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Effect of Promoter Modification on Mosquitocidal *cryIVB* Gene Expression in *Synechococcus* sp. Strain PCC 7942

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The impact of promoter modification on the expression of the mosquitocidal *Bacillus thuringiensis* subsp. *israelensis cryIVB* gene when used to transform the cyanobacterium *Synechococcus* sp. strain PCC 7942 has been examined. Maximal transcript and protein abundances were achieved by the addition of the *lacZ* promoter upstream of the *cryIVB* sequence. Replacement of the endogenous corresponding *Bacillus* sequences with the *Synechococcus petF1* promoter, ribosome binding site, and initiation codon also resulted in increased expression of the *cryIVB* gene relative to the expression obtained with the *Bacillus* promoter alone but decreased expression relative to the expression achieved with the tandem array of the *Bacillus* and *lacZ* promoters. *Synechococcus* cells carrying plasmids in which the expression of the *cryIVB* gene was regulated by either the *lacZ* or the *petF1* promoter were readily consumed by first-instar *Culex restuans* larvae and proved to be toxic for these organisms.

Many strains of the gram-positive soil bacterium *Bacillus thuringiensis* produce parasporal crystalline inclusion bodies with highly specific lepidopteran, dipteran, and coleopteran larvicidal activities (5, 32). When ingested by susceptible larvae, the crystal proteins are solubilized in the insect mid-gut, in which they cause structural deformation of the epithelial cells, a process that eventually leads to larval death from starvation. Sequence analysis of crystal protein genes (*cry* genes) has resulted in a proposed classification system based on protein sequence homology and range of insecticidal activity (16). These genes are grouped into four classes: Lepidoptera specific (*cryI*), Lepidoptera and Diptera specific (*cryII*), Coleoptera specific (*cryIII*), and Diptera specific (*cryIV*).

The ovoid crystalline inclusions of *B. thuringiensis* subsp. *israelensis* are toxic when ingested by larvae of mosquitoes and black flies. As these two types of organisms are important vectors of several human and animal diseases, there is considerable interest in the use of this bacterium as an effective agent for insect control (8, 22, 27). It is now apparent that the *B. thuringiensis* subsp. *israelensis* parasporal crystal contains at least four mosquitocidal polypeptides encoded by the genes located on a 72-MDa (112-kb) plasmid (15). The mosquitocidal genes that have been isolated and sequenced, *cryIVA*, *cryIVB*, *cryIVC*, and *cryIVD*, encode proteins with predicted molecular masses of 135, 128, 78, and 72 kDa, respectively (2, 11, 25, 29).

Although *B. thuringiensis* subsp. *israelensis* spore preparations are now used as a biological control agent for mosquitoes, the application of these preparations as biolarvicides has two major disadvantages. The spores of the bacterium settle rapidly to the bottom of aquatic habitats and thus are removed from the larval feeding zone. Therefore, applications may need to be repeated frequently. An additional safety concern is the presence of the *cytA* gene product (10, 31), a 27-kDa, noninsecticidal protein component of the *B. thuringiensis* subsp. *israelensis* parasporal crystal that has been shown to be hemolytic for erythrocytes from many species, including humans (28). It has been suggested that one way to overcome these problems would be to express only the *cryIV* toxin gene(s) in an appropriate bacterial host that would have a longer residence time in the larval feeding zone and that would be a palatable, natural food source for mosquito larvae. As native sources of food for mosquito larvae, the unicellular, transformable cyanobacteria would seem to fulfill these two criteria and would seem to be a suitable host for the expression of *cryIV* toxin genes (22, 27).

Several initial attempts to express mosquitocidal proteins in cyanobacteria met with limited success. In an early study, a mosquitocidal gene isolated from *B. sphaericus* 1593M and encoding a cell wall- or membrane-bound toxin was expressed in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (26). Angsuthanasombat and Panyim (3) reported the transformation of the cyanobacterium *Agmenellum quadruplicatum* PR-6 with the *cryIVB* mosquitocidal gene on a vector containing a phycocyanin promoter. In another study (7), several copies of the *cryIVB* gene under the control of the tobacco *psbA* promoter were integrated into the chromosome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. In each of these studies, although the transformation of the cyanobacterium was successful, only limited expression of the various *Bacillus* genes was obtained and high concentrations of cell lysates were required for larvicidal activity. Intracellular concentrations of the *cry* gene products were sufficiently low that larvae feeding on intact cells were not affected. It is apparent that if the cyanobacteria are to be useful hosts for the *B. thuringiensis* subsp. *israelensis cryIVB* toxin gene, there must be a considerable increase in the levels of expression. Recently, the expression of another toxin gene, *cryIVD*, in the unicellular cyanobacterium *A. quadruplicatum* PR-6 was reported (21). Mosquitocidal levels of expression in intact cells were achieved by fusing the coding region of the *cryIVD* gene to the promoter and initial coding sequence of the highly expressed phycocyanin β-subunit gene (*cpcB*). In the study reported here, we

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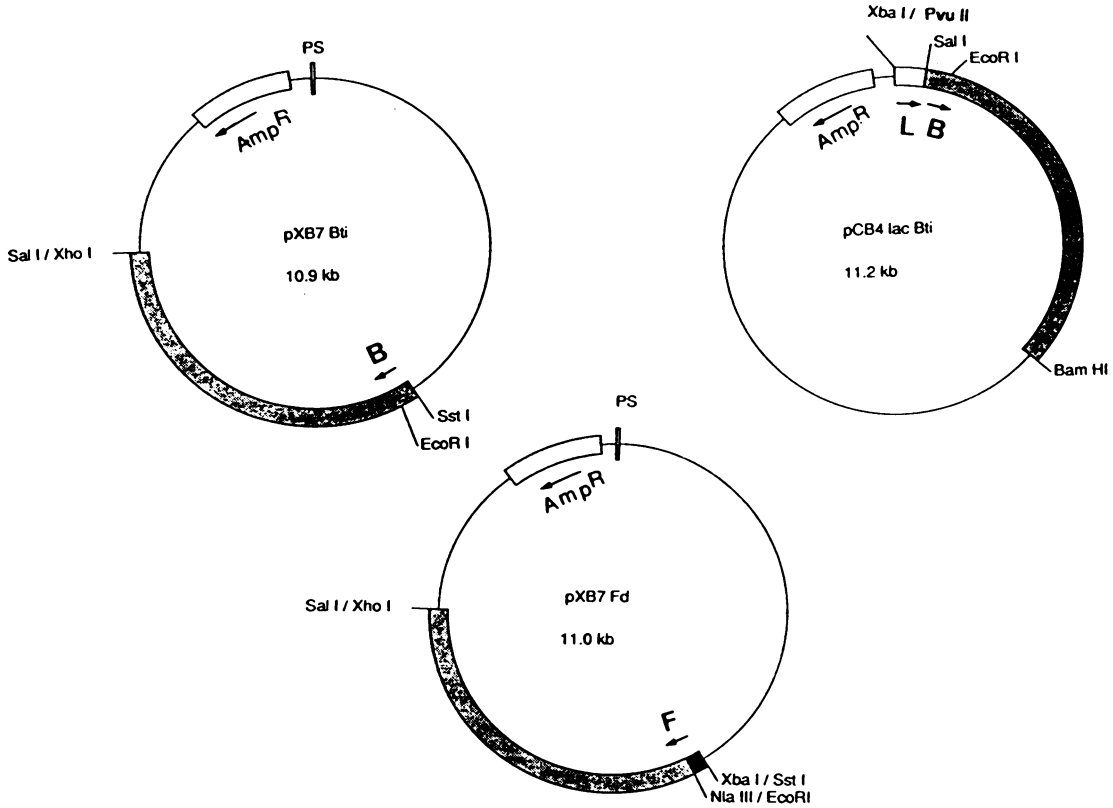

FIG. 1. Construction of plasmids pXB7Bti, pCB4lacBti, and pXB7Fd. The shaded segment indicates the position of the *cryIVB* gene. The presence of the endogenous *Bacillus* promoter region is indicated by B, and an arrow shows the direction of transcription. The position of the *petF1* promoter region and initiation codon flanking the modified *cryIVB* gene is indicated by a black box labelled F. An open box labelled L indicates the position of the *lacZ* promoter region 5′ of the intact *cryIVB* gene. The positions of the polycloning site (PS) and the various restriction endonuclease sites used in cloning are also indicated.

attempted to maximize the expression of the *cryIVB* toxin gene under the control of different promoter constructions in the cyanobacterium *Synechococcus* sp. strain PCC 7942. In addition to studies on expression and *cryIVB* protein stability, we showed clearly that the intact, transformed cyanobacteria are toxic when ingested by mosquito larvae.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* JM101 and DH5α (19) were used in all cloning experiments. Cells were grown at 37°C in Luria broth, and transformants were selected on the same medium supplemented with 50 μg of ampicillin ml^{-1}.

The unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 was maintained on BG-11 (1) medium solidified with 1.0% Bacto agar or grown as an aerated liquid batch culture at 28°C and with a light intensity of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. Transformation of *Synechococcus* sp. strain PCC 7942 and isolation of cyanobacterial DNA were carried out as described previously (14). Transformants were selected on BG-11 plates supplemented with 1 μg of ampicillin ml^{-1}.

Plasmid construction. Standard cloning procedures were used, as described previously (19). Restriction endonucleases, Klenow polymerase, and T4 DNA ligase were used in accordance with the suggestions of the individual suppliers.

The *cryIVB* crystal protein gene from *B. thuringiensis* subsp. *israelensis* was subcloned in both orientations as a 3,786-bp *Xba*I fragment from plasmid pRX80 (9) into the *Xba*I site of pUC18 to provide additional cloning sites. The *Xba*I DNA fragment includes a sequence that is located 160 bp 5′ of the initiation codon and that contains the −10 and −35 regions, the structural gene sequence, and a sequence that is located 118 bp 3′ of the stop codon and that contains the transcription termination loop. The two constructs cloned into pUC18 were designated p2/17 and p20. A 3.8-kb *Bam*HI-*Sal*I fragment from construct p20 was inserted into the *Synechococcus* sp. strain PCC 7942-*E. coli* shuttle vector pCB4 (13), which previously had been modified by the addition of the *lacZ* promoter region (isolated as a 220-bp *Pvu*II-*Bam*HI fragment from pBS [Stratagene Inc., La Jolla, Calif.]) upstream of the cloning site. This procedure resulted in the generation of the 11.2-kb vector pCB4lacBti (Fig. 1).

A 3.8-kb *Sac*I-*Sal*I fragment from p2/17 was inserted into the *Sac*I-*Xho*I sites of pXB7, which is a derivative of the *Synechococcus-E. coli* shuttle vector pCB4 and which contains an extra polycloning site but no additional promoter regions (18). The resulting plasmid was designated pXB7Bti (Fig. 1).

Vector pXB7Bti was used to generate an additional construct in which the region upstream of *cryIVB*, the ribosome binding site, and the initiation codon were removed and replaced with a portion of the *Synechococcus* sp. strain PCC 7942 ferredoxin gene (*petF1*) (23). The region of *petF1* used

in this construct was isolated from clone pFDE1 (23) as a 100-bp *Xba*I-*Nla*III fragment and was fused to an *Nla*III-*Eco*RI linker and then ligated to the *Sac*I (modified with T4 DNA polymerase) and *Eco*RI sites of pXB7Bti. The resulting vector, pXB7Fd, contained the cyanobacterial ferredoxin promoter, the ribosome binding site, and the ATG codon fused in frame to the second amino acid of the *cryIVB* crystal protein gene (Fig. 1).

Protein isolation and Western blotting (immunoblotting). Fifty-milliliter cultures of cyanobacteria were harvested by centrifugation (8,000 × *g*; 10 min), resuspended in 3 ml of 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.5) containing 10 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 1 mM benzamidine, and lysed in a prechilled French pressure cell at 20,000 lb/in^2. The lysates were centrifuged briefly (2 min; 3,000 × *g*) to remove unbroken cells and then prepared for electrophoresis by the addition of dithiothreitol and Na_2CO_3 each to a final concentration of 0.1 M and 1 volume of denaturing buffer (5% sodium dodecyl sulfate [SDS], 0.1% bromphenol blue, 30% sucrose). Samples were placed in a boiling water bath for 2 min prior to being loaded on the gel. Bacterial protein samples were prepared from 3-ml overnight cultures by the same protocol. Proteins were separated on an SDS–10% polyacrylamide gel with the Laemmli buffer system (17), electrotransferred to nitrocellulose, and probed with a rabbit antiserum directed against the *cryIVB* polypeptide as described previously (4). Specifically bound primary immunoglobulin G was detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G.

Antibody production. Crystal protein inclusions of *B. thuringiensis* subsp. *israelensis* NRC 5993 were isolated as described by Thomas and Ellar (28) and electrophoresed on an SDS–10% polyacrylamide gel. The 128- and 135-kDa bands corresponding to the *cryIVB* and *cryIVA* gene products were cut out from the gel, and the polypeptides were electroeluted. Polyclonal antibodies were produced in a female New Zealand White rabbit by a technique developed for small doses of immunogens (30).

RNA isolation and slot blotting. Three-hundred-milliliter cultures of cyanobacteria were harvested during the mid-log phase of growth, and the RNA was isolated by a method described previously (23). Following denaturation with formaldehyde, 5-μg aliquots (as determined by spectrophotometry) of the appropriate RNA samples were transferred to nitrocellulose with a slot blot apparatus, and the blots were prehybridized for 6 h at 42°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) containing 50% (vol/vol) formamide, 1% (wt/vol) skim milk powder, and 0.1% (wt/vol) SDS (hybridization solution) (19). For the generation of probe DNA, agarose gel-purified *cryIVB* DNA fragments or higher-plant ribosomal DNA was labelled with [α-^{32}P]dCTP by the random-primer procedure and added to fresh hybridization solution at a final concentration of 10^6 cpm · ml^{-1} (12). Following 16 h of hybridization at 42°C, the blots were washed at room temperature twice for 20 min each time with 2× SSPE containing 0.1% SDS and twice for 20 min each time with 0.1× SSPE containing 0.1% SDS. The extent of hybridization was determined by autoradiography.

***cryIVB* protein stability assays.** Aliquots of *E. coli* cell lysates containing pXB7Bti were incubated with increasing amounts of *Synechococcus* sp. strain PCC 7942 cell lysates for various periods of time at 28°C. At appropriate intervals, samples were removed, denatured, and electrophoresed as described above, and the proteins were transferred to nitrocellulose. The blots were then probed with antisera directed against the *cryIVB* protein.

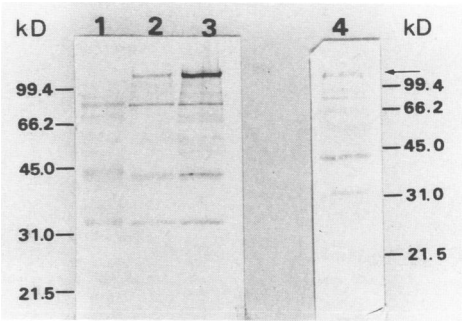

FIG. 2. Western blot analysis of total protein extracts from *E. coli* transformed with the *cryIVB* gene. Soluble proteins were separated by SDS-polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose, and probed with a rabbit antiserum directed against *cryIVA* and *cryIVB* proteins. The Western blot shows results obtained with extracts from *E. coli* cells containing vector pXB7 alone (lane 1), pCB4lacBti (lane 2), pXB7Fd (lane 3), and pXB7Bti (lane 4). The arrow on the right shows the *cryIVB* protein. The positions of molecular mass markers are indicated on the left and right.

Toxicity assays. First-instar larvae of *Culex restuans* mosquitoes were obtained from eggs collected from local artificial ponds and hatched in the laboratory. Following hatching, single larvae were placed into individual wells of microtiter plates and exposed to various concentrations of wild-type and *cryIVB*-expressing *Synechococcus* cells. Larvae were fed daily with new additions of cyanobacteria, and larval viability was monitored by observation in situ with a dissection microscope.

RESULTS

Vector construction and *cryIVB* expression in *E. coli*. The cloning procedures described in Materials and Methods generated three plasmids in which the expression of the *B. thuringiensis* subsp. *israelensis cryIVB* gene is directed from three different promoter constructions: the *B. thuringiensis* subsp. *israelensis cryIVB* promoter itself, a tandem array of both *lacZ* and *Bacillus* promoters, and the *Synechococcus* sp. strain PCC 7942 *petF1* promoter (Fig. 1). The last plasmid is actually a translation fusion construct in which, along with the promoter region and ribosome binding site, the ferredoxin initiation codon is fused to the second amino acid of the *cryIVB* coding region.

The level of *cryIVB* expression in *E. coli* JM101 cells containing the three plasmids was determined by Western blot analysis (Fig. 2). Maximal accumulation of the 128-kDa protein was achieved with the cyanobacterial *petF1* promoter directing *cryIVB* expression (Fig. 2, lane 3). The tandem array of the *lacZ* and *cryIVB* promoters resulted in less efficient expression of the 128-kDa polypeptide (Fig. 2, lane 2), whereas the plasmid containing the *cryIVB* promoter alone was capable of only very limited expression (Fig. 2, lane 4). Overstaining of the immunoblot resulted in the appearance of three other proteins that were also present in cells containing the vector alone (Fig. 2, lane 1) and as such were not products of *cryIVB* expression.

Western blot analysis of the *Synechococcus cryIVB* protein. The abundance of the 128-kDa protein was also determined for *Synechococcus* sp. strain PCC 7942 cells transformed

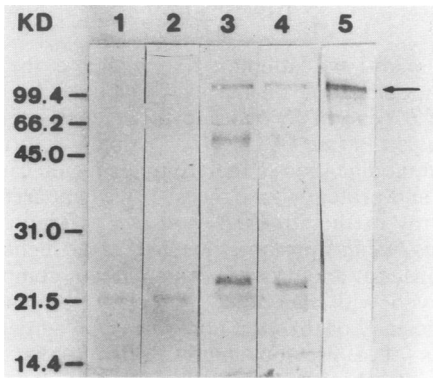

FIG. 3. Western blot analysis of total protein extracts from *Synechococcus* cells transformed with vector pXB7 alone (lane 1), pXB7Bti (lane 2), pCB4lacBti (lane 3), and pXB7Fd (lane 4). A soluble protein extract obtained from *E. coli* cells transformed with pXB7Fd and expressing the *cryIVB* protein is shown in lane 5. Following gel electrophoresis and blotting, the nitrocellulose was probed with antisera directed against the *cryIVA* and *cryIVB* proteins. The position of the toxin protein is indicated with an arrow. The positions of the molecular mass markers are also indicated.

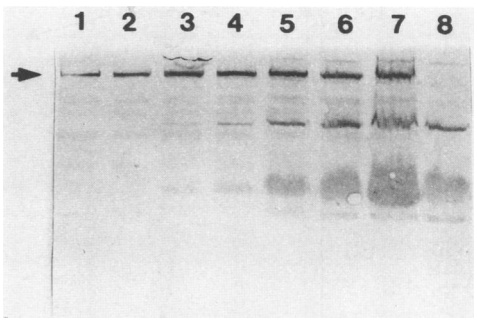

FIG. 4. *cryIVB* protein stability in *Synechococcus* cell lysates. Aliquots of bacterial lysates containing the full-length, *E. coli*-expressed *cryIVB* protein were incubated for 24 h at 28°C with increasing amounts of wild-type *Synechococcus* cell lysates. Following incubation, samples were removed and proteins were separated by SDS gel electrophoresis, transferred to nitrocellulose, and probed with antisera directed against the *cryIVB* protein. Shown is an immunoblot profile for *E. coli* cell lysates containing the *cryIVB* protein alone (lane 1), after 24 h of incubation alone (lane 2), and after incubation for 24 h with 1, 2, 5, 10, and 15 μg of wild-type *Synechococcus* cell lysate protein (lanes 3, 4, 5, 6, and 7, respectively). The position of the toxin protein is indicated with an arrow. Lane 8 shows the immunoblot profile generated for 10 μg of wild-type *Synechococcus* cell lysate protein.

with the same three shuttle vector constructs (Fig. 1). The presence of each plasmid in ampicillin-resistant colonies of *Synechococcus* transformants was confirmed by Southern blot analysis (data not shown). A Western blot indicating the levels of the 128-kDa protein is shown in Fig. 3. No detectable *cryIVB* protein production was seen in cells transformed with pXB7Bti, in which the expression of the toxin is driven by the endogenous *B. thuringiensis* subsp. *israelensis* promoter (Fig. 3, lane 2). In contrast, a significant amount of the 128-kDa protein was found in cells transformed with pCB4lacBti (Fig. 3, lane 3).

The accumulation of the *cryIVB* gene product in *Synechococcus* sp. strain PCC 7942 cells containing pXB7Fd, the ferredoxin translation fusion construct was lower than that observed in cell lines containing the *lacZ* and *Bacillus* tandem promoters; a faint signal was obtained for the 128-kDa protein on the Western blot (Fig. 3, lane 4). Overall, there was a significant decrease in *cryIVB* gene expression in *Synechococcus* sp. strain PCC 7942 cells with all three constructs relative to the levels of the 128-kDa protein in *E. coli* (compare Fig. 3, lanes 2 to 4, with Fig. 2 and Fig. 3, lane 5). In addition to the decrease in expression, a number of smaller, immunopositive bands were detected in cell extracts of cell lines containing the *lacZ* and *Bacillus* tandem promoter construct as well as the *petF1* translation fusion vector. These smaller proteins were not present in the *Synechococcus* cell line containing the vector alone (Fig. 3, lane 1) or in *E. coli* cells expressing high levels of the 128-kDa polypeptide (Fig. 3, lane 5).

***cryIVB* protein stability in cyanobacterial lysates.** *E. coli* lysates containing high levels of the *cryIVB* protein were incubated at 28°C for 24 h with increasing concentrations of wild-type *Synechococcus* cell lysates, and the products of these reactions were analyzed by Western blotting (Fig. 4). Little if any proteolysis of the intact 128-kDa *cryIVB* protein occurred, and no accumulation of distinct, smaller, immunopositive bands was detected. This result is in contrast to the Western blot profile obtained for *Synechococcus* sp. strain PCC 7942 cells expressing the *cryIVB* gene (compare Fig. 3 and 4).

RNA slot blot analysis of *Synechococcus cryIVB* mRNA. The relative abundances of *cryIVB* mRNA in *Synechococcus* sp. strain PCC 7942 cells containing the three plasmid constructs are shown in Fig. 5. Total RNA was isolated from the three cell lines as well as two control cultures, slot blotted, and probed with the *cryIVB* gene. Hybridization with a ribosomal DNA probe was used to ensure that equal amounts of RNA were present in each slot. The data showed that the mosquitocidal gene was transcribed in all three cell lines containing the *cryIVB* plasmids but that the levels of expression exhibited by the constructs were quite different. Maximal *cryIVB* mRNA abundance was achieved with the plasmid containing both *lacZ* and *Bacillus* promoters and then with pXB7Fd and pCB4Bti (Fig. 5A, lanes 3, 4, and 5). No significant hybridization signal was observed for RNA samples obtained from wild-type cells or from cells transformed with the shuttle vector alone (Fig. 5A, lanes 1 and 2).

Toxicity assays. Newly hatched larvae of *C. restuans* were exposed to low concentrations of *Synechococcus* cells containing shuttle vector pXB7 alone, plasmid pCB4lacBti, or

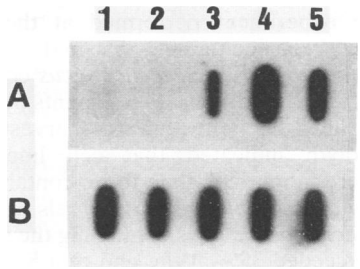

FIG. 5. RNA slot blot analysis of *cryIVB* expression in *Synechococcus* sp. strain PCC 7942 cells. Slot blots of 5 μg of total RNA isolated from wild-type cells (lane 1) or cells transformed with vector pXB7 alone (lane 2), pXB7Bti (lane 3), pCB4lacBti (lane 4), and pXB7Fd (lane 5) were probed with the radiolabelled *cryIVB* coding sequence (A) or a 5.8-kb *Eco*RI fragment containing a portion of the 18S-23S ribosomal DNA sequence isolated from soybean (B).

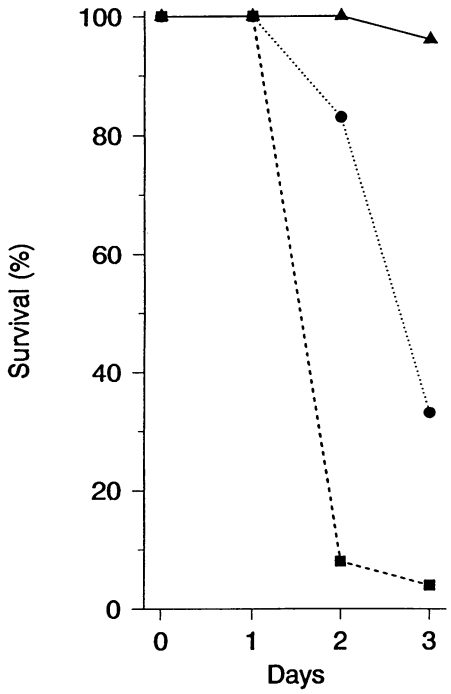

FIG. 6. Mortality of 72 *C. restuans* larvae fed *Synechococcus* cells transformed with vector pXB7 alone (▲), pCB4lacBti (●), or pXB7Fd (■) (n = 24 each). Larvae were hatched at time zero and placed in individual microtiter wells, in which they were fed once daily with the appropriate culture. The total number of larvae used was 72; 24 individuals were tested with each cell line.

plasmid pXB7Fd (Fig. 6). For these experiments, cyanobacterial cultures were harvested at the mid- to late-log phase of growth (approximately 1.5×10^8 cells · ml^{-1}), and 100-μl aliquots were added to microtiter plate wells containing individual larvae and 100 μl of water. The larvae were observed to rapidly consume the cyanobacteria and initially appeared to exhibit normal feeding behavior. Larvae feeding on *Synechococcus* cells expressing the *cryIVB* protein began dying on the second day of exposure and exhibited structural deformation of the mid-gut consistent with *B. thuringiensis* toxin damage. The data shown in Fig. 6 were obtained from one experiment in which the toxicity of each cell line was tested with 24 larvae over a period of 4 days. In this trial and in a replicate experiment performed at the same time, *Synechococcus* cells containing plasmid pXB7Fd were found to be more toxic than *Synechococcus* cells containing plasmid pCB4lacBti. Additional experiments performed with the same cell lines but with cultures harvested at earlier stages of growth indicated that cell lines containing pCB4lacBti were more toxic than those containing pXB7Fd (data not shown). In all experiments, levels of mortality for larvae fed *Synechococcus* cells containing the shuttle vector alone never exceeded 35% at the end of a 5-day period. In addition, these dead larvae did not exhibit the mid-gut structural deformities found in larvae killed by toxin exposure. Lower concentrations of cyanobacteria containing the *B. thuringiensis* subsp. *israelensis cryIVB* gene also were found to be effective as larvicides. A 10-fold decrease in cell concentration was found to generate similar rates of mortality among the *C. restuans* larvae (data not shown).

DISCUSSION

In this study, we attempted to evaluate the effect of promoter modification on the expression of the mosquitocidal *cryIVB* gene in *E. coli* and the cyanobacterium *Synechococcus* sp. strain PCC 7942. The data described show clearly that modification of the promoter region can increase transcript and protein abundances. It was apparent that the tandem array of the strong *E. coli lacZ* promoter and the endogenous *Bacillus* promoter resulted in the highest level of *cryIVB* transcript and protein accumulation, compared with that achieved with the other two plasmids. The demonstrated efficiency of the *lacZ* construct is in agreement with the results of a previous study showing that promoter sequences matching the *E. coli* promoter consensus sequence can be very effective in cyanobacteria and can generate high rates of transcription (24). It has also been shown that intact *lacZ* is transcribed and translated in a closely related cyanobacterium (6). As vector pCB4lacBti contains both the *lacZ* and the *cryIVB* transcription initiation sites, it is possible that the enhanced rate of toxin production is the result of the synthesis of two mRNA species. It is also possible that the enhanced transcriptional activity of *cryIVB* in this vector is similar to an observation made for *E. coli*: increased numbers of tandemly arranged penicillinase promoters resulted in a corresponding increase in protein production (20). In two earlier studies on the cyanobacterial expression of *cryIVB*, the authors observed additional immunopositive bands on Western blots of total cyanobacterial proteins and concluded that in vivo degradation of the large mosquitocidal proteins was at least partially responsible for the reduced level of toxicity (3, 7). We have also observed the generation of low-molecular-mass immunopositive signals; however, the inability of cyanobacterial lysates to degrade the intact *E. coli*-expressed *cryIVB* protein would suggest that these smaller immunopositive proteins are the result of premature termination of either cyanobacterial transcription or cyanobacterial translation and are not the result of in situ proteolysis.

It is interesting that the *lacZ-Bacillus* construct was more effective in the cyanobacterium than was a construct that contained a *Synechococcus* sp. strain PCC 7942 promoter and ribosome binding site. We had assumed that the addition of an endogenous promoter region and ribosome binding site would maximize the expression of the *cryIVB* gene. It has been suggested, however, that many of the characterized genes that are expressed in the vegetative cells of another cyanobacterium, *Anabaena* sp. strain PCC 7120, including *petF1*, have relatively weak promoters (24). This suggestion may account for the lower-than-expected levels of *cryIVB* expression, even though the native *petF1* promoter region and ribosome binding site were present. In addition, the pattern of expression of pXB7Fd is presumably similar to that of the native *petF1* product and would be controlled by the same set of parameters, such as light fluence, nutritional status of the cells, and culture age. These parameters may not have been optimal for maximum expression of the pXB7Fd construct, whereas the expression of the *lacZ-Bacillus* construct would be relatively independent of many environmental effects.

In the recent paper by Murphy and Stevens (21), it was shown that high levels of expression of another *B. thuringiensis* subsp. *israelensis* toxin gene, *cryIVD*, in the cyanobacterium *A. quadruplicatum* PR-6 can be achieved with a similar gene fusion strategy. This strategy involved ligation of the gene coding for the 67-kDa *cryIVD* protein behind the

promoter, ribosome binding site, and six initial codons of the *A. quadruplicatum* PR-6 phycocyanin β-subunit gene (*cpcB*). The *cpcB* gene product is a major component of the cyanobacterial phycobilisome, and as such, its expression is driven by the strong *cpcB* promoter. The addition of this strong promoter to the *cryIVD* coding region results in the expression of the toxin protein at sufficiently high levels that the transformed cyanobacteria are lethal when consumed by *Culex pipiens* mosquito larvae.

In agreement with the work of Murphy and Stevens on *cryIVD* (21) and in contrast to earlier work on the *cryIVB* toxin gene (3, 7), our data show that it is possible to achieve larvicidal effects with *cryIVB* expression in intact cyanobacteria. Both constructs pCB4lacBti and pXB7Fd were toxic when consumed by *C. restuans* larvae, with levels of mortality averaging 70 to 95% 2 to 3 days after the commencement of feeding. The relative toxicity of each construct seemed to be somewhat culture age dependent. Cells containing pXB7Fd were more toxic when harvested at the mid- to late-log stage of growth, whereas pCB4lacBti-containing cells exhibited a higher level of toxicity when harvested at lower cell densities. These results may explain the discrepancy between the RNA slot blot and Western blot studies and the toxicity trials. RNA and protein isolation experiments were performed with cultures in the early- to mid-log growth phase, whereas the toxicity trials shown in Fig. 6 were done with older cultures. A more detailed study of *cryIVB* expression patterns in each cell line is now in progress. This present study, however, clearly shows that levels of expression of the *cryIVB* gene can be increased by promoter manipulation and that it is possible to generate cyanobacteria that are toxic when consumed by mosquito larvae.

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