Note

Insecticidal Activity of the Products of Two 130-kDa Protein Genes

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Strains of *Bacillus thuringiensis* produce parasporal crystal proteins known as δ -endotoxin during sporulation. The crystals of *B. thuringiensis* var. *israelensis* (*BTI*) are toxic to dipteran insects like mosquitoes and black flies,¹⁾ and those of *B. thuringiensis* var. *kurstaki* (*BTK*) and several other varieties are toxic to lepidopteran insects.²⁾

We have reported the cloning and nucleotide sequencing of the two 130-kDa protein genes of strain *BTI* HD522 in our previous paper.³⁾ The amino acid sequences deduced for the two 130-kDa proteins, ISRH3 and ISRH4, are identical in their C-terminal 467 amino acids. Several research groups have reported the cloning of 130 kDa insecticidal protein genes of other *BTI* strains and the insecticidal activities of the cloned gene products against mosquito larvae.⁴⁻⁷⁾

Here, we report the insecticidal activity of the two 130-kDa proteins (ISRH3 and ISRH4) and that of the C-terminal half of ISRH3.

A 5.2-kb *Hind*III fragment coding for ISRH3 on the plasmid pBGH3³⁾ was subcloned into the *Hind*III sites of pUC13⁸⁾ and pKK223-4⁹⁾ to construct plasmid pUCH3 and plasmid pKKH3, respectively (Fig. 1A). The direction of transcription of the ISRH3 gene in pUCH3 and pKKH3 was the same as that of the β -galactosidase gene. A 5.0-kb *Hind*III fragment coding for ISRH4 on the plasmid pBGH4³⁾ was subcloned into the *Hind*III site of pUC13 to construct plasmid pUCH4 (Fig. 1A). The direction of transcription of the ISRH4 gene in pUCH4 was opposite to that of the β -galactosidase gene. An enzyme-linked immunosorbent assay (ELISA) indicated that amounts of ISRH3 or ISRH4 gene products in *Escherichia coli* JM109¹⁰⁾ cells harboring pUCH3, pKKH3, and

FIG. 1. (A): Restriction Maps of Cloned DNA Fragments from the Large Plasmids of *BTI*.

Cloned DNA fragments of pUCH3, pUCH4, and pKKH3 were derived from *BTI* HD522, and a cloned DNA fragment of pUCX1 was derived from *BTI* 4Q1. With pUCH3, pUCH4, and pUCX1, pUC13 was used as a vector. To construct pKKH3, pKK223-4 was used as a vector. All recombinant plasmids were used to transform *E. coli* JM109 by the method of Hanahan.¹⁶⁾ On the right, the sizes of cloned DNA fragments are shown. Thick arrows indicate the direction of transcription and the coding stretch of ISRH3 and ISRH4. The filled-in area of the thick arrow indicates a stretch where ISRH3 and ISRH4 are identical in their amino acid sequences. The hatched area in the thick arrows indicates a stretch where homology was relatively low.

(B): Restriction Map of pUCX1'.

A 1.8 kb *Xba*I fragment derived from *BTI* 4Q1 plasmid DNAs, the nucleotide sequence of which was identical to that of the 1.8 kb *Xba*I fragment on pBGH3,³⁾ was cloned into the *Xba*I site of pUC13, providing pUCX1. pUCX1 was digested with the restriction enzyme *Sal*I, filled in, and ligated, providing pUCX1'. pUCX1', which encoded the N-terminal of β -galactosidase and the C-terminal 575 amino acids of ISRH3, produced a fused protein in *E. coli* JM109 cells in the presence of 1 mM IPTG. An arrow indicates the direction of transcription and the coding stretch of the fused protein. H, *Hind*III; P, *Pst*I; X, *Xba*I; C, *Cla*I; E, *Eco*RI.

pUCH4 were increased by isopropyl β -D-thiogalactoside (IPTG); (data not shown). We have confirmed the synthesis of the two 130-kDa proteins when the cloned ISRH3 and ISRH4 genes were used to transform *E. coli* (results

TABLE I. INSECTICIDAL ACTIVITY ON MOSQUITO LARVAE OF PROTEIN EXTRACTS OF *E. coli* JM109 CELLS CARRYING PLASMIDS

Test sample ^a	LC ₅₀ (μg/ml) ^b	
	24 hr	48 hr
JM109/pUC13	—	— ^c
JM109/pUCH3	39	32
JM109/pUCH4	36	30
JM109/pUCX1'	36	25
JM109/pKK223-4	—	— ^c
JM109/pKKH3	40	25

^a Cells of *E. coli* JM109 carrying plasmids were cultured at 28°C for 24 hr in L broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.2) containing 50 μg/ml ampicillin and 1 mM IPTG. The cells were harvested and washed with 1 M NaCl by centrifugation. Cell pellets were suspended in 0.1 M 2-mercaptoethanol and ultrasonicated. The suspension was mixed with an equal volume of 0.2 M glycine-NaOH (pH 10.5), incubated at 37°C for 3 hr, adjusted to pH 7.5 with 1 N HCl, and centrifuged. Proteins in the supernatant were precipitated with 50% saturated ammonium sulfate. The precipitates were suspended again in 0.1 M glycine-NaOH (pH 10.5) containing 0.05 M 2-mercaptoethanol, dialyzed against Dulbecco's PBS (without Ca²⁺ and Mg²⁺) (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄), and centrifuged. Mosquito larvicidal activity of the supernatant was analyzed basically by the procedure of Schnell *et al.*¹⁷⁾ Protein extracts were diluted in 1 ml of 0.1 M Tris-HCl (pH 7.4) and mixed with 10 μl of latex beads (0.8 μm, 10% solids; Sigma Chemical Co.). The mixture was left at room temperature for 1 hr, and then added to 24 ml of distilled water in which there were 20 third-instar larvae of the mosquito *Culex pipiens*. Mortality was scored after incubation at 26°C for 24 hr and 48 hr.

^b LC₅₀: Lethal concentration (50% end point) is shown in a final concentration of protein extract.

^c At the concentration of 240 μg/ml of protein extract, no larvae died.

will be reported elsewhere).

Because 467 amino acids in the C-terminal region (about 40% of the 130-kDa proteins) of ISRH3 and ISRH4 are identical, we expect that this region might be important in the insecticidal activity. Therefore, we constructed a recombinant plasmid producing the C-terminal half of the 130-kDa protein in *E. coli* JM109. A 1.8 kb *Xba*I fragment of a plasmid, pUCX1', was derived from a 70 Md plasmid, pBTI-6, in strain *BTI* 4Q1¹¹⁾ (Fig. 1B). The nucleotide sequence of the 1.8 kb *Xba*I fragment from *BTI* HD522 was identical to that from *BTI* 4Q1, so we expected that *E. coli* JM109 carrying pUCX1' would

produce the 575 C-terminal amino acids of the ISRH 3 protein. A fused protein synthesized in *E. coli* JM109 carrying pUCX1' was detected by immunoblotting (data not shown).

Crude extracts of *E. coli* cells carrying pUCH3, pKKH3, or pUCH4 were toxic to mosquito larvae, but those of *E. coli* cells carrying the vector pUC13 or pKK223-4 were not (Table I). The results indicate that both ISRH3 and ISRH4 130-kDa proteins were mosquitoicidal. Extracts prepared from *E. coli* cells carrying pUCX1' were also toxic to mosquito larvae (Table I). These results indicate that the C-terminal half of ISRH3 has insecticidal activity, and that there might be a mosquitoicidal domain in the C-terminal region completely conserved between ISRH3 and ISRH4.

Several investigators have reported that there is an insecticidal domain in the N-terminal half of the 130-kDa protein of *BTK*¹²⁻¹⁵⁾ toxic to lepidopterans. There is homology at the middle region of the N-terminal half between the 130-kDa proteins of *BTI* and *BTK* (data not shown). Therefore, the N-terminal half of *BTI* may also have insecticidal activity. However, it is not known whether the N-terminal halves of the 130-kDa proteins of *BTI* are insecticidal. The 130-kDa proteins of *BTI* may have two or more insecticidal domains.

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