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Mosquito Larvicidal Activity of Transgenic *Anabaena* Strain PCC 7120 Expressing Combinations of Genes from *Bacillus thuringiensis* subsp. *israelensis*

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Various combinations of the genes *cryIVA* (*cry4A*), *cryIVD* (*cry11A*), and *p20* from *Bacillus thuringiensis* subsp. *israelensis* were introduced into the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 by means of *Escherichia coli-Anabaena* shuttle vector pRL488p and were expressed under control of two tandem strong promoters, a cyanobacterial promoter (*PpsbA*) and an *E. coli* T7 promoter (P_{A1}). Two of the clones carrying *cryIVA* plus *cryIVD*, one with *p20* and one without *p20*, displayed toxicity against third-instar larvae of *Aedes aegypti* at levels greater than any level previously reported for transgenic cyanobacteria.

Bacillus thuringiensis subsp. *israelensis* has been used widely to control mosquitoes and blackflies, which are vectors of human infectious diseases (19). The larvicidal activity of this organism is contained in a parasporal crystalline inclusion (δ-endotoxin) synthesized during sporulation (7). A 75-MDa plasmid includes all of the genes responsible for δ-endotoxin production (4). *B. thuringiensis* subsp. *israelensis* is widely accepted as a biological pesticide because of its highly specific activity against dipteran insects without adverse effects on other organisms and because no resistance has been developed by the target mosquito larvae (14).

Current applications of *B. thuringiensis* subsp. *israelensis* for mosquito control are limited by the short half-life of existing preparations under field conditions (33). One way to overcome this limitation is by cloning the genes coding for the *B. thuringiensis* subsp. *israelensis* toxin in organisms inhabiting the breeding zones of mosquitoes and used by them as a food source (5, 27). Cyanobacteria have been considered attractive candidates for this purpose (5, 6, 33). Several attempts have been made during the last decade to produce transgenetic mosquitocidal cyanobacteria (2, 9, 17, 22, 23, 25, 31). Some success has been achieved in expressing single *cry* genes in unicellular strains, but the intact transformed cells displayed levels of mosquito larvicidal activity below the level required for effective and practical use as bioinsecticides in a natural aquatic environment (2, 9, 17, 22). Attempts to enhance expression of *cryIV* genes in cyanobacteria by strong inherent promoters (e.g., P_{psbA} [9] and P_{cpcB} [17]) were not satisfactory, probably because the transcription or translation products were unstable (22, 23). An engineered organism carrying a combination of toxin genes might be expected to be a substantial improvement due to possible synergistic interactions, such as those that occur in *Escherichia coli* (3). In addition, when more than one gene is responsible for larvicidal activity, the concern that resistance might develop in mosquitoes is diminished (14).

In the present study, combinations of the genes *cryIVA* (*cry4A*), *cryIVD* (*cry11A*), and *p20*, previously cloned and expressed in *E. coli* (3) as operons (in pUHE), were introduced into the nitrogen-fixing filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. The most toxic clones were selected for further study.

P20 improved the expression level of *cytA* (*cyt1Aa*), *cryIVA* (*cry4A*), and *cryIVD* (cry11A) in *E. coli* (1, 3, 28, 32) and in an acrystalliferous strain of *B. thuringiensis* (8, 30), probably by acting as a chaperonin for stabilizing the gene products. Synergism among purified CryIVA, CryIVB, and CryIVD was clearly demonstrated (10, 18), with the highest degree of synergism occurring between CryIVA and CryIVD.

Construction of shuttle vector for expression. Recombinants pHE4-ADR and pHE4-AD (containing *cryIVA* plus *cryIVD*, with and without *p20*, respectively [3]) were found by *Kpn*I-*Pst*I double digestion to contain a single *Kpn*I site located downstream of *cryIVA* (data not shown). This site was deleted to produce plasmids pHE4-ADR(−K) (Fig. 1) and pHE4-AD (−K), which were necessary for further construction (see below), by digesting pHE4-ADR and pHE4-AD with *Kpn*I and filling the sticky ends with T4 polymerase to form blunt ends, which were ligated by T4 DNA ligase. The other vector, pBS-SK/N, had been constructed previously to include a promoter and operator (P/O), a ribosome binding site (RBS), and an *Nco*I site by inserting a 135-bp *Pst*I-*Xho*I fragment from pUHE24-2 (3) into the polylinker of pBlueScriptII SK$^+$ (24). P/O is a repressible promoter derived from the early T7 promoter P_{A1} combined with two tandem *lacO* operators (3). DNA modification enzymes and restriction enzymes were used as recommended by the suppliers (New England BioLabs and United States Biochemical, respectively). Plasmids were introduced into *E. coli* HB101, DH5α, or XL-Blue MRF′ by transformation. Competent cells were prepared, and plasmids were isolated by standard procedures (20).

The *cryIVADR* and *cryIVAD* operons (containing *cryIVA* and *cryIVD*, with and without *p20*, respectively) were transferred from pHE4-ADR(−K) and pHE4-AD(−K) into pBS-SK/N by *Nco*I-*Pst*I double digestion to produce pSBJ1 and pSBW1, respectively, which were then united with pRL488p at their unique *Kpn*I sites (see above). The latter plasmid had been prepared by inserting a 1-kb *Sal*I-*Kpn*I fragment containing the strong constitutive promoter P_{psbA} from pRL435K (kindly sup-

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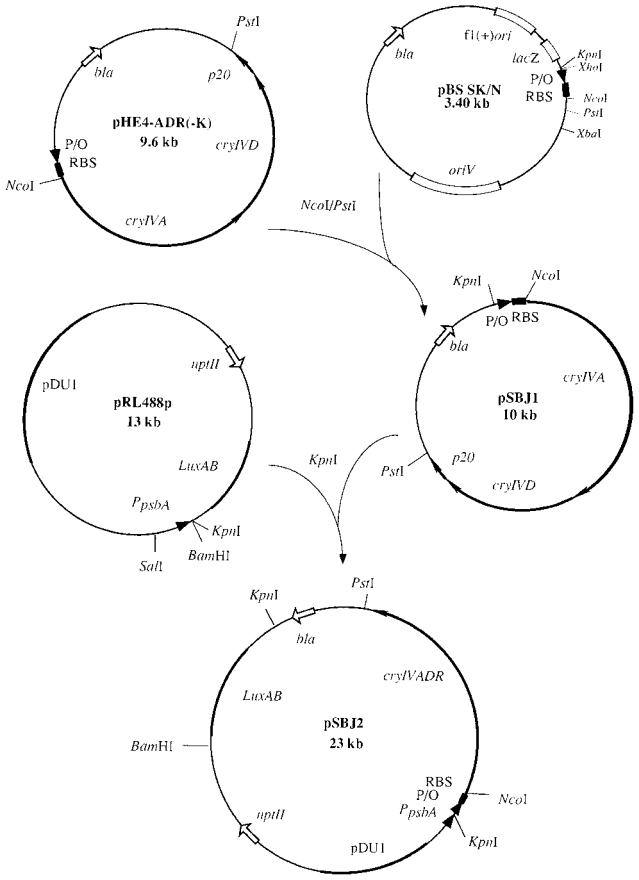

FIG. 1. The *cryIVADR* operon was transferred from pHE4-ADR(−K) to pBS-SK/N by *Nco*I-*Pst*I double digestion to form pBSJ1, which was then joined with expression shuttle vector pRL488p at its unique *Kpn*I site. The resulting hybrid recombinant plasmid was designated pSBJ2.

plied by Y. Hirshberg) into the shuttle vector pRL488 (13). The final constructs (about 23 and 22 kb long) with the *cryIVADR* and *cryIVAD* operons under control of the tandem promoters were designated pSBJ2 and pSBW2, respectively.

The clone with *cryIVA* alone was constructed from pHE4-ADR like pSBJ1 and pSBW1 were constructed, but without removal of the *KpnI* site (downstream of *cryIVA*) (see above), as follows. The *cryIVADR* operon was transferred from pHE4-ADR into pBS-SK/N by *Nco*I-*Pst*I double digestion to produce pSBJ1(+K). A 3.6-kb *Kpn*I fragment containing *cryIVA* (with P/O and a RBS) was isolated and inserted into the unique *Kpn*I site of pRL488p to create pSBJ3 (16.6 kb).

Similarly, pSB4-D, pSB4-DR, and pSB4-A were constructed by joining pUCE4-D, pUCE4-DR, and pUCE4-A (3), respectively, with pRL488p at their unique *Kpn*I sites. These plasmids contained a single promoter, P_{psbA}, and were used as control constructs for comparison.

Introduction of *cryIV* genes into *Anabaena* sp. strain PCC 7120. The recombinant shuttle expression vectors containing *cryIV* genes were introduced into *Anabaena* sp. strain PCC 7120 by triparental conjugation mating with *E. coli* (12). *E. coli* HB101 harboring each of the constructed plasmids and helper plasmid pRL528 (12) was mixed with *E. coli* J53 containing conjugal plasmid RP4 and with *Anabaena* sp. strain PCC 7120 pregrown in BG11 liquid medium at 28°C without stirring under cool white fluorescent light illumination. The mixed suspensions were spread onto nitrocellulose membranes over BG11 agar plates. After 24 h of incubation, the membranes were transferred to selective plates containing neomycin (25 μg ml^{-1}), and colonies that formed on the membranes about 10 days later were inoculated into 5 ml of BG11 medium supplemented with neomycin. After 1 week, when true exconjugant clones had grown, each clone was inoculated into a flask and cultivated under the same conditions. Among the approximately 100 clones tested (see below), two independent pSBJ2 clones carrying *cryIVADR* (designated clones 7 and 11) were found to be toxic to larvae of *Aedes aegypti*. Similarly, four pSBW2 clones containing *cryIVAD* (designated clones 1, 2, 5, and 6) and one pSBJ3 clone containing *cryIVA* (designated clone 16) were selected for further study. The clones were then freed of contaminating *E. coli* by repeated streaking on agar plates and were shown to grow well in BG11 liquid medium containing neomycin, as measured by the concentration of chlorophyll in methanol extracts (16).

Mosquito larvicidal activities. For bioassays, cells (of *E. coli* DH5α and *Anabaena* sp. strain PCC 7120) without *lacI* were cultivated in the appropriate liquid medium, harvested by centrifugation, and resuspended in distilled water. Samples were then added together with 10 or 20 third-instar larvae of *Aedes aegypti* to disposable cups containing 50 or 100 ml of sterile tap water, respectively, and the larvicidal activities were determined after incubation for 24 h at 28°C (3, 15). The concentrations of cells which killed 50% of the exposed population in a standard bioassay (LC_{50}) were determined by probit analysis by performing duplicate bioassays at each of six doses.

Six clones of *Anabaena* sp. strain PCC 7120, two carrying *cryIVADR* (pSBJ2) and four carrying *cryIVAD* (pSBW2), exhibited very high toxicity against *Aedes aegypti* larvae, as did their *E. coli* counterparts harboring the same plasmids (Table 1). The LC_{50} of clones containing *cryIVADR* and *cryIVAD* were equal to or less than 9×10^4 cells ml^{-1} (0.8 μg of total soluble protein ml^{-1}) at 24 h and 3×10^4 cells ml^{-1} (0.27 μg of total soluble protein ml^{-1}) at 48 h, which is the lowest reported value for engineered cyanobacterial cells with *B. thuringiensis* subsp. *israelensis* toxin genes (2, 9, 17, 22, 23).

Even though P_{psbA} was reported to be a strong cyanobacterial promoter (11, 26), construct pSB4-A with this promoter alone was much less toxic than pSBJ3 with two tandem promoters (P_{psbA} and P_{A1}) (Table 1). Similarly, neither pSB4-D nor pSB4-DR showed any toxicity. The phenomenon of improved expression of *cry* genes with tandem promoters has been described previously (22).

Plasmid recovery and stability. Plasmid pSBJ2 was isolated from several colonies of *E. coli* XL-Blue MRF′ cells transformed with total DNA prepared from clone 11. Selection of transformed cells was done on Luria-Bertani agar plates containing ampicillin and kanamycin (each at a concentration of

TABLE 1. Toxicities of recombinant clones with combinations of *cryIV* genes against third-instar *Aedes aegytpti* larvae

Plasmid	Gene(s)	LC_{50} (10^5 cells ml^{-1}) in[a]:	
		E. coli	*Anabaena* sp.
pSBJ3	*cryIVA*	16	53
pSBW2	*cryIVA*, *cryIVD*	2.3	0.85
pSBJ2	*cryIVA*, *cryIVD*, *p20*	3.3	0.9[c]
pSB4A[b]	*cryIVA*	$>10^7$	$>10^7$

[a] Values are averages from three bioassays performed as described in the text.
[b] pSB4A was a construct with a single promoter (P_{psbA}).
[c] Equivalent to 0.8 μg of total cell soluble protein ml^{-1} or to 30 ng of chlorophyll ml^{-1}.

50 μg ml^{-1}). The plasmids extracted from the cells were found to be structurally intact. Cultures of *E. coli* transformed with the isolated plasmids were as toxic as the original clone (data not shown).

The *Anabaena* sp. strain PCC 7120 cultures carrying the various combinations of *cryIV* genes were repeatedly subcultured for 1 year under selective pressure. The toxicities of the subcultures were assayed at every passage (about six generations), and no major differences were found (data not shown). Cells of clone 11 carrying the largest plasmid with high toxicity grew as vigorously as the wild-type cells (data not shown), indicating that pSBJ2 at least is quite stable inside *Anabaena* sp. strain PCC 7120 and that the constitutive expression of *cryIV* toxins is not harmful to the host cells.

The plating efficiency (fraction of CFU on selective plate out of the total viable cells) of clone 11 after growth on nonselective medium was more than 95% (data not shown). The toxicity was slightly lower, possibly due to a decrease in the number of plasmid copies during growth in nonselective medium for at least 1 month (6 generations) (data not shown).

All of the recombinant plasmids in the clones of *Anabaena* sp. strain PCC 7120 contained the pDU1-based replicon, which was originally isolated from the filamentous cyanobacterium *Nostoc* sp. (12). This replicon seems to be stable in *Anabaena* sp. strain PCC 7120, but upon expression of genes 51 and 42 of *Bacillus sphaericus* 2297 its toxicity was found to decrease slightly when the organism was grown without selection (31). It is not clear whether this decrease in toxicity is attributable to a loss of plasmids or to modification of the plasmids or toxin products.

Comparison to previous studies. This is the first report of constitutive expression of high mosquito larvicidal activity by transgenic *Anabaena* sp. strain PCC 7120 with combinations of two δ-endotoxin genes (*cryIVA* and *cryIVD*) and regulatory gene *p20* of *B. thuringiensis* subsp. *israelensis*. The cyanobacterium *Agmenellum quadruplicatum* PR-6, transformed with *cryIVD* behind its own strong phycocyanin promoter (P_{cpcB}), produced inclusion bodies and was mosquitocidal, but the onset of toxicity with *Culex pipiens* larvae (fed at 12-h intervals from hatching) was delayed (high mortality occurred only after 5 days [17]). The mortality of second-instar *Aedes aegypti* larvae after 48 h of exposure to transgenic *Agmenellum quadruplicatum* PR-6 expressing *cryIVB* (10 mg of total protein ml^{-1}) under the same promoter (P_{cpcB}) was 45% (2). When *cryIVB* was expressed in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 from the same promoter that was used here (P_{psbA}), the levels of the toxic polypeptide were very low and whole cells were not mosquitocidal at a concentration of 4×10^8 cells ml^{-1} (9). Using tandem promoters for expression of *cryIVB* (its own promoter and *lacP*) in *Synechococcus* sp. strain PCC 7942 increased the mosquitocidal activity, but the activity remained relatively low. Mortality of first-instar larvae of *Culex restuans* fed once daily became apparent after only 2 days (22).

The toxicities of our clones (expressing *cryIVA* and *cryIVD*, with and without *p20*, under P_{psbA} and P_{A1} in *Anabaena* sp. strain PCC 7120) were substantially higher; the LC_{50} after 24 h was about 9×10^4 cells ml^{-1} or 0.8 μg of total soluble protein ml^{-1}. Assuming that expressed toxin protein accounts for less than 5% of the total soluble proteins in *Anabaena* sp. strain PCC 7120 (as estimated from the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblot hybridization [data not shown]), an LC_{50} of 0.8 μg of total soluble protein ml^{-1} is equivalent to an LC_{50} of 40 ng of inclusion protein ml^{-1}, which is not far from the LC_{50} of 10 ng ml^{-1} obtained for purified inclusions from *B. thuringiensis* subsp. *israelensis* (18), especially considering the absence of CytA and other toxins. This high toxicity demonstrates that the observed synergistic interaction between CryIVA and CryIVD (3, 10, 18) is not confined to *E. coli*; the toxicity of a clone expressing both CryIVA and CryIVD is greater than that of a clone expressing CryIVA alone (Table 1) (18). In this study, no significant difference was found between the toxicities and stabilities of clones with and without *p20*, even though a role for P20, probably as a chaperonin, has been proposed for high levels of expression in other systems (1, 3, 28, 32).

Several reasons other than synergism and strong tandem promoters could explain the high toxicity levels obtained in this study. The appropriate RBS and the corresponding codon usage should increase translation efficiency in the transgenic organism. The codon usage of the four *cry* genes of *B. thuringiensis* subsp. *israelensis* (23) resembles that of *Anabaena* sp. (29) but not that of *Synechocystis* sp. strain PCC 7942 (23, 29).

In this study a high level of toxicity was observed with recombinant clones of *Anabaena* sp. strain PCC 7120 suitable for mosquito control. However, laboratory strains with foreign genes cannot succeed in nature, because their adaptive values are low compared with those of indigenous species. So far, a dry powder prepared by lyophilization of recombinant cells has retained the same high toxicity as the original culture (data not shown). Use of such a preparation should facilitate wide application of the transgeneric strains. However, the present formulation may not be economic. We plan to reintroduce an indigenous strain, such as *Anabaena siamensis*, containing the constructs into its natural habitat for effective control of mosquitoes (5, 6, 33). In the meantime, a recombinant strain lacking competitiveness and therefore bound to have a short life in the field is safe to use, since the probability of recombinant DNA leaking into other organisms by horizontal gene flow is much lower than the probability that this will occur with long-lasting recombinants. Field application of the latter organisms would certainly require much more stringent measures for control and gene containment, such as a recently reported method (21) in which workers used a recombinase-mediated system for elimination of the antibiotic resistance gene marker from genetically engineered *B. thuringiensis* strains.

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REFERENCES

1. **Adams, L. F., J. E. Visick, and H. R. Whiteley.** 1989. A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. J. Bacteriol. **171:** 521–530.
2. **Angsuthanasombat, C., and S. Panyim.** 1989. Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadruplicatum* PR-6. Appl. Environ. Microbiol. **55:**2428–2430.
3. **Ben-Dov, E., S. Boussiba, and A. Zaritsky.** 1995. Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **177:**2851–2857.
4. **Ben-Dov, E., M. Einav, N. Peleg, S. Boussiba, and A. Zaritsky.** 1996. Restriction map of the 125-kilobase plasmid of *Bacillus thuringiensis* subsp. *israelensis* carrying the genes that encode delta-endotoxins active against mosquito larvae. Appl. Environ. Microbiol. **62:**3140–3145.
5. **Boussiba, S., and X. Q. Wu.** 1995. Genetically engineered cyanobacteria as a *BTI* toxin genes delivery system: a biotechnological approach to the control of malaria mosquitoes, p. 49–64. *In* Combating malaria. Proceedings of a UNESCO/WHO meeting of experts. UNESCO, Paris, France.
6. **Boussiba, S., and A. Zaritsky.** 1992. Mosquito biocontrol by the δ-endotoxin genes of *Bacillus thuringensis* var. *israelensis* cloned in an ammonium excreting mutant of rice field isolate of the nitrogen-fixing cyanobacterium

Anabaena siamensis, p. 89. *In* W. J. Whelan, F. Ahmad, H. Bialy, S. Black, M. L. King, M. B. Rabin, L. P. Solomonson, and I. K. Vasil (ed.), Advances in gene technology: feeding the world in the 21st century. Proceedings of The 1992 Miami BioTechnology Winter Symposium. IRL Press/Oxford University Press, Oxford, United Kingdom.

7. **Bulla, L. A., Jr., D. B. Bechtel, K. J. Kramer, Y. I. Shethna, A. I. Aronson, and P. C. Fitz-James.** 1980. Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*. Crit. Rev. Microbiol. **8:**147–204.
8. **Chang, C., S.-M. Dai, R. Frutos, B. A. Federici, and S. S. Gill.** 1992. Properties of 72-kilodalton mosquitocidal protein from *Bacillus thuringiensis* subsp. *morrisoni* PG-14 expressed in *B. thuringiensis* subsp. *kurstaki* by using the shuttle vector pHT3101. Appl. Environ. Microbiol. **58:**507–512.
9. **Chungjatupornchai, W.** 1990. Expression of the mosquitocidal protein genes of *Bacillus thuringiensis* subsp. *israelensis* and the herbicide-resistance gene *bar* in *Synechocystis* PCC6803. Curr. Microbiol. **21:**283–288.
10. **Crickmore, N., E. J. Bone, J. A. Williams, and D. J. Ellar.** 1995. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. **131:**249–254.
11. **Elhai, J.** 1993. Strong and regulated promoters in the cyanobacterium *Anabaena* PCC 7120. FEMS Microbiol. Lett. **114:**179–184.
12. **Elhai, J., and C. P. Wolk.** 1988. Conjugal transfer of DNA to cyanobacteria. Methods Enzymol. **167:**747–754.
13. **Elhai, J., and C. P. Wolk.** 1990. Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*. EMBO J. **9:**3379–3388.
14. **Georghiou, G. P., and M. C. Wirth.** 1997. Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. **63:**1095–1101.
15. **Khawaled, K., E. Ben-Dov, A. Zaritsky, and Z. Barak.** 1990. The fate of *Bacillus thuringiensis* var. *israelensis*-killed pupae of *Aedes aegypti*. J. Invertebr. Pathol. **56:**312–316.
16. **Mackinney, G.** 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. **140:**315–322.
17. **Murphy, R. C., and S. E. Stevens, Jr.** 1992. Cloning and expression of the *cryIVD* gene of *Bacillus thuringiensis* subsp. *israelensis* in the cyanobacterium *Agmenellum quadruplicatum* PR-6 and its resulting larvicidal activity. Appl. Environ. Microbiol. **58:**1650–1655.
18. **Poncet, S., A. Delécluse, A. Klier, and G., Rapoport.** 1995. Evaluation of synergistic interactions among the CryIVA, CryIVB, and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. **66:**131–135.
19. **Porter, A. G., E. W. Davidson, and J.-W. Liu.** 1993. Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiol. Rev. **57:**838–861.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Sanchis, V., H. Agaisse, J. Chaufaux, and D. Lereclus.** 1997. A recombinase-mediated system for elimination of antibiotic resistance gene marker from genetically engineered *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. **63:**779–784.
22. **Soltes-Rak, E., D. J. Kushner, D. D. Williams, and J. R. Coleman.** 1993. Effect of promoter modification on mosquitocidal *cryIVB* gene expression in *Synechococcus* sp. strain PCC 7942. Appl. Environ. Microbiol. **59:**2404–2410.
23. **Soltes-Rak, E., D. J. Kushner, D. D. Williams, and J. R. Coleman.** 1995. Factors regulating *cryIVB* expression in the cyanobacterium *Synechococcus* PCC 7942. Mol. Gen. Genet. **246:**301–308.
24. **Stratagene Co.** 1994. Stratagene catalog. Stratagene Co., La Jolla, Calif.
25. **Tandeau de Marsac, N., F. de la Torre, and J. Szulmajster.** 1987. Expression of the larvicidal gene of *Bacillus sphaericus* 159M in the cyanobacterium *Anacystis nidulans*. Mol. Gen. Genet. **209:**396–398.
26. **Thiel, T.** 1994. Genetic analysis of cyanobacteria, p. 581–611. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
27. **Thiery, I., L. Nicolas, R. Rippka, and N. Tandeau de Marsac.** 1991. Selection of cyanobacteria isolated from mosquito breeding sites as a potential food source for mosquito larvae. Appl. Environ. Microbiol. **57:**1354–1359.
28. **Visick, J. E., and H. R. Whiteley.** 1991. Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* on production of the CytA protein by *Escherichia coli*. J. Bacteriol. **173:**1748–1756.
29. **Wada, K., Y. Wada, F. Ishibashi, T. Gojobori, and T. Ikemura.** 1992. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. **20:**2111–2118.
30. **Wu, D., and B. A. Federici.** 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J. Bacteriol. **175:**5276–5280.
31. **Xudong, X., K. Renqiu, and H. Yuxiang.** 1993. High larvicidal activity of intact cyanobacterium *Anabaena* sp. PCC 7120 expressing gene 51 and gene 42 of *Bacillus sphaericus* sp. 2297. FEMS Microbiol. Lett. **107:**247–250.
32. **Yoshisue, H., K.-I. Yoshida, K. Sen, H. Sakai, and T. Komano.** 1992. Effects of *Bacillus thuringiensis* var. *israelensis* 20-kDa protein on production of the *Bti* 130-kDa crystal protein in *Escherichia coli*. Biosci. Biotechnol. Biochem. **56:**1429–1433.
33. **Zaritsky, A.** 1995. Biotechnology in the service of pesticide production and use for integrated control of mosquitoes, p. 31–48. *In* Combating malaria. Proceedings of a UNESCO/WHO meeting of experts. UNESCO, Paris, France.