

**GENETIC ENGINEERING OF *BACILLUS THURINGIENSIS*
VAR. *ISRAELENIS* GENES FOR BIOCONTROL OF MOSQUITO
VECTORS OF DISEASES IN TROPICAL REGIONS**

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ABSTRACT

To make efficient application of the endotoxin proteins for biocontrol of mosquito vectors of diseases in Thailand we attempted to identify mosquito-larvicidal endotoxin genes of *B.t.i.* by molecular cloning of the DNA fragments in *E. coli*. Two genes encoding ~130 kd proteins, each highly toxic to *Aedes aegypti* larvae were obtained. The complete nucleotide sequence of one gene revealed a regulatory sequence and the structural gene encoding 1136 amino acids. By deletion analysis of the gene, the toxic moiety of the 130 kd was located within a peptide of approximately 70 kd. Introduction of the 130 kd gene into a local isolate of photobacteria and *Agmenellum quadruplicatum* PR-6 to construct an organism that reproduces and persists in the environment where mosquito larvae breed, or that grows well in a tropical condition is being carried out.

INTRODUCTION

Bacillus thuringiensis var. *israelensis* (*B.t.i.*) produces parasporal crystal proteins, delta-endotoxin, which are specifically toxic to mosquito and blackfly larvae (Goldberg and Margalit, 1977). The toxin consists of several protein components with those of 130 kDa, 65 kDa and 25-28 kDa predominating (Chesterkhina et al., 1985), each of which had been shown to exhibit mosquito-larvicidal activity (Ward et al., 1984; Hurley et al., 1985; Wu and Chang, 1985). Identification of genes encoding the delta-endotoxin should permit genetic engineering of the genes for biocontrol of mosquitoes. Genes encoding 28 kDa toxin (Ward et al., 1984; Waalwijk et al., 1985), 72 kDa (Thome et al., 1986) and 130 kDa (Bourgouin et al., 1986; Angsuthanasombat et al., 1987) have been identified by molecular cloning in *E. coli*. In this communication we report attempts on genetic-engineering of 130 kDa delta-endotoxin gene for biocontrol of mosquitoes.

Molecular cloning of *B.t.i.* kDa delta-endotoxin gene in *E. coli*

B.t.i. 110 kb plasmid was fragmented with XbaI and ligated to pUC12 vector. Screening of 800 recombinants with endotoxin-mRNA-enriched RNA yielded 32 *E. coli* clones, 17 of which gave positive immunoreactivity with antiserum to the whole *B.t.i.* toxin. *E. coli* clones which killed *A. aegypti* larvae had restriction endonuclease maps as shown in Figure 1. Two (pMU14 and pMU388) contained identical 3.8 kb *B.t.i.* DNA inserts in opposite orientation. The products from clones pMU14, pMU90 and pMU388 were analysed by Western blot (Burnette, 1981) as shown in Figure 2, where a major protein was 130 kDa. By phase-contrast microscopy, *E. coli* containing pMU388 showed a small bright particle which could be extracted to yield 130 kDa protein in SDS-PAGE. The solubilized protein was toxic to *A. aegypti* larvae with LC₅₀ of 200 ng/ml. Another clone (pMU500-1) produced endotoxin slightly smaller than 130 kDa which also killed *A. aegypti* larvae.

TABLE I
Summary of deleted delta-endotoxin gene

Clones harbouring plasmids	DNA fragments (kb)	Major protein product ^(a) (kDa)			Antigenicity ^(b)	Toxicity ^(c) (LD ₅₀ :cell/ml)
		130	115	105		
pMU 388	3.68 (1-3648)	130	115	105		3.2 × 10 ⁵
		90	70			
NBal 14	3.48 (203-3648)	130	115	105	+	8.0 × 10 ⁵
		90	70			
PvX 1	2.31 (263-2572)	90	70		+	4.2 × 10 ⁶
NBal 55	3.40 (286-3583)	130	115	105	+	>10 ⁹
		90	70			
NBal 20	1.50 (x-3648)		50		+	-
Cla 2	3.06 (1-3067)	105	82	72	+	7.5 × 10 ⁵
AccB 49	2.47 (1-2472)	90	70		+	3.8 × 10 ⁶
CBal 151	2.18 (1-2181)	70			+	7.8 × 10 ⁶
CBal 112	1.99 (1-1994)		ND		-	>10 ⁹
Bal 24	1.86 (1-1862)		58 ^(d)		-	-
pUC 12	0				-	-

Restriction endonuclease analysis clearly distinguished the insert of pMU500-1 from that of pMU388 or pMU14. The results demonstrated presence of two closely related endotoxin genes in *B.t.i.*

The complete nucleotide sequence of a 130 kDa gene

The DNA sequence of pMU388 was determined by the chain termination method (Sanger et al., 1977) and shown in Figure 3. The 3648 (base pair) sequence consisted of 150 bp 5'-non-translating, 126 bp 3'-region and 3408 bp coding sequence. The deduced 1136 amino acids had molecular mass of 127,863 Da, slightly smaller than that estimated by SDS-PAGE. The 5'-non-translating region contained SD-sequence at the bases 139th-144th. The 3'-end contained a loop-stem of 18 bp starting from 3583rd bp which was probably a strong transcription terminator.

Identification of the toxic part of the gene

To determine if all 1136 amino acids are essential for mosquito larvicidal activity we constructed truncated 130 kDa genes by deletions of the 5'- and 3' ends, analysed the toxin product by Western blot and tested the toxicity, as summarized in Table I. A maximum of 39 amino acids could be deleted from the NH₂-terminal without a significant loss of the toxicity. Further deletion of 7 more amino acids, however, gave non-toxic protein. As many as 459 amino acids could be removed from the carboxylic end without a significant loss of the toxicity. The deletion analysis located the toxic portion of the gene between the amino acids 39th and 677th. The analysis also revealed that the immuno-reactivity portion of the toxin resided in the carboxy terminal half since the clone containing the amino acids 1-560 was not immunogenic, but that containing 561-1136 was as immunogenic as the complete 1136 amino acid toxin.

Construction of photobacteria containing the 130 kDa gene

Photobacteria are commonly found where mosquito larvae breed in tropical regions and thus constitute a potential biocontrol of the larvae. *A. quadruplicatum* PR-6, a well-studied photobacterium (Stevens and Porter, 1980) was employed to uptake the 130 kDa endotoxin gene. As

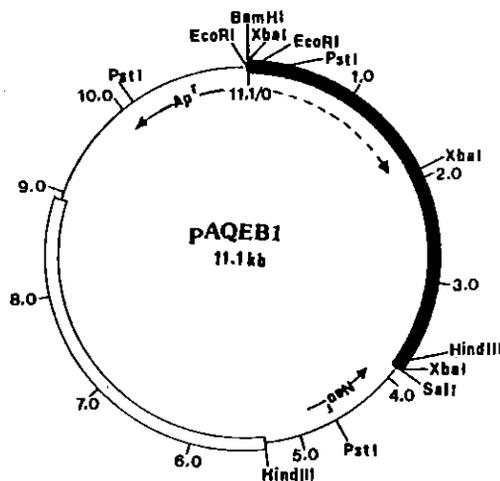


Fig. 4. Restriction endonuclease map of pAQEB1. Thin line represents map of pAQE19 vector. Thick line represents 3.8 Kb endotoxin gene. Dashed arrow is transcriptional direction of the endotoxin gene.

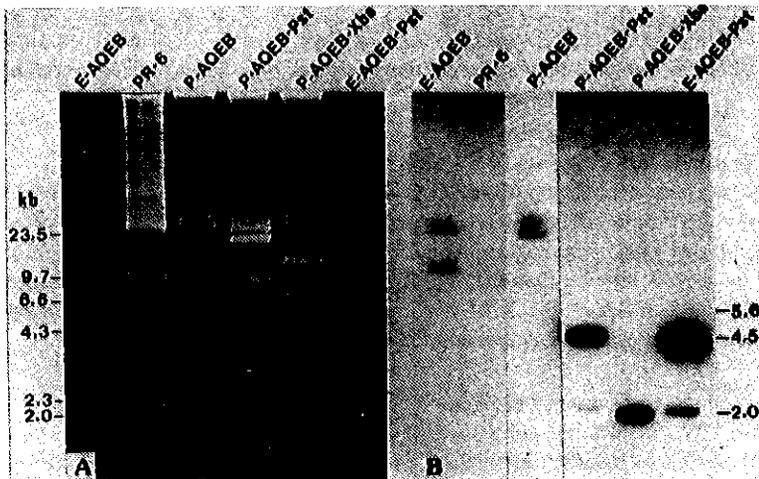


Fig. 5. Southern blot analysis of existence of 130 kDa gene in transformed *A. quadruplicatum* PR-6. Plasmids were extracted, cut with PstI or XbaI, separated on agarose gel, transferred onto nitrocellulose and subsequently hybridized with ^{32}P -labelled 130 kDa gene. (A) Ethidium bromide staining patterns; (B) autoradiographic patterns of (A) E-AQEB is plasmid pAQEB1 extracted from *E. coli*. Pr-6 is the indigenous plasmid of *A. quadruplicatum* PR-6. P-AQEB is plasmid extracted from the transformant. P-AQEB-Pst and P-AQEB-Xba are P-AQEB plasmids cut with PstI and XbaI respectively. E-AQEB-Pst is E-AQEB cut with PstI.

diagrammed in Figure 4, the gene was ligated to pAQE19 vector at the BamHI/SalI site to construct pAQEB1. The recombinant was introduced into the *A. quadruplicatum* PR-6 and selected on kanamycin medium. $1.5 \times 10^2/\mu\text{g}$ transformation efficiency was obtained. Analysis of plasmids by Southern blot demonstrated presence of 130 kDa gene as shown in Figure 5. However, the transformants were not toxic to *A. aegypti* larvae, and did not produce the 130-kDa protein when probed by the Western blot analysis. The intact pAQEB1 could be rescued from the transformed *A. quadruplicatum* PR-6, indicating its extrachromosomal presence. The results demonstrate a successful construction of a photobacterium containing the 130 kDa gene. By inserting the 130 kDa gene under the control of a strong promoter it should be possible to construct the photobacteria to produce the endotoxin and kill mosquito larvae.

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REFERENCES

- Angsuthanasombat, C., Chungjatupornchai, W., Kertbundit, S., Luxananl, P., Settasalan, C., Wilairat, P. and Panyim, S. 1987. Cloning and expression of 130kd mosquito-larvicidal delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis* in *Escherichia coli*. *Mol. Gen. Genet.* 208:384-389.
- Bourgouln, C., Klier, A. and Rapoport, G. 1986. Characterization of the genes encoding the haemolytic toxin and the mosquitocidal delta-endotoxin of *Bacillus thuringiensis israelensis*. *Mol. Gen. Genet.* 205:390-397.

- Burnette, W.N. 1981. Western blotting: Electrophoretic transfer of protein from SDS-PAGE to unmodified nitrocellulose and radiographic detection with antibody and radiiodinated protein. *A. Anal. Biochem.* 112:195-203.
- Chesterkhina, G.G., Zalunin, I.A., Bormatova, M.E., Klepikova, F.S., Khodava, O.M. and Stepanov, V.M. 1985. Structural features of crystal-forming proteins produced by *Bacillus thuringiensis* subspecies *israelensis*. *FEBS Lett.* 190:345-348.
- Goldberg, L.J. and Margall, J. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranatania uniuiculata*, *Culex univittatus*, *Aedes aegypti* and *Culex pipiens*. *Mos. News* 37:353-358.
- Hurley, J.M., Lee, S.G., Andrews, R.W., Klowden, M.J. and Bulla, L.A. 1985. Separation of the cytolytic and mosquitocidal proteins of *Bacillus thuringiensis* subsp. *israelensis*. *Biochem. Biophys. Res. Commun.* 126:961-965.
- Sanger, F., Nicklen, S. and Coulter, A.R. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Stevens, Jr., S.E. and Porter, R.D. 1980. Transformation in *Agmenellum quadruplicatum*. *Proc. Natl. Acad. Sci. USA* 77:6052-6056.
- Thorne, L., Garduno, F., Thompson, T., Decker, D., Zounes, M., Wild, M., Walfield, A.M. and Pollock, T.J. 1986. Structural similarity between the lepidoptera and diptera specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. *israelensis* and *kurstaki*. *J. Bacteriol.* 166:801-811.
- Waalwijk, C., Dulleman, A.M., Van Workman, M.E.S. and Vlsser, B. 1985. Molecular cloning and the nucleotide sequence of the Mr 28,000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. *Nucleic Acids Res.* 13:8207-8217.
- Ward, E.S., Ellar, D.J. and Todd, J.A. 1984. Cloning and expression in *Escherichia coli* of insecticidal delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. *FEBS Lett.* 175:377-382.
- Wu, D. and Chang, F.N. 1985. Synergism in mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis* crystal. *FEBS Lett.* 190:232-236.