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Biosynthesis of 130-Kilodalton Mosquito Larvicide in the Cyanobacterium *Agmenellum quadruplicatum* PR-6

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The 130-kilodalton mosquito larvicidal gene, cloned from *Bacillus thuringiensis* var. *israelensis*, was introduced into the cyanobacterium *Agmenellum quadruplicatum* PR-6 by plasmid transformation. Transformed cells synthesized 130-kilodalton delta-endotoxin protein and showed mosquito larvicidal activity. Results demonstrate a potential use of a cyanobacterium for biological control of mosquitoes.

Bacillus thuringiensis var. *israelensis* produces a parasporal protein, delta-endotoxin, which is specifically toxic to larvae of mosquitoes and blackflies (8). Delta-endotoxin, which is the factor lethal to mosquito larvae, consists of several proteins with molecular masses of 130 to 135, 65 to 70, and 28 kilodaltons (kDa) as the major composition (2, 5, 12). The 130- to 135-kDa protein is highly toxic to mosquito larvae and contains no hemolytic activity (12, 16). Thus, it represents the most promising candidate for an effective and environmentally safe mosquito larvicide. Two different 130-kDa delta-endotoxin genes have been cloned (1, 3, 13, 14, 17) and sequenced (14, 15, 17, 18), and the mosquito larvicidal moiety of one of the genes was located at the 72-kDa amino-terminal fragment (11). Application of *B. thuringiensis* var. *israelensis* for biological control of mosquitoes encounters several drawbacks, including the presence of the hemolytic 28-kDa protein (2, 3, 6, 12, 16) and the requirement that the organism be applied frequently because it is rapidly lost in the mud and thus removed from the larval feeding zone (10). A potential approach to circumvent the problems is to genetically engineer microorganisms living in the upper layers of aquatic habitats of mosquito larvae to synthesize the 130-kDa mosquito larvicide. The microorganisms producing the larvicide should persist in the aquatic habitats and thus reach target mosquito larvae more effectively. A cyanobacterium is a strong candidate for such a procedure, since it is widely distributed in the upper layers of water. Moreover, a cyanobacterium requires simple growth media because of its photosynthetic capability. *Agmenellum quadruplicatum* PR-6 is a well-characterized cyanobacterium with a transformation system and gene expression that have been established (4). In this study, *A. quadruplicatum* PR-6 was thus chosen for synthesizing a 130-kDa mosquito larvicide and testing its larvicidal activity.

A recombinant plasmid containing the 130-kDa gene was constructed from a biphasic cloning vector, pAQE19LPC ΔSal, which expresses neomycin and ampicillin resistances in both *Escherichia coli* and *A. quadruplicatum* PR-6, and pMU388, which expresses the 130-kDa mosquito larvicidal protein in *E. coli* (1). pAQE19LPCΔSal was cut with *Bam*HI and *Sal*I and then ligated to the *Bam*HI and *Sal*I 130-kDa gene fragment from pMU388 by a standard protocol (9) (Fig. 1). The ligated product was amplified in *E. coli* JM107, and the recombinant plasmid pAqPc388i was selected on the basis of ampicillin and kanamycin resistances and the presence of the 130-kDa gene. The plasmid was further characterized by restriction enzyme analysis. The plasmid pAqPc388i, isolated from the *E. coli*, was used to transform *A. quadruplicatum* PR-6 by adding the plasmid DNA to a late-log-phase liquid culture (4×10^7 cells per ml) as described by Buzby et al. (4). Transformants were selected on an A medium agar plate (4) containing 200 μg of kanamycin per ml. Approximately 1.5×10^2 transformants per μg of DNA were obtained. The presence of pAqPc388i in transformed cells was not evident from analysis by rapid alkaline extraction and agarose gel electrophoresis (Fig. 2A, lane 3). However, the presence of resident plasmids of *A. quadruplicatum* PR-6 was clearly detectable (Fig. 2A, lanes 2 and 3). Southern blot analysis of plasmids with *Xba*I fragments of the 130-kDa gene from pMU388 as the probe (Fig. 2B, lanes 3 to 5) exhibited the presence of the 130-kDa gene in the transformed *A. quadruplicatum* PR-6. By using plasmid DNA extracted from the transformed *A. quadruplicatum* PR-6 to transform *E. coli* JM107, the intact pAqPc388i plasmid was recovered from *E. coli* transformants (results not shown), indicating that pAqPc388i existed as autonomous plasmid DNA in transformed *A. quadruplicatum* PR-6. Calculations based on intensity from the autoradiography gave an estimate of one plasmid copy per cell.

Protein extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis with rabbit antiserum against the 130-kDa protein, followed by [^{125}I]protein A detection, as previously described (1). Figure 3 shows the appearance of the 130-kDa protein from the extracts of *A. quadruplicatum* PR-6 containing pAqPc388i (lane 2) and of *E. coli* containing the same plasmid (lane 1). The 130-kDa protein was absent from the cyanobacterium transformed with the pAQE19LPC ΔSal vector (Fig. 3, lane 3). Biosynthesis of the 130-kDa protein from the pAqPc388i plasmid in *E. coli* was much stronger than in *A. quadruplicatum* PR-6. A relatively low plasmid copy number in *A. quadruplicatum* PR-6 (one copy per cell) probably accounted for the small amount of the 130-kDa protein. Greater degradation of this gene product in *A. quadruplicatum* PR-6 (Fig. 3, arrowhead) than in *E. coli* was apparent.

The mosquito larvicidal activity of pAqPc388i-containing cells is shown in Table 1. The cells were sonicated and then fed in triplicate to 10 second-instar *Aedes aegypti* larvae as previously described (1). *A. quadruplicatum* PR-6 containing pAqPc388i significantly killed mosquito larvae, whereas the cyanobacterium containing the vector alone was relatively

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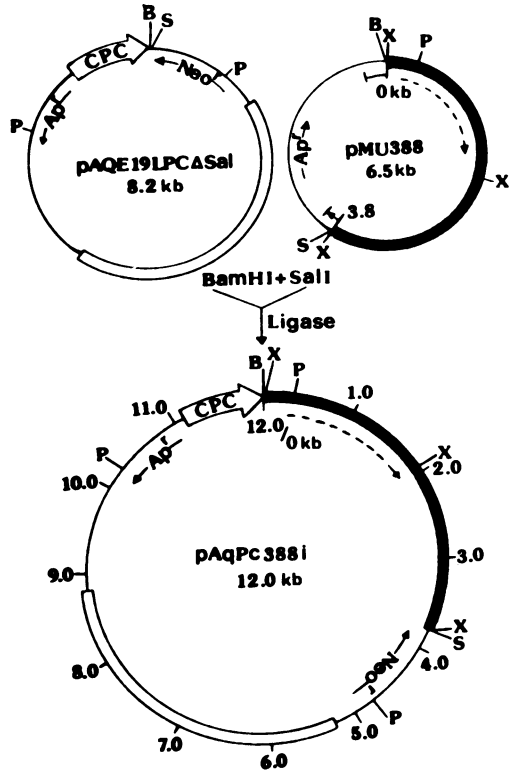

FIG. 1. Construction of pAqPc388i recombinant plasmid from pMU388 and pAQE19LPCΔSal. Thick line, 130-kDa gene from pMU388; CPC, cyano-phycocyanin promoter; dashed arrow, transcriptional direction of 130-kDa gene; B, P, S, and X, restriction enzymes *Bam*HI, *Pst*I, *Sal*I, and *Xba*I, respectively; kb, kilobase; Ap^r, ampicillin resistance; Neo^r, neomycin resistance.

harmless. The larvicidal activity of *E. coli* containing pAqPc388i was more effective than that of its *A. quadruplicatum* PR-6 counterpart. *E. coli* containing pAqPc388i expressed a larger amount of the 130-kDa protein (Fig. 3, lane 1) than did the corresponding *A. quadruplicatum* PR-6. A

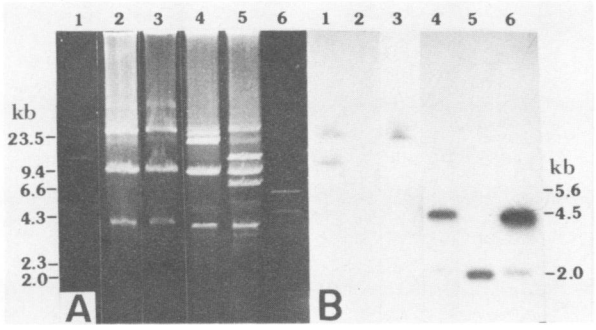

FIG. 2. Demonstration of the presence of the 130-kDa gene. (A) Ethidium bromide staining pattern; (B) autoradiogram of Southern blot hybridization of panel A probed with the 130-kDa gene fragment. Lanes 1 and 6, 50 ng of pAqPc388i plasmid extracted from *E. coli* (lane 6 is cut with *Pst*I); lane 2, 1 μg of plasmid extract of *A. quadruplicatum* PR-6 containing the pAQE19LPCΔSal vector; lanes 3 to 5, plasmid extracts (1 μg each) of *A. quadruplicatum* PR-6 containing the pAqPc388i plasmid (lane 4 is digested with *Pst*I, and lane 5 is digested with *Xba*I). kb, Kilobase.

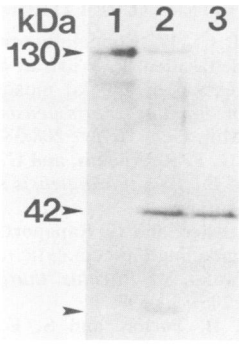

FIG. 3. Autoradiogram of Western blot analysis of protein products. Total protein (50 μg) of *E. coli* harboring plasmid pAqPc388i (lane 1) and total protein (250 μg) of *A. quadruplicatum* PR-6 harboring plasmid pAqPc388i (lane 2) and pAQE19LPCΔSal (lane 3) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, probed with rabbit antiserum against the 130-kDa protein, and subjected to [^{125}I]protein A detection. The last arrowhead indicates a degradation product.

larger quantity of undegraded 130-kDa mosquito larvicidal protein in *E. coli* than in *A. quadruplicatum* PR-6 (Fig. 3) probably accounted for the higher larvicidal activity.

The results reported here demonstrate that the 130-kDa protein can be synthesized in a cyanobacterium and that its mosquito larvicidal activity can be expressed. Expression of the larvicidal gene of *Bacillus sphaericus* 1593M in the cyanobacterium *Anacystis nidulans* R2 (7) supports the notion that a cyanobacterium is a promising organism found at the water surface for biosynthesis of a mosquito larvicidal protein. Greater expression of undegraded 130-kDa delta-endotoxin will be necessary for paving the way to genetically engineer a local cyanobacterium found in mosquito habitats for effective control of tropical disease vectors.

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TABLE 1. Mosquito larvicidal activity of sonicated cells

Microorganism	Plasmid	Total protein concn (mg/ml)	Mortality (%) at:	
			24 h	48 h
B. thuringiensis subsp. *israelensis* (4Q272)	—[a]	10^{-4}	95	100
E. coli JM107	pMU388	1	70	100
E. coli JM107	pAqPc388i	1	15	60
E. coli JM107	pAQE19LPCΔSal	1	0	5
A. quadruplicatum PR-6	pAqPc388i	10	ND[b]	45
A. quadruplicatum PR-6	pAQE19LPCΔSal	10	ND	5

[a] —, A 110-kilobase endogenous plasmid.
[b] ND, Not determined.

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