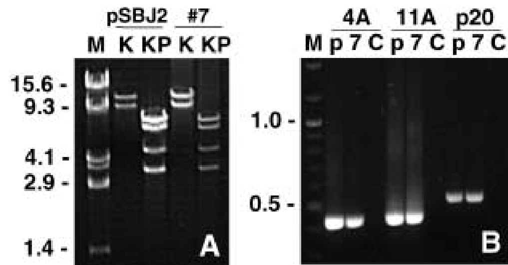

Fig. 2A,B Characterization of a plasmid recovered from *A*.7120 clone 7 through cloning in *Escherichia coli*. **A** Restriction pattern of the recovered plasmid and pSBJ2. The plasmids were either cut with *Kpn*I (*K*) or with both *Kpn*I and *Pst*I (*KP*). **B** PCR amplification of fragments of the three *Bti* genes from the recovered plasmid (*7*). pSBJ2 (*p*) was used as the positive control. *C* Negative control (no DNA added), *M* marker. Sizes are in kb

by Wu et al. (1997) to construct the cargo vector pSBJ2, consisting of the parent plasmids, pRL488p and pSBJ1. Restriction of the plasmid with *Kpn*I yielded fragments of the size expected for pRL488p (13 kb) and pSBJ1 (10 kb; Fig. 2A). Restriction of the same plasmid with both *Kpn*I and *Pst*I yielded a fragment (one of four fragments) of about 6.5 kb (Fig. 2A), the size expected for the construct containing the three *Bti* genes originally assembled and expressed in *E. coli* by Ben-Dov et al. (1995) and later transferred to *A*.7120 (Wu et al. 1997). The restriction patterns were identical to those of pSBJ2 (Fig. 2A). When this plasmid was used as template for PCR amplification of the *Bti* genes, the PCR products were of the size expected for *cry4Aa*, *cry11Aa*, and *p20* fragments (Fig. 2B). Thus, pSBJ2 was apparently stable and structurally intact in clone 7.

That pSBJ2 remained autonomously replicating in clone 7 is consistent with the observation that cultures maintained in neomycin-free medium for about 1 year partially lost their resistance to neomycin and their toxicity to mosquito larvae, but recovered both neomycin resistance and their toxicity to mosquito larvae when exposed to neomycin (see below).

Attempts to recover pSBJ2 from clone 11 using the same protocol used successfully to recover the plasmid from clone 7 were all unsuccessful. This result, coupled with the findings that, through 4 years of selection-free culture, the *Bti* genes stably existed in clone 11 and that the clone remained strongly larvicidal, indicates that the *Bti* genes had integrated into the chromosome in clone 11.

Larvicidal activity of recombinant clones

Because the toxicity of the recombinant clones may be affected by growth conditions, these were grown in both slow-growth (flask cultures, see Materials and methods) and fast-growth conditions (glass-column cultures); and samples were harvested for toxicity assays after 3 days and 7 days (representing the late-log and late-stationary growth phases of glass-columns cultures).

Table 1 Larvicidal activity (LC_{50}) against *Aedes aegypti* of 3-day-old and 7-day-old cultures (in glass columns or flasks) of *Anabaena* PCC7120 (*A*.7120) strains (see Fig. 1 for growth curves). The values are averages of two (flasks) or four (glass columns) replicates (±SD). *Chl-a* Chlorophyll a, *Nm* neomycin, *nt* not toxic, *WT* wild type

A.7120 strain	Chl-a (ng ml^{-1})		Protein (ng ml^{-1})	
	3-day-old	7-day-old	3-day-old	7-day-old
Glass columns				
WT	nt	nt	nt	nt
11 +Nm	25.7±6.4	20.5±4.6	349.6±25.6	264.0±65.2
11 –Nm	30.7±5.2	12.9±1.4	341.2±27.1	206.0±78.9
7 +Nm	22.7±5.9	14.2±2.0	223.8 ±78.0	166.6±55.7
7 –Nm	nt	nt	nt	nt
Flasks				
WT	nt	nt	nt	nt
11 +Nm	15.5±7.6	7.6±2.2	232.5±74.2	91.2±40.7
11 –Nm	16.7±6.1	7.5±0.7	200.4±56.0	119.7±15.8
7 +Nm	7.8±4.6	5.5±3.1	81.0±48.4	75.1±32.8
7 –Nm	nt	nt	nt	nt

While the wild type was not toxic to larvae (maximum chl-a concentration tested: 2 μg chl-a ml^{-1}), the recombinant clones, constructed 4 years ago, remained highly toxic (Table 1). Clone 11, whether or not it was maintained under neomycin selection, was highly larvicidal; and the lowest LC_{50} recorded was ca. 7.5 ng chl-a ml^{-1} (within 24 h of feeding). Cells grown in flasks were about 2-fold more toxic than those grown in glass columns. Seven-day-old cultures, both in flasks and in glass columns, were also considerably more toxic (1.3- to 2.4-fold) than the 3-day-old cultures, whether toxicity was expressed in chl-a or total protein.

Clone 7 also continued to be highly toxic, but only when the cells were maintained under neomycin selection (Table 1). These cells were generally more toxic than clone 11 cells, although the difference varied with the culture system and with the age of the cultures. The difference was most pronounced between 3-day-old flask-cultures of the two clones; and those of clone 7 were about 2-fold (chl-a basis) or 3-fold (total protein basis) more toxic than those of clone 11. The older cultures of clone 7 (maintained under antibiotic selection) also showed stronger larvicidal activity: 7-day-old cultures were about 1.4- to 1.6-fold more toxic than 3-day-old cultures.

To determine whether the clone 7 would lose its larvicidal activity in the absence of selection, cultures that had been continuously maintained under selection were subcultured into neomycin-free medium. The results showed that it did not lose its larvicidal activity after being subcultured four times over a period of 1 month (Table 2).

Clone 7 cultures that were continually subcultured in neomycin-free medium for about 1 year lost their toxicity (up to the maximum concentration tested, equivalent to 80 ng chl-a ml^{-1}; Table 1), although the *Bti* genes were still detectable by PCR (Fig. 1B). To determine whether

Table 2 Larvicidal activity (LC_{50}) against *A. aegypti* of *A.*7120 clone 7 maintained either with or without antibiotic selection for 1 year, when transferred to new culture conditions (in glass columns)

Growth condition	Subculture	Day after start of subculture	LC_{50} (ng Chl-a ml^{-1}; ±SD)
Clone 7 grown with neomycin for 4 years, transferred to non-selective medium	1	–	12.0±2.1
	2	7	15.9±6.4
	3	8	19.5±3.5
	4	10	10.0[c]
Clone 7 grown without neomycin for 1 year, transferred to selective medium	1[a]	6	19.5±2.1
	2[b]	6	3.2±2.3

[a] The growth curve is shown in Fig. 4A [b] The growth curve is shown in Fig. 4B [c] one of the two replicates was lost

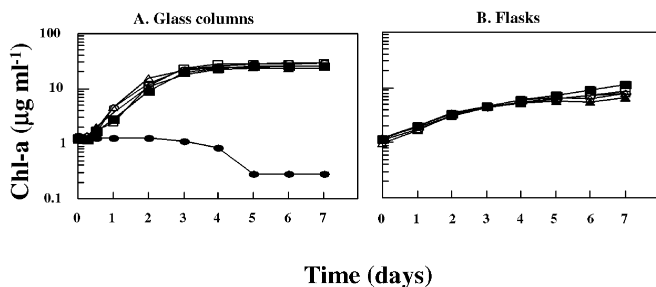

Fig. 3 Growth of transgenic and wild-type clones of *A.*7120 in glass columns (**A**) and in flasks (**B**), expressed in Chl-a. Cultures were grown with (*closed symbols*) or without (*open symbols*) neomycin (25 µg ml^{-1}), as described in Materials and methods. ●, ○ Wild type, ■, □ clone 7, ▲, △ clone 11

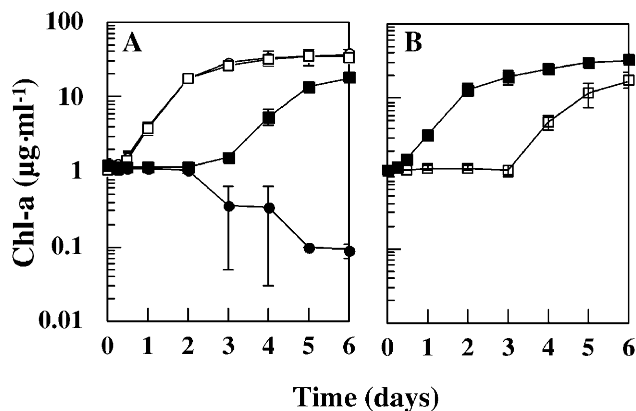

Fig. 4A,B Growth of *A.*7120 clone 7, maintained in neomycin-free medium for 1 year, upon transfer to a medium containing neomycin (25 µg ml^{-1}). Cultures of wild-type cells maintained in neomycin-free medium and cultures of clone 7 in medium containing neomycin were used as controls. **A** Growth upon exposure to neomycin: ●, ○ wild-type cells grown in medium with or without neomycin, respectively, ■, □ clone 7, maintained in neomycin-free medium for 1 year and then grown in a medium with or without neomycin, respectively. **B** Growth of subcultures of clone 7 upon exposure to neomycin. Subcultures from (**A**) with (■) and without (□) neomycin, respectively, transferred into media containing neomycin. *Chl-a* Chlorophyll a

these cultures would recover their larvicidal activity when exposed to antibiotic selection, cells were transferred to a medium containing neomycin and allowed to grow under optimal conditions (i.e., in the glass columns). As shown in Fig. 4A, the cultures exposed to neomycin for the first time after 1 year of selection-free culture were able to grow, but only after a 3-day lag (the cultures maintained previously under neomycin-selection did not show this lag), during which cell lysis apparently occurred, as indicated by the leaching of phycocyanin into the medium (data not shown). When the cultures exposed to neomycin selection in the first experiment were subcultured into fresh media containing neomycin, the subcultures resumed growth without going through a lag phase (Fig. 3B). Assays also indicated that 6-day old cultures from the first and second experiments were strongly larvicidal (Table 2). These observations support the hypotheses that: (1) the *Bti* genes in clone 7 continued to be plasmid-borne, and (2) in the absence of antibiotic selection, most (but not all) cells in the cultures lost the plasmid and thus were non-larvicidal. Apparently, when the cultures were challenged with neomycin, only those cells that retained the plasmid were able to survive and initiate growth of the culture.

Growth of the recombinant and wild-type *A.*7120 clones

The growth of the recombinant and wild-type clones was compared, using flask cultures (slow-growth conditions) and glass-column cultures (fast-growth conditions). Under fast-growth conditions, the doubling-time [in the log phase (first 3 days), based on chl-a measurements] of clone 7 grown with or without neomycin (ca. 23 h and 19 h, respectively) and the doubling-time of clone 11 grown with or without neomycin (ca. 20 h and 19 h, respectively) were comparable with that of the wild type (ca. 20 h; Fig. 4A). Under slow-growth conditions, the doubling times of clone 7 in the presence or absence of neomycin (ca. 38 h and 32 h, respectively) and clone 11 in the presence or absence of neomycin (ca. 37 h) were also comparable with, if not slightly faster than, that of the wild type (ca. 39 h; Fig. 4B). The growth curves based on total-protein and dry-weight measurements were similar to those based on chl-a measurements (data not shown). The maximal cell densities attained by the transgenic and wild-type cultures were also higher in the glass columns (up to 30 µg chl-a ml^{-1}) than in the flasks (ca. 10 µg chl-a ml^{-1}).

Discussion

In this report, we provide evidence that *A.*7120 expressing *Bti* mosquitocidal toxin genes are suitable for biotechnological exploitation.

The long-term stability of the *Bti* genes is likely enhanced by their integration into the chromosome. Our data indicate that clone 11 carries the *Bti* genes in its chromosome (see Results). This clone continues to carry the genes and to be strongly mosquitocidal even when continually subcultured in the absence of antibiotic selection for 4 years. Integration of genes into the chromosome apparently ensures their stable presence in the cells and obviates the need to keep cultures under selection (Kalman et al. 1995). Techniques for introducing genes into the chromosome in cyanobacteria have already been described (e.g., see Elhai 1994; Taroncher-Oldenburg and Stephanopoulos 2000).

Our results also indicate that the *Bti* genes borne by an autonomously replicating vector are stable in *A.*7120, provided the cultures are maintained under selection. Clone 7, from which plasmids containing the *Bti* genes can be rescued, continues to carry the genes and to be lethal to mosquito larvae 4 years after its construction, but only when the cultures are maintained under continuous antibiotic selection. When maintained without selection for 1 year, clone 7 lost its toxicity (Table 1), as most cells in the cultures apparently lost the plasmids (Fig. 1). This is consistent with reports that cyanobacterial strains with plasmid-borne toxin genes tend to loss toxicity when grown without selection (Sangthongpitag et al. 1997; Xu et al. 1993).

The toxicity assays indicate that, 4 years after their construction, the larvicidal activity of clone 7 (kept under antibiotic selection) and clone 11 (either kept or not kept under antibiotic selection) has remained high. The LC_{50} values were generally less than 30 ng chl-a ml^{-1}, essentially similar to values reported earlier (Wu et al. 1997). Interestingly, cultures of clone 7 continuously maintained under selection were slightly to moderately more toxic, up to ~3-fold when toxicity is expressed in terms of total protein, than clone 11. This difference in toxicity may have resulted from the difference in copy number of the endotoxin genes in the two clones, the *cry* genes being located on a plasmid in the former and in the chromosome in the latter, as our data suggest. Our hypothesis is consistent with the observation of Elhai (1993) that luciferase activity in *A.*7120 bearing *luxAB* (under the control of the *rbc* promoter) on a plasmid is about 3-fold higher than in *A.*7120 bearing the same construct in the chromosome, which is indicative of the higher copy number of pDU1-based plasmids relative to the chromosome.

The toxicity of the clones to mosquito larvae is apparently dependent on the growth conditions of the cells, as the older (late-stationary phase) and slower-growing cultures tend to be more toxic. Whether this higher toxicity results from increased gene expression in these growth conditions or from accumulation of proteins, or both, has not yet been specifically investigated. Preliminary characterization of clone 11 indicated that Cry11Aa polypeptides are stable in this clone (data not shown). Soltes-Rak et al. (1993, 1995) found that the *Bti* CryIVB polypeptides are not readily degraded by proteases and are apparently stable in *Synechococcus* PCC7942; and they tend to accumulate in the cells until the early stationary phase. *Bti* CryIVD (Cry11Aa) polypeptides are also stable in *Synechococcus* PCC7002 (Murphy and Stevens 1992), as is *B. sphaericus* binary toxin in *Anabaena* sp. FACHB (Xu et al. 2000) and in *Synechococcus* PCC6301 (Sangthongpitag et al. 1997). It would seem that cellular accumulation of endotoxin polypeptides could, at least partially, account for higher larvicidal activity of the older or slower-growing cultures.

Growth of the recombinant strains is apparently not compromised by expression of the toxin genes. The growth rate of the recombinant (and larvicidal) clones, cultured in the presence of neomycin, was comparable to that of the wild type (grown without the antibiotic), not only under slow-growth (i.e., maintenance) conditions but particularly under fast-growth conditions, confirming the observations made by Wu et al. (1997) that the constitutive expression of the endotoxin genes did not adversely affect the growth of *A.*7120 and that the cry proteins were not toxic to the cells. In contrast, *Synechococcus* PCC7002 expressing Cry11Aa grew slower than the strain that did not carry the gene (although carrying the same vector; Stevens et al. 1994).

The ability of recombinant clones to grow as fast as the wild type and to remain highly toxic in fast-growth conditions is another indication of the suitability of the clones for mass production. The use of the glass-column system in this study is the first step towards mass production of recombinant *A.*7120 clones. We have successfully used the same system for optimization of culture conditions prior to scaling up the production of other filamentous cyanobacteria, using outdoor photobioreactors (Boussiba 1993; Querijero-Palacpac et al. 1990).

Our data indicate the suitability of *A.*7120 strains expressing *Bti* toxin genes for long-term culture and mass production. We have started developing techniques to scale up the production of the strains. Development of different formulations of the biomass (primarily as nonviable microbials) is also underway.

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