Development of a Cyanobacterial Biolarvicide

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Results of studies on a larvicidal cyanobacterium that expresses a Bti cryIVD *gene fusion are reported. Genetically altered* Agmenellum quadruplicatum *PR-6 is shown to be toxic to larvae of three major genera of disease-bearing mosquitos. Factors affecting expression of Bti genes in cyanobacteria are discussed.*

Key words: *Agmenellum quadruplicatum* - *Synechococcus* - cyanobacteria - *Bacillus thuringiensis* var. *israelensis* - mosquitocidal - larvacide

The cyanobacteria represent the largest and most diverse group of the photosynthetic bacteria (Stanier & Cohen-Bazire 1977). They are found in virtually every sunlit aquatic environment on earth, and various species represent a significant portion of mosquito larvae diets. These facts, along with the acknowledgement that current *Bacillus thuringiensis* subsp. *israelensis* (Bti)-based larvicides, while potent, express limited field lifetimes, have led us and others (de Marsac et al. 1987, Angsuthanasombat & Panyim 1989, Chungjiatupornchai 1990, Murphy & Stevens 1992, Soltes-Rak et al. 1993, Xudong et al. 1993, Stevens et al. 1994) to become interested in developing certain cyanobacteria as biolarvicides for targeting specific toxins to mosquito larvae. Two factors have been especially helpful in this regard. The first is that several cyanobacterial species have been sufficiently developed as genetic models such that methods are available for genetically manipulating them to accept and express foreign DNA. The second is that the most effective current means of controlling mosquitos consists of several unmodified proteins produced by a *Bacillus* bacterium. These factors suggest it would be feasible to transfer a *Bacillus* mosquito toxin gene into a natural food source cyanobacterium, and in so doing produce an effective biolarvicide for the control of mosquitos. Our efforts have focussed on expressing the Bti *cryIVD* gene in the cyanobacterium *Agmenellum quadruplicatum* PR-6 (PR-6).

Since the discovery of its larvicidal properties in the late 1970's, Bti has become a preferred agent for mosquito control in many countries (Goldberg & Margalit 1977, de Barjac 1978, Margalit & Dean 1985). The bacterium's acceptance as a method for controlling these insects is based largely on the selective nature of its killing activity, which appears limited to larvae of the dipteran order. Bti's larvicidal activity is due to a family of proteins which are produced and assembled into parasporal crystalline inclusion bodies during the bacterium's sporulation cycle (Hofte & Whiteley 1989, Federici et al. 1990). Upon ingestion by larvae, the crystal proteins are solubilized in the larval midgut where they act to disrupt the epithelium cells of the larval midgut region. Although comparative toxicity studies of the four major crystalline proteins have shown widely varying estimates for their absolute and even relative LD_{50}'s, the accumulating evidence seems to indicate that the *cryIVD* gene product is at least as toxic, and probably more so, than the other crystalline proteins (Federici et al. 1990).

Larval feeding studies in which *Aedes aegypti* larvae were allowed to feed and develop on a diet of various cyanobacterial species indicated the unicellular cyanobacterium *A. quadruplicatum* PR-6 as a preferred food source for the larvae (Stevens et al. 1994). Similar in structure and genetic complexity to that of other gram-negative bacteria, PR-6 possesses several attributes that make the species especially suitable to genetic manipulation and analysis. Among these attributes PR-6 lists a relatively short generation time, the ability to be maintained in the laboratory on both solid and liquid defined medium, and a natural transformation ability which allows the species to take up both linear and whole plasmid DNA from its environment. Several biphasic shuttle vectors, based on a fusion between the *Escherichia coli* pBR322 plasmid and pAQ1, the smallest indigenous PR-6 plasmid, have been developed to exploit the latter property (Buzby et al. 1983). These vectors allow for cloning and general genetic manipulations to be car-

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ried out in *E. coli* strains prior to their introduction and maintenance in the cyanobacterium.

We report here results of studies on a larvicidal cyanobacterium that expresses the Bti *cryIVD* gene translationally fused to the highly expressed PR-6 *cpcB* gene. The cyanobacterium is shown to be toxic to larvae of the three major genera of disease-bearing mosquitos. Factors that may affect expression of Bti genes in cyanobacteria are discussed.

MATERIALS AND METHODS

Strains, culture conditions and transformation - *A. quadruplicatum* PR-6 (*Synechococcus* PCC 7002) was maintained in liquid culture and on 1.5% w/v agar plates in medium A as previously described (Stevens & Porter 1980). Plasmid isolation, purification and transformations of *E. coli* and PR-6 were performed as descibed previously (Murphy & Stevens 1992).

PCR and DNA manipulations - Polymerase chain reaction (PCR) amplification of the Bti *cryIVD* gene with added flanking sequences employed the procedure of Saiki et al. (1985) and has been previously described (Murphy & Stevens 1992). DNA modifying enzymes and agarose gel electrophoresis were employed using standard procedures.

Larvicidal assays - Larvicidal assays of *Culex pipiens* and *Ae. aegypti* feeding on transformed PR-6 have been previously described (Murphy & Stevens 1992). Larvicidal assays of *C. quinquefasciatus, Ae. taeniorhynchus* and *A. quadrimaculatus* were performed as follows. Ninety-six freshly hatched larvae of each species were separated and placed into the individual microtiter wells of a 96-well culture dish containing 0.25ml of distilled water. Stationary phase PR-6 cultures were harvested by centrifugation and washed with modified medium A (Murphy & Stevens 1992). Feeding occurred at 12 hr intervals, with alternate wells in each microtiter plate receiving either control (pAQE19ΔSal) or pAQRM56-bearing PR-6 cells. Prior to each feeding, all larvae were observed *in situ* under a disection microscope to check for larva viability.

DNA analysis software - An Intelligenetics DNA sequence analysis program "SEQ.COD" was employed to analyze the codon usage of all known PR-6 gene sequences recorded in either GENEBANK and/or EMBL (search names *Synechococcus* and *A. quadruplicatum*).

RESULTS

A major advance toward expressing foreign genes in PR-6 has been the development of a PR-6 expression plasmid based on PR-6/ *E. coli* biphasic shuttle vectors. Plasmid pAQE19 Δ Sal, a derivative of the *E. coli*: PR-6 biphasic expression vector pAQE19LPC (Buzby et al. 1990), provides both *E. coli* and PR-6 with resistance to the antibiotics ampicillin and kanamycin, and carries the promoter and initial protein coding sequence of the highly expressed PR-6 *cpcB* (phycocyanin β subunit) gene (de Lorimier et al. 1984). 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 polymerase chain reaction (PCR) DNA fragment bearing the entire Bti *cryIVD* gene was generated using primers derived from the known *cryIVD* sequence (Donovan et al. 1988). Additional nuclectides containing *Sal*I restriction sites were added to the 5' side of both primers such that restriction of the generated PCR fragment and its subsequent ligation with *Sal*I-restricted pAQE19Δ Sal would produce an in-frame *cpcB/cryIVD* translational gene fusion (Fig. 1). The resulting plasmid,

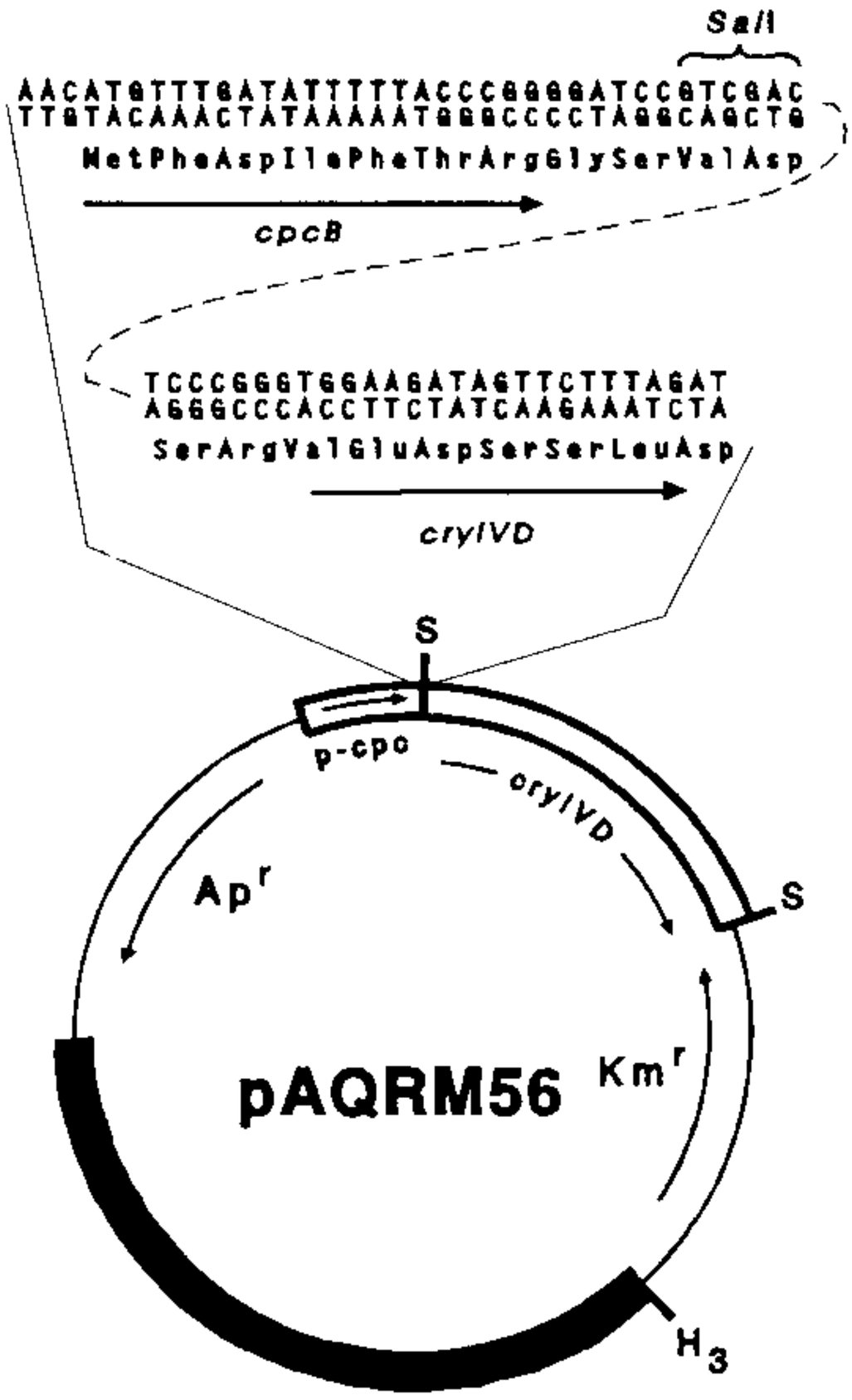

Fig. 1: plasmid pAQRM56 bearing a *cpcB/cryIVD* gene fusion. Thick solid line indicates PR-6 pAQ1 DNA sequence. Ap and Km represent ampicillin and kanamycin resistance-conferring genes, respectively. p-cpc represents the PR-6 *cpc* operon promoter and initial *cpcB* coding sequence. Restriction sites: S = *Sal*I, H3 = *Hind*III.

pAQRM56, was used to transform PR-6 cells for expression and larvicidal analysis.

Expression of the *cpcB/cryIVD* fusion product was detectable by both Coomassie blue and immunostaining following SDS-PAGE and western blotting, respectively, of PR-6 whole cell extracts bearing plasmid pAQRM56 (Murphy & Stevens 1992). When polypeptides of whole cell lysates from PR-6 cells bearing either pAQE19Δ Sal or pAQRM56 were size-fractionated via SDS-PAGE and stained with Coomassie blue, the presence of an extra 67-kDA polypeptide from the extracts of those PR-6 cells harboring plasmid pAQRM56 was clearly visible. This result is consistent with the finding that the *cryIVD* gene product shows an apparent molecular weight of 65 to 68-kDa on SDS polyacrylamide gels, and supports the notion that the *cpcB/cryIVD* gene fusion is being expressed in these cells. Further support for this notion was obtained from western blot analysis of the size-fractionated polypeptides from the PR-6 cell lysates. Antibodies raised against the Bti *cryIVD* gene product were employed to determine whether the observed 67-kDa polypeptide produced by pAQRM56-bearing cells was antigenically related to the CryIVD protein. Following immunostaining of SDS-PAGE size-fractionated polypeptides blotted onto transfer membrane, a single strong signal coinciding with the Coomassie blue-stained 67-kDa polypeptide was observed in those cells harboring pAQRM56 while no signal was detected from cells harboring the control plasmid (Murphy & Stevens 1992). Both the presence of an extra polypeptide with a molecular weight matching that predicted for the *cpcB/cryIVD* gene fusion product and the finding that this polypetide is antigenically related to the Bti CryIVD protein provided strong evidence that the *cpcB/cryIVD* gene fusion was being expressed in PR-6 cells harboring plasmid pAQRM56.

In order to determine whether PR-6 cells expressing the *cpcB/cryIVD* gene fusion product could be lethal to mosquito larvae feeding on the cells, laboratory hatched *C. pipiens* larvae were fed PR-6 cells bearing either plasmid pAQRM56 or the control plasmid pAQE19ΔSal. Initially, newly hatched larvae readily ingested pAQE19Δ Sal- and pAQRM56-bearing PR-6 cells, but within two days those larvae feeding on the pAQRM56-bearing cells began to stop feeding. Microscopic observation revealed severe midgut distortions in these larvae, all of which died within six days. In contrast, more than 90% of the control larvae remained alive, continuing to feed and maintain a healthy appearance throughout the same period. PR-6 cells expressing the *cpcB/cryIVD* gene fusion were toxic to *C. pipiens* mosquito larvae based on these results (Murphy & Stevens 1992). Subsequent experiments have

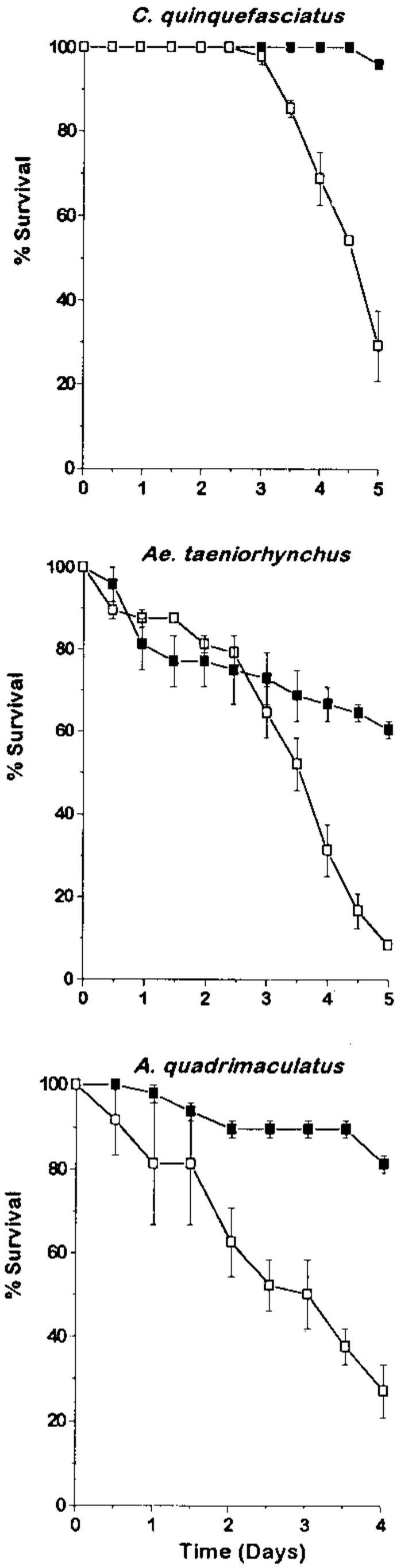

Fig. 2: survival rates of *Culex quinquefasciatus*, *Aedes taeniorhynchus* and *Anopholes quadrimaculatus* fed either pAQE19Δ Sal- (dark squares) or pAQRM56-bearing (open squares) PR-6 cells. Larvae hatched at time zero were placed into separate microtiter wells for feeding as described in Materials and Methods.

shown that pAQRM56-bearing PR-6 cells are also toxic to *Ae. aegypti* (Stevens et al. 1994), *Ae. taeniorhynchus, C. quinquefasciatus* and *A. quadrimaculatus* mosquito larvae (Fig. 2).

DISCUSSION

The results from our SDS-PAGE, western blot and larvae survival assays make clear that PR-6 cyanobacterial cells are expressing a larvicidal CryIVD moiety. A common theme to the larvicidal assays is the observation that several days are required for complete killing activity to be manifested. Whether this is a function of digestive rate or relatively low toxin dosage to the midgut is not known. Soltes-Rak et al. (1993) have found in their work with *Synechococcus* sp. PCC 7942 bearing a *cryIVB* gene fusion that increasing the cyanobacterial promoter efficiency can speed the rate of killing *Culex* larvae feeding on the cells. This supports our view that increasing toxin production in transformed cyanobacteria is possible and should increase their effectiveness as biolarvicides, and we are currently examining a number of methods for increasing expression of the *cpcB/cryIVD* gene fusion in PR-6.

The literature and our own observations indicate that several factors may be important in optimizing toxin production in transformed cyanobacteria. One seems to be the importance of employing translational gene fusions, continuing a trend first noticed for Bt gene expression in transgenic plants (Höfte & Whiteley 1989). We have also noticed, as have others, that the toxicity of Bti gene-bearing cyanobacterial cells can vary with culture conditions. Soltes-Rak et al. (1993) have reported for example, that the toxicity of cyanobacterial cells bearing the Bti *cryIVB* gene varied as a function of cell culture age. We have observed that PR-6 cells bearing *cryIVD* driven by the PR-6 *cpc* operon promoter are more toxic in stationary phase than during log growth. This may be a direct gene dosage effect, reflecting the fact that pAQ1-sized PR-6 plasmid levels tend to increase as the cells enter stationary phase (Roberts & Koths 1976). The design or discovery of a PR-6 plasmid exhibiting higher copy numbers could significantly increase the toxicity of cyanobacterial cells bearing plasmid-borne Bti genes.

The previously mentioned work of Soltes-Rak et al. (1993) demonstrates the potential of promoter manipulation as a means of increasing cyanobacterial toxicity. We have become more interested in the rate of translation as a possible limiting factor in cyanobacterial toxin protein production. It is now well established that different species prefer the use of certain codons over others for encoding certain amino acids. It is also known that the prevalence of any species' preferred codons within the coding sequence of a gene can be correlated with the expression levels of that gene; that is, the higher the expression level of a gene, the more prevalent the appearance of preferred codons within that gene's coding sequence (Ikemura 1981). Preferred codon usage analysis of PR-6, for example, indicates that the isoleucine-encoding AUA codon is rarely used (Table), while 19 AUA codons are found in the Bti *cryIVD* gene coding sequence. If PR-6 has difficulty translating through AUA codons, their prevalence in the foreign Bti gene could prove a substantial hindrance to cyanobacterial toxin production. Whether this is in fact happening can be tested by selectively altering codons in the cyanobacterial *cpcB/cryIVD* gene fusion, a project we are currently pursuing.

There are now several published reports on cyanobacterial species made toxic to mosquito

TABLE

Isoleucine-encoding codons in Pr-6

PR-6 gene	#AUU	#AUC	#AUA
cpcA	1	5	0
cpcB	3	5	0
cpcC	9	3	0
cpcD	3	1	0
cpcE	8	5	0
cpcF	7	8	0
glnA	17	7	0
gltX	4	3	0
isiA	12	7	0
isiB	6	4	0
M.Aqu1A	5	6	5
M.Aqu1B	4	1	0
mpeA	1	6	0
mpeB	3	6	0
ndhF	19	21	0
petA	9	13	0
petB	10	10	0
petC	4	5	0
petD	9	5	0
petF	3	3	0
petH	12	10	0
psaA	25	24	0
psaB	14	28	0
psaC	2	2	0
psaE	4	2	0
psbD2	9	8	0
recA	17	10	0
Total	220	208	5

larvae via transformation employing genes from Bti or *B. sphaericus* (Murphy & Stevens 1992, Soltes-Rak et al. 1993, Xudong et al. 1993). Considering the relatively short time cloned toxin genes have been available, results of research in this area show much promise that effective cyanobacterial larvicides can be developed. With the prospect of more genes and cyanobacterial expression systems becoming available, and as expression is enhanced through further manipulations at the genetic level, it seems not unreasonable to be optimistic that the future will bear out that promise.

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