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Cloning and Expression of the *cryIVD* Gene of *Bacillus thuringiensis* subsp. *israelensis* in the Cyanobacterium *Agmenellum quadruplicatum* PR-6 and Its Resulting Larvicidal Activity

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A mosquitocidal cyanobacterium has been developed by introducing the mosquito-toxic *cryIVD* gene from *Bacillus thuringiensis* subsp. *israelensis* into the unicellular cyanobacterium *Agmenellum quadruplicatum* PR-6 (*Synechococcus* sp. strain PCC 7002). The *cryIVD* gene was introduced into the cyanobacterium on a derivative of the PR-6 expression vector pAQE19ΔSal in which the *cryIVD* gene was translationally fused to the initial coding sequence of the highly expressed PR-6 *cpcB* gene. Coomassie blue staining and immunoblot analysis of gel-fractionated cell extract polypeptides indicate that the *cpcB-cryIVD* gene fusion is expressed at high levels in the cyanobacterial cells, with little or no apparent degradation of the *cryIVD* gene product. Larvicidal assays revealed that freshly hatched *Culex pipiens* mosquito larvae readily ingested the transformed cyanobacteria and that the cells proved to be toxic to the larvae.

The gram-positive soil bacterium *Bacillus thuringiensis* subsp. *israelensis* is one of many *B. thuringiensis* strains able to produce insecticidal proteins (for reviews, see references 2, 10, and 19). These proteins, expressed during the sporulation cycle, assemble into parasporal crystalline inclusion bodies. The parasporal crystal produced by *B. thuringiensis* subsp. *israelensis* is toxic when ingested by the larvae of mosquitoes and black flies. Upon ingestion, crystal proteins are solubilized in the larval midgut and act in a manner not yet clearly defined to disrupt the epithelium of the larval midgut region. Swelling and/or lysis of the epithelial cells is followed by larval death from starvation.

Mosquitoes are the major vectors for a number of human and animal diseases, including malaria, yellow fever, viral encephalitis, dengue fever, and filariasis. In the larval stage, mosquitoes are filter feeders that feed primarily on microscopic algae and bacteria. In view of these facts and because of the very selective nature of the toxic effects of the *B. thuringiensis* subsp. *israelensis* crystal proteins, there has been interest in employing *B. thuringiensis* subsp. *israelensis* as an agent for mosquito control. There are, however, two major difficulties encountered in attempting to employ *B. thuringiensis* subsp. *israelensis* as both an effective and environmentally safe biolarvicide. First, one protein component of the *B. thuringiensis* subsp. *israelensis* crystal, the *cytA* gene product, expresses hemolytic activity and has expressed cytolytic activity in the presence of cultured mammalian cells (7, 11, 17). Second, sprayed *B. thuringiensis* subsp. *israelensis* spores demonstrate a limited field life as insecticides; *B. thuringiensis* strains are not natural competitors in the aquatic environments in which all mosquitoes breed, and the spores quickly sink out of the upper few decimeters of lakes and streams, where most larval feeding occurs.

In order to overcome both of these difficulties and to present feeding larvae with a continuous pesticide exposure, there has been interest in transferring individual genes coding for nonhemolytic mosquito-toxic *B. thuringiensis* subsp. *israelensis* proteins into bacterial species that could more successfully compete in aquatic larval feeding locales. We believe that the cyanobacterium *Agmenellum quadruplicatum* PR-6 (*Synechococcus* sp. strain PCC 7002) is an attractive candidate for such a role. Most important, mosquito larvae readily feed on this unicellular cyanobacterial species (15a). PR-6 cells have limited nutritional requirements, and, as photoautotrophs, they remain accessible to larvae in the upper level of aquatic habitats. The species is adaptable to both fresh- and saltwater environments and has a wide temperature tolerance. It is readily transformed with chromosomal or plasmid DNA, and an expression plasmid has been developed for the expression of foreign genes by it.

Several proteins assemble to form *B. thuringiensis* subsp. *israelensis* crystals (10). The *cytA* gene product is a 27-kDa protein that shows little or no larvicidal activity but, as mentioned above, is hemolytic. Although none of the other crystal proteins expresses hemolytic activity, each has been found to exert larvicidal effects on at least some mosquito species (5, 7). The *cryIVD* (we follow here the terminology suggested by Höfte and Whiteley [10]) gene has most recently been cloned and sequenced (8). A major component of the *B. thuringiensis* subsp. *israelensis* crystal complex, the *cryIVD* gene product is a 72-kDa protein that has been shown to be highly toxic to mosquito larvae (8).

We report here the first instance of a genetically altered cyanobacterium that is lethal to mosquito larvae feeding directly on living cyanobacterial cells. High expression levels of the *B. thuringiensis* subsp. *israelensis* CryIVD protein, as determined by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis, were obtained in PR-6. The *cryIVD* gene was introduced into the cyanobacterium on a plasmid in which the *cryIVD* gene was translationally fused to the initial coding sequence of the highly expressed PR-6 *cpcB* gene. Thus, *cryIVD* expression is under direct control of the strong PR-6 phycocyanin operon promoter in the altered strain.

MATERIALS AND METHODS

Strains, culture conditions, and transformation. The unicellular cyanobacterium *A. quadruplicatum* PR-6 was main-

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tained in liquid culture and on 1.5% agar plates in medium A as described previously (16). Following transformation, PR-6 cells were adapted to 0% NaCl via successive liquid subculturings in medium A containing 0.3% stepwise reductions in NaCl concentrations (modified medium A). Transformation of PR-6 was carried out as described by Stevens and Porter (16). Transformants were selected and maintained on media containing ampicillin at concentrations of 2.0 and 4.0 μg/ml, respectively. All PR-6 transformations were verified by back-transforming *Escherichia coli* with PR-6 plasmid isolates and subsequent restriction analysis of plasmids recovered from the *E. coli* transformants.

E. coli high-subcloning-efficiency HB101 competent cells were obtained from BRL Life Technologies, Inc., and were used for all *E. coli* plasmid transformations according to the protocol provided. *E. coli* transformants were selected and maintained on 50 and 100 μg of ampicillin per ml, respectively, in Luria-Bertani medium.

Plasmids. Plasmid pEG218 is a pBR322 derivative harboring the entire *B. thuringiensis* subsp. *israelensis cryIVD* gene (8). Plasmid pAQE19ΔSal is a derivative of the *E. coli*:PR-6 biphasic expression vector pAQE19LPC (4). Plasmid pAQE19ΔSal, which is based on a fusion between the *E. coli* plasmid pBR322 and the smallest indigenous PR-6 plasmid pAQ1, provides both *E. coli* and PR-6 with resistance to the antibiotics ampicillin and kanamycin. Plasmid pAQE19ΔSal also carries the promoter region for the PR-6 phycocyanin operon. A more detailed description of this plasmid is given in the legend to Fig. 1 and in Results.

Polymerase chain reaction (PCR) and DNA manipulations. Amplification of the *B. thuringiensis* subsp. *israelensis cry IVD* gene with added flanking restriction sites was done by the procedure of Saiki et al. (15) by employing a DNA thermal cycler and a DNA amplification reagent kit with *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). Oligonucleotide primers were synthesized on an LKB-Pharmacia Gene Assembler Plus synthesizer. Amplification was allowed to proceed for a total of 25 thermal cycles, with a minimum hybridization temperature of 55°C. Oligonucleotide primers employed for amplification of the *cryIVD* gene were a 45-mer 5′-CGCAGCTCGCGTCGACTCCCGGGTG GAAGATAGTTCTTTAGATAC-3′ containing a *Sal*I restriction site (nucleotides [nt] 11 to 16) and the second through twenty-third nucleotides of the *cryIVD* protein coding sequence (nt 24 to 45) and a 40-mer 5′-CGATGC TCGCGACAAGTCGACTCACTCCTCTTGTGCTAAC-3′ containing a *Sal*I restriction site (nt 16 to 21) and 21 nt (nt 20 to 40) complementary to sequence downstream from the *cryIVD* termination codon (nt 2000 to 1980 of Donovan et al. [8]).

DNA-modifying enzymes and agarose gel electrophoresis were employed by using standard procedures (13). Restriction endonucleases and T4 DNA ligase were purchased from BRL Life Science Technologies, Inc.; calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals. Small-scale *E. coli* plasmid preparations were done by alkaline lysis (3). Small-scale plasmid preparations from PR-6 were done as previously described (14).

Crude extract preparations and SDS-PAGE. PR-6 cells harboring either pAQE19ΔSal or pAQRM56 were grown in liquid modified (0% NaCl) medium A with 4.0 μg of ampicillin per ml to early stationary phase. One-milliliter aliquots were frozen at −20°C and thawed at room temperature. Cells were harvested by centrifugation, resuspended in lysis solution (50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA), and allowed to incubate for 5 min at room temperature in the presence of 5 mg of hen egg white lysozyme (Sigma) per ml. The resulting spheroplasts were microcentrifuged for 1 min at 14,000 × *g*, and the pellet was washed once with 0.1 ml of lysis solution before being resuspended in 0.05 ml of 1 mM phenylmethylsulfonyl fluoride–10 mM EDTA–2% SDS. The samples were frozen at −80°C for 10 min and thawed at 37°C, and the suspension was twice sonicated for 5 s. NaOH was added to 0.05 N, and extracts were maintained at 37°C for 30 min just prior to the addition of 3.3 volumes of dissociation buffer (60 mM Tris-Cl, pH 6.8; 100 mM dithiothreitol; 2% SDS; 16% glycerol; 0.01% bromophenol blue). Immediately following the addition of dissociation buffer, samples were placed into a boiling water bath for 2 min and cooled to room temperature before 20-μl samples were loaded into gel wells. Samples were separated by electrophoresis through a 1.5-mm-thick 7.5 to 15% linear gradient polyacrylamide gel (acrylamide/bis-acrylamide, 30:0.8) as described by Laemmli (12) on an LKB 2050 midget electrophoresis unit.

Western blotting and immunostaining. Electrophoretic blotting of polypeptides fractionated by SDS-PAGE onto an Immobilon (Millipore) transfer membrane was carried out by the procedure of Towbin et al. (18). After blotting, the transfer paper was immunostained by the procedure suggested by Amersham for immunogold silver staining with their Auroprobe BLplus gold-labelled goat anti-mouse immunoglobulin G plus immunoglobulin M (H + L) as secondary antibody. Mouse antisera raised against the *B. thuringiensis* subsp. *israelensis* CryIVD protein (a gift of William Donovan, Ecogen Corporation) was diluted 500-fold prior to use.

Larvicidal assays. Adult female *Culex pipiens* mosquitoes were obtained locally and allowed to lay eggs in a glass petri dish containing 40 ml of distilled water. After hatching, larvae were separated and placed into individual microtiter wells in 96-well culture dishes; each well contained 0.25 ml of distilled water and one larva. Between feedings, culture dishes were covered with plastic wrap to reduce evaporation. PR-6 cultures were grown to stationary phase, harvested by centrifugation, washed once in modified medium A, and restored to their original volume in fresh modified medium A. At each feeding, all live larvae were provided with 3.0 to 5.0 μl of the washed PR-6 cells. Feeding occurred at 12-h intervals, with alternate wells in each microtiter plate receiving control (wild-type or pAQE19ΔSal-bearing PR-6 cells) or pAQRM56-bearing PR-6 cells. Just prior to each feeding, all larvae were observed in situ under a dissection microscope to check for viability and, if the larva was alive, the general condition of the larva.

RESULTS

Construction of a PR-6 expression plasmid bearing a *cpcB-cryIVD* translational gene fusion. Plasmid pAQE19ΔSal is an *E. coli*:PR-6 biphasic expression vector that carries the promoter and initial protein coding sequence of the PR-6 phycocyanin β (*cpcB*) subunit (Fig. 1). A multiple cloning site containing *Sma*I, *Bam*HI, and *Sal*I restriction sites is located immediately following the initial six codons of the *cpcB* coding sequence. A 2.0-kbp PCR DNA fragment bearing the entire *cryIVD* gene was generated from plasmid pEG218 by employing oligonucleotide primers based on the known *cryIVD* gene sequence. Both primers also carried additional 5′ nucleotides bearing *Sal*I endonuclease restriction sites; the *Sal*I site on the primer bearing the 5′ portion of the *cryIVD* gene was located such that ligation between the

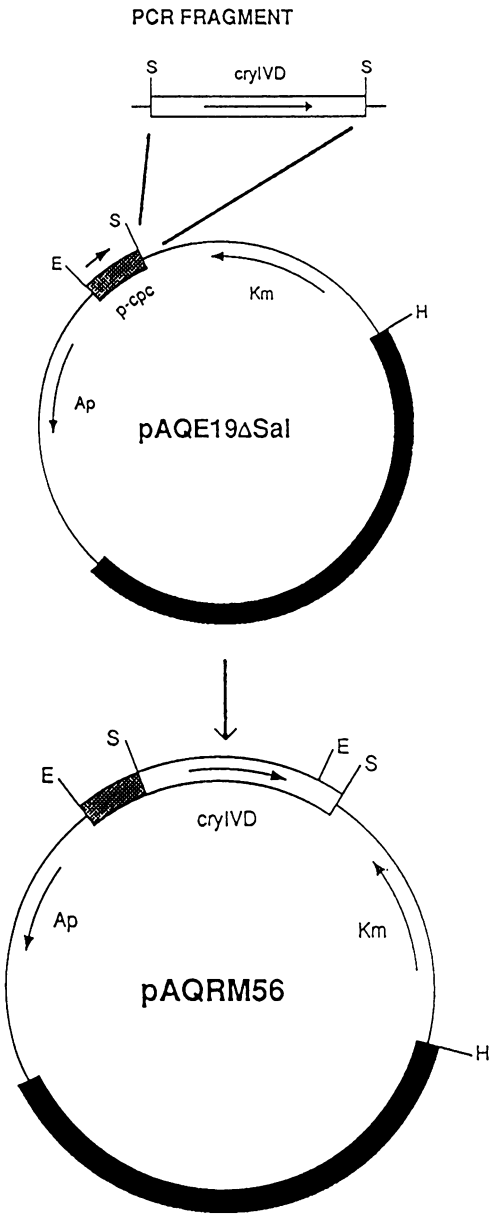

FIG. 1. Construction of plasmid pAQRM56. *Sal*I-restricted PCR DNA fragment harboring the *B. thuringiensis* subsp. *israelensis* *cryIVD* gene was ligated with *Sal*I-restricted pAQE19ΔSal plasmid. Thick solid lines indicate PR-6 plasmid pAQ1 sequence; shaded thick lines (p-cpc) indicate regions carrying the PR-6 phycocyanin operon promoter and initial *cpcB* coding sequence. Arrows give location and orientation of genes coding for ampicillin and kanamycin resistance. Abbreviations: S, *Sal*I; E, *Eco*RI; H, *Hin*dIII.

*Sal*I-restricted PCR fragment and plasmid pAQE19ΔSal, which carries a single *Sal*I site in its multicloning region, would produce the desired in-frame *cpcB-cryIVD* gene fusion (Fig. 2). *Sal*I-restricted pAQE19ΔSal was treated with calf intestinal alkaline phosphatase prior to ligation with the PCR-generated fragment in order to prevent self-ligation of the plasmid.

E. coli cells transformed with the ligation product were selected on ampicillin. Plasmid DNA isolated from selected transformants was subjected to restriction analysis to verify both insertion of the 2.0-kbp *Sal*I fragment bearing the *cryIVD* gene and its proper orientation in plasmid pAQE19ΔSal (data not shown). Two such plasmids, designated pAQRM56a and pAQRM56b, were subsequently employed to transform PR-6. Although both transformed cultures were used in expression and larvicidal assays, there was no discernible difference between the two PR-6 cultures and the two plasmids were subsequently designated pAQRM56.

Expression and immunological studies. The predicted molecular mass of the *cpcB-cryIVD* gene fusion product is 74 kDa. In an attempt to determine whether expression of the protein occurred in transformed PR-6 cells, whole-cell extracts were prepared from cultures of cyanobacterial cells harboring either pAQRM56 or the control plasmid pAQE19ΔSal. The polypeptides were size fractionated via SDS-PAGE, and the gel was stained with Coomassie brilliant blue. The presence of a 67-kDa polypeptide is clearly evident from extracts of those PR-6 cells harboring plasmid pAQRM56, whereas no such polypeptide is seen in those cells harboring the control plasmid (Fig. 3, lanes 2 and 3). These results are consistent with previous reports that the 72-kDa CryIVD protein shows an apparent molecular mass of 65 to 68 kDa on SDS-polyacrylamide gels.

Antibodies raised against the *B. thuringiensis* subsp. *israelensis* CryIVD protein were employed to determine whether the observed 67-kDa polypeptide produced in the cyanobacterial cells was antigenically related to the CryIVD protein. Following immunostaining of SDS-PAGE-size-fractionated polypeptides blotted onto a transfer membrane, a single strong signal coincidental with the Coomassie brilliant blue-stained 67-kDa polypeptide was observed in those PR-6 cells harboring plasmid pAQRM56; no signal was detectable in PR-6 cells carrying the control plasmid (Fig. 3, lanes 4 and 5). Both the observation that PR-6 cells harboring plasmid pAQRM56 produce a polypeptide not observed in extracts of PR-6 cells carrying plasmid pAQE19ΔSal and the finding that this polypeptide retains the antigenic integrity of the *B. thuringiensis* subsp. *israelensis* CryIVD protein argue strongly for the notion that these cyanobacterial cells are in fact expressing the *cpcB-cryIVD* gene fusion provided by the presence of plasmid pAQRM56.

Larvicidal assays. *C. pipiens* mosquito larvae hatched in the laboratory were observed to readily ingest both wild-type and pAQE19ΔSal-bearing PR-6 cells and were found to be able to pupate and emerge as adults when these cyanobacteria were provided as the sole food source. In order to determine whether cyanobacterial cells expressing the *cpcB-cryIVD* gene fusion product could prove to be lethal to mosquito larvae feeding on the cells, newly hatched mosquito larvae were separated and fed either wild-type or pAQRM56-bearing PR-6 cells as described in Materials and Methods.

Initially, the newly hatched *C. pipiens* larvae readily ingested both wild-type and pAQRM56-bearing PR-6 cells. However, within 2 days those larvae feeding on the pAQRM56-bearing cells began to stop feeding, became sluggish and nonresponsive to physical probing, and displayed internal structural deformities in the midgut region that occasionally resulted in complete disruption of the body cavity. Although microscopic observation revealed that many larvae remained alive in such a state for several days, all of these larvae died within 6 days of hatching (Fig. 4). In contrast, more than 90% of the control larvae remained alive, continuing to feed and maintain a healthy appearance throughout the same period. A similar experiment (terminat-

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5'——cpcB————————                                    5'——cryIVD————————3'
ATG TTT GAT ATT TTT ACC CGG GGA TCC GTC GAC TCC CGG GTG GAA GAT AGT TCT TTA

MET PHE ASP ILE PHE THR arg gly ser val asp ser arg val GLU ASP SER SER LEU
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FIG. 2. Translational *cpcB-cryIVD* gene fusion sequence of plasmid pAQRM56. *Sal*I endonuclease restriction site of fusion between *cryIVD*-bearing PCR fragment and plasmid pAQE19ΔSal is indicated by nucleotides in boldface type. Nucleotides under cpcB and cryIVD represent PR-6 phycocyanin β subunit and *B. thuringiensis* subsp. *israelensis cryIVD* sequence, respectively.

ed after 4 days) utilizing pAQE19ΔSal-bearing PR-6 cells as controls yielded almost identical results; 4 days after hatching, 94% (90 of 96) of larvae fed pAQE19ΔSal-bearing cells were alive versus 51% (49 of 96) of larvae fed pAQRM56-bearing cyanobacterial cells. In both experiments, it was noted that those control larvae that died did not present symptoms similar to those observed for larvae fed the pAQRM56-bearing cyanobacteria. Rather, these larvae appeared generally healthy and active at one feeding but were found dead 12 h later.

DISCUSSION

We have described here a mosquitocidal cyanobacterium created by the introduction of a gene encoding a mosquito-toxic *B. thuringiensis* subsp. *israelensis* protein into PR-6. The *B. thuringiensis* subsp. *israelensis cryIVD* gene was translationally fused to the PR-6 *cpcB* gene on a biphasic plasmid and, upon introduction into the cyanobacterium, was apparently expressed under the direct control of the strong PR-6 phycocyanin operon promoter.

Expression of the *cpcB-cryIVD* gene fusion product was easily observed following Coomassie blue staining of fractionated PR-6 cell extract proteins on an SDS-polyacrylamide gel. Although the predicted molecular mass of the gene fusion product is 74 kDa, the protein appeared to run at approximately 67 kDa on the gel. This is consistent with previous reports that the 72-kDa *B. thuringiensis* subsp. *israelensis* protein shows an apparent molecular mass of 65 to 68 kDa as determined by SDS-PAGE. The reason for this discrepancy, whether a specific cleavage event or anomalous SDS binding, is not known. Certainly neither SDS-PAGE nor Western blot analysis gave any indication of gross degradation of the CryIVD protein in the cyanobacterial cell extracts. Immunostaining of blotted proteins with antiserum raised against the CryIVD protein revealed a single, very clean band. Upon severe overstaining, immunoblots showed secondary bands that correlated in general with banding patterns observed for Coomassie blue-stained gels. However, close examination of the CryIVD Coomassie blue-stained band reveals it to be in fact two very close but distinct bands. The fact that both bands were observed from different extracts on multiple gels indicates that their appearance is not due to either sample or gel artifacts. The possibility that these two bands represent the whole *cpcB-cryIVD* fusion product vis-à-vis one processed via breakage at the fusion point exists but awaits confirmation.

The high expression level and lack of degradation observed for the CryIVD protein in PR-6 are perhaps surprising in view of the results reported from previous attempts to introduce *B. thuringiensis* subsp. *israelensis* genes encoding mosquito-toxic proteins into cyanobacterial species. Angsuthanasombat and Panyim (1) have introduced the 130-kDa *B. thuringiensis* subsp. *israelensis* endotoxin (*cryIVB*) gene

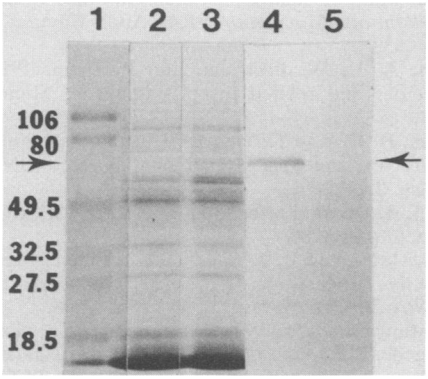

FIG. 3. Expression studies with PR-6 cells harboring plasmids pAQE19ΔSal and pAQRM56. Coomassie brilliant blue-stained polypeptides of whole-cell extracts separated by PAGE in the presence of SDS are shown in lanes 2 and 3. Whole-cell extracts were prepared from PR-6 bearing plasmids pAQE19ΔSal (lane 2) and pAQRM56 (lane 3). The 67-kDa polypeptide apparently expressed from plasmid pAQRM56 is indicated by the arrows on both sides of the figure. Identically loaded but unstained lanes from the same gel were electroblotted onto an Immobilon transfer membrane. Blotted polypeptides from PR-6 bearing pAQRM56 (lane 4) and pAQE19ΔSal (lane 5) were exposed to mouse polyclonal antiserum directed against the *B. thuringiensis* subsp. *israelensis* CryIVD protein and immunostained via silver amplification of gold-labelled goat anti-mouse antibody. Relative mobilities of standards (lane 1) are represented in kilodaltons.

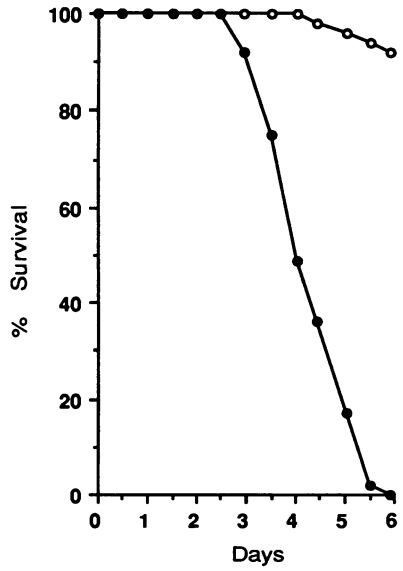

FIG. 4. Survival rates of *C. pipiens* mosquito larvae fed either wild-type (○) or pAQRM56-bearing (●) PR-6 cells. Larvae hatched at time zero were placed into separate microtiter wells for feeding as described in Materials and Methods. Total larva number represented is 191; 96 larvae were fed wild-type cells and 95 larvae were fed transformed PR-6 cells.

into PR-6, and Chungjatupornchai (6) has reported integrating the *cryIVD* gene into the *Synechocystis* sp. strain PCC 6803 chromosome. In both cases, expression levels were deemed too low for toxic effects to be observed on larval feeding, while immunostaining of gel-fractionated cyanobacterial extracts revealed degradation of the CryIVB protein. The low expression levels observed for *cryIVB* in PR-6 are particularly interesting, as the expression plasmid employed was the same as in this study; however, introduction of the *cryIVB* gene was based on a transcriptional fusion, whereas the work described here employed a translational gene fusion. The apparent difference between expression of translational gene fusions versus that of intact *B. thuringiensis* subsp. *israelensis* genes in non-*B. thuringiensis* subsp. *israelensis* species has been previously noted (10) but remains unexplained. More understandable perhaps is the difference in degradation observed for the CryIVB and CryIVD proteins expressed in cyanobacterial cells. Donovan et al. (8) have reported that *Bacillus megaterium* cells carrying the cloned *cryIVD* gene harbored crystallike inclusions composed of the 72-kDa CryIVD protein, and we have observed phase-bright objects in pAQRM56- but not pAQE19ΔSal-bearing PR-6 cells. As Chungjatupornchai (6) reported that the 130-kDa CryIVB protein did not form inclusions in *Synechocystis* sp. strain PCC 6803, it seems possible that the difference in degradation observed between CryIVB and CryIVD proteins in the respective cyanobacterial strains may be due to the protective association of CryIVD proteins in crystal formations. If this is indeed the case, the ability of CryIVD crystal formation to maintain the proteins' integrity and, hence, toxicity could prove to be a decided advantage in employing the *cryIVD* gene over other *B. thuringiensis* subsp. *israelensis* mosquito-toxic genes for creating genetically engineered biolarvicides.

B. thuringiensis subsp. *israelensis* crystal proteins have been shown to be toxic to mosquito larvae of the *Culex*, *Aedes*, and *Anopheles* genera (9). We have reported here that PR-6 cells harboring plasmid pAQRM56 and expressing the *B. thuringiensis* subsp. *israelensis cryIVD* gene are toxic to *C. pipiens* mosquitoes, and preliminary results in our laboratory indicate the cells to be at least as toxic to *Aedes aegypti* larvae (data not shown). As with larvae fed wild-type or pAQE19ΔSal-bearing PR-6 cells, freshly hatched *C. pipiens* larvae readily consumed the PR-6 cells harboring plasmid pAQRM56; the distinctive blue-green color of cyanobacterial cells was very apparent in the larval midgut shortly after the first feeding, and it was quite easy to mark the progression of the cells up the midgut tract. However, unlike the former, which continued to feed and remain active, all of those larvae feeding on cyanobacterial cells expressing the *cryIVD* gene eventually stopped feeding and later died. When the larvae were viewed under magnification, distortions of the midgut region in these larvae were quite striking, supporting previous findings that the *B. thuringiensis* subsp. *israelensis* proteins primarily affect the epithelial cells of this region. Also interesting was the observation that although test larvae were fed only PR-6 cells expressing the *B. thuringiensis* subsp. *israelensis* protein, none of the symptoms eventually observed (e.g., cessation of feeding, lethargy, and midgut distortion) appeared immediately but rather were observed in the larvae only after a lag period of roughly 2 to 3 days. The reason for this apparent delay in toxicity is not presently known. The use of independently derived clones limits but does not preclude the possibility that mutations were introduced into the *cryIVD* portion of the gene fusion during the PCR step. It is also possible that the 14 N-terminal amino acids resulting from the fusion of the *cpcB* and *cryIVD* genes might have a deleterious effect on the toxicity of the *cryIVD* moiety. Donovan et al. (8) have reported that *B. megaterium* cells harboring the *cryIVD* gene have a 24-h 50% lethal dose of about 5 μg of cells (wet weight) per ml in *A. aegypti*. We have obtained similar results in studies with PR-6 cells harboring the *cpcB-cryIVD* gene fusion with *C. pipiens*. However, our studies of the 50% lethal dose for cyanobacterial cells carrying pAQRM56 have been complicated by two factors. First, the protein products of the *cpc* genes are functional parts of the phycobilisome, which is the major light-harvesting antenna of cyanobacteria. We are currently investigating the effects of culture conditions on expression of the *cpcB-cryIVD* fusion product in PR-6 cells, as expression from the *cpc* operon promoter is likely to vary with cell density and light availability. Second, even PR-6 cells harboring the gene for β-lactamase expression show a very low tolerance (2 to 4 μg/ml) for ampicillin. Thus, comparative plating studies in our laboratory indicate that only a fraction of cells grown in liquid culture may actually be carrying the pAQRM56 plasmid. We are currently working on a new plasmid construction that will not require ampicillin selection of the expression plasmid in PR-6 cells.

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