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Cloning and Analysis of δ-Endotoxin Genes from *Bacillus thuringiensis* subsp. *alesti*

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Bacillus thuringiensis subsp. alesti produced only CryIA(b)-type protoxins, and three cryIA(b) genes were cloned. One was cryptic because of an alteration near the 5′ end, and the other two were very similar to each other. The protoxin encoded by one of the latter genes differed from other CryIA(b) protoxins in its greater stability and relative toxicity for two members of the order Lepidoptera.

Bacillus thuringiensis forms intracellular inclusions containing protoxins (δ-endotoxins) active on the larvae from at least three insect orders (11, 15). Most lepidopteran-active strains produce multiple protoxins with a major class designated CryI on the basis of deduced amino acid sequences and specificities (11). Several related genes of one such class called *cryIA*(b) have been cloned from at least three *B. thuringiensis* subspecies (7, 9, 11, 25). The encoded protoxins are similar in toxicity for selected lepidopterans and in their lack of stability due to a 26-amino-acid deletion in the carboxyl half (6). The CryIA(b) protoxin from *B. thuringiensis* subsp. *aizawai* IC1 is unique, however, in that toxicity for either certain lepidopterans or dipterans depends upon the source of proteases (larval gut extract) used for converting the protoxin to toxin (8, 9). Two residues near the carboxyl end of the toxin were required for this novel property (8), indicating that just a few key amino acids in these protoxins could account for specificity differences.

B. thuringiensis subsp. *alesti* appeared to contain only CryIA(b) protoxins on the basis of reaction with only Cry IA(b)-specific monoclonal antibodies (10). In addition, the sequences of several tryptic peptides were identical to those from CryIA(b) protoxins, but some were unique (4), suggesting that novel CryIA(b)-related protoxins may be present. A further study was made, therefore, of the protoxin gene complement of this subspecies. One of the cloned genes was sequenced, and the toxicity of this protoxin for two test lepidopterans was examined.

Total RNA was extracted from sporulating cultures (1) of *B. thuringiensis* subsp. *alesti* (27) and hybridized to gene-specific oligonucleotides (3) (Fig. 1). There was hybridization with a *cryIA*(b) probe (Fig. 1C; would also react with *cryIC* mRNA) but with neither *cryIA*(a) nor *cryIA*(c) probes (Fig. 1A and B).

Protoxin gene complexity was measured by hybridizing Southern transfers of *Hin*dIII-digested total DNA with a 1.95-kb *Nsi*I-*Bcl*I fragment cloned from the 5′ end of the *cryIA*(c) gene (26). Fragments of 1.4 and 5.3kb hybridized, but only the latter is characteristic of *cryIA*(b) genes (12). Subsequently, it was found that the novel 1.4-kb fragment was due to an extra *Hin*dIII site near the amino end of a *cryIA*(b) gene (Fig. 2). Overall, the DNA and RNA hybridization data were consistent with the presence of only the *cryIA*(b) gene(s) in *B. thuringiensis* subsp. *alesti*.

*Nde*I-digested total DNA (18) was ligated into pBR322 and electroporated (5) into *Escherichia coli* DH5α with a Bio-Rad Gene Pulser. Fifteen colonies (of a total of about 5,000) hybridized (17) with the 1.95-kb *Nsi*I-*Bcl*I fragment, and the plasmids from each were digested with various restriction enzymes in order to determine the minimum number of different cloned genes. Three patterns were found, and two containing 1.4-kb *Hin*dIII fragments (which hybridized with the probe) were very similar. These related clones, designated pAC2 and pAC16, also had similar *Nde*I-plus-*Pst*I restriction enzyme digest patterns, which differed from a third pattern found in a clone designated pAC6.

The insert from plasmid pAC2 was sequenced (21) (Sequenase kit; U. S. Biochemical Corp.) after two *Nde*I-*Pst*I fragments of 2.5 and 4.0 kb were subcloned into M13mp19 (Fig. 2). There were 14 amino acid differences between residues 1 to 281 (highly conserved region among *cryIA* genes) from the *B. thuringiensis* subsp. *berliner* and subsp. *kurstaki* HD1 CryIA(b) protoxins (7, 25), and most of these were conserved. The most extensive differences among the CryIA protoxins are in the so-called variable region between residues 282 and ca. 610 (11, 15). In the amino-terminal half of this region, the pAC2 protoxin had 10 amino acid differences from the *B. thuringiensis* subsp. *berliner* CryIA(b) protoxin, and only one of these was conserved. There was only one difference in the second halves of the variable regions of these toxins.

In general, the amino acid sequences of the carboxyl halves of all CryI protoxins are very highly conserved (ca. 90%) (11, 15). The pAC2 protoxin differed by 29 amino acids from the *B. thuringiensis* subsp. *berliner* CryIA(b) protoxin in this region and did not contain either the 26-amino-acid deletion found in all other CryIA(b) protoxins (7, 11, 25) or the 4-amino-acid deletion found in CryIA(a) and CryIA(c) protoxins (dashed lines in Fig. 2).

The other two clones, pAC6 and pAC16, were sequenced between nucleotides 1190 and 1311 within the variable region by using the CE509 oligonucleotide primer (Fig. 2). All three had identical sequences except for one nucleotide difference (also one amino acid difference) in the pAC6 gene.

For bioassays (Table 1), *B. thuringiensis* inclusions were purified on Renografin (Squibb) gradients (3, 22) and the proteins were solubilized in 0.03 M Na_2CO_3–2% beta-mercaptoethanol, pH 9.6. The soluble fractions were dialyzed for 16 h at 4°C versus 0.03 M $NaHCO_3$, pH 8.5, and the protein content was determined with the BCA reagent

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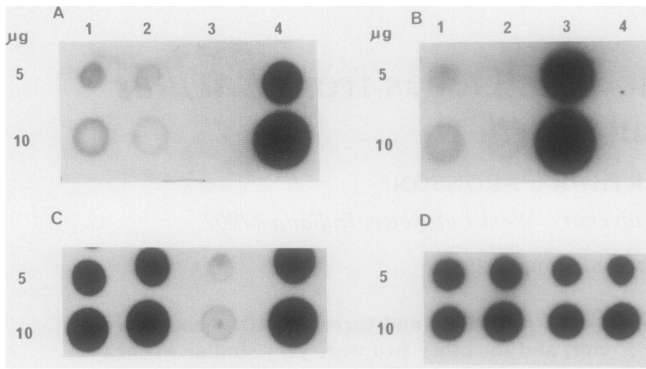

FIG. 1. Dot blots of RNA from sporulating cultures (80% of the cells contained phase-bright endospores) of *B. thuringiensis* subsp. *alesti* (lane 1), subsp. *kurstaki* HD1-9, which contains only a *cryIA*(b) gene (19) (lane 2), subsp. *kurstaki* HD73, which contains only a *cryIA*(c) gene (3, 11) (lane 3), and subsp. *sotto* HD6 (from the U.S. Department of Agriculture, Peoria, Ill.), which contains a *cryIA*(a) gene and probably others (11) (lane 4). Hybridization was with ^{32}P-oligonucleotide 200A specific for the *cryIA*(a) gene (3) (A); ^{32}P-oligonucleotide 355A specific for the *cryIA*(c) and *cryIA*(a) genes (3) (but not the *cryIA*(a) gene from *B. thuringiensis* subsp. *sotto* [23]) (B); ^{32}P-oligonucleotide 356A specific for the *cryIA*(b) (and *cryIC*) genes (C); and the N_7 probe containing the entire coding region of the *cryIA*(c) gene (3) (D). There is extensive sequence conservation among all of the *cryIA* genes (11, 15), so N_7 serves as a general probe. Labeling, hybridization conditions, and washing were as previously described (3, 17).

(Pierce Biochemicals). *E. coli* cells containing clone pAC2, pAC16, EB1 [*B. thuringiensis* subsp. *berliner cryIA*(b) gene], or BP10 [*B. thuringiensis* subsp. *kurstaki* HD1 *cry IA*(b) gene] or the cloning vehicle pBR322 were harvested from LB medium (17) plus 20 µg of ampicillin ml^{-1} and lysed by sonication, and the pellets were extracted with 0.10 N NaOH at 27°C for 30 min. Following neutralization with 2 M Tris (pH 8.0) and centrifugation, the supernatants were dialyzed and protein was measured as described above. Various concentrations were spread on the surface of an artificial diet for bioassays with first-instar larvae of *Heliothis virescens* and *Trichoplusia ni* (3). Mortality was determined after 7 days, and 50% lethal dose (LD_{50}) values were calculated by probit analysis (16).

B. thuringiensis subsp. *alesti* inclusion extracts [at least two CryIA(b) protoxins] were somewhat more toxic for *H. virescens* than for *T. ni* larvae, in contrast to inclusion extracts from *B. thuringiensis* subsp. *kurstaki* HD1-9, which contained only one species of CryIA(b) protoxin (3, 19) (Table 1). Extracts of *E. coli* clones containing pAC2 or pAC16 may be even more selective for *H. virescens*, so there may be additional protoxins in inclusions from *B. thuringiensis* subsp. *alesti* which have somewhat different specificities for these two lepidopterans.

Another *cryIA* gene (pAC6) appears to be cryptic, since it was not toxic and there was no detectable protoxin antigen in extracts from an *E. coli* clone (14). There was a novel sequence near the amino end of the pAC6 gene (determined with the 282B oligonucleotide primer [Fig. 2]) with no open reading frame and an apparent lack of a ribosome binding site. This sequence contained a *Kpn*I site not found in the pAC2 clone [nor in other *cryIA*(b) gene sequences]. As a result, a unique 2.1-kb *Kpn*I fragment hybridizing with the N_7 probe (as in Fig. 1) was present in this clone and in digests of total DNA from *B. thuringiensis* subsp. *alesti*, so

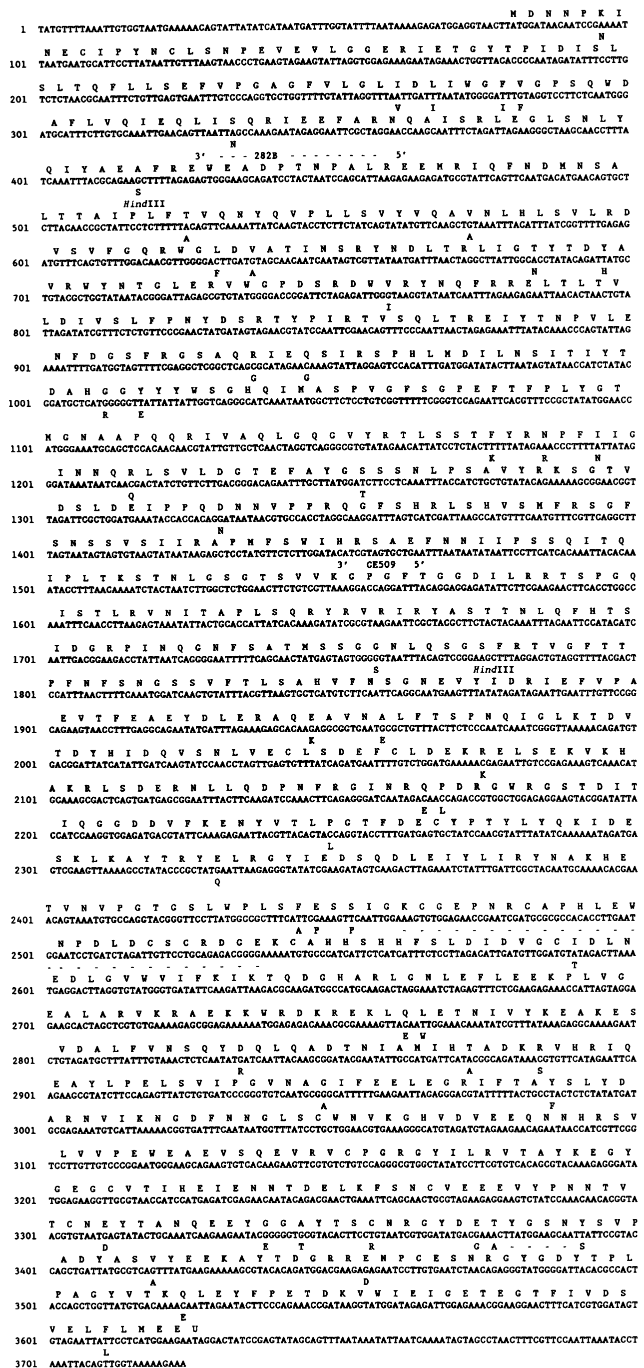

FIG. 2. Nucleotide and amino acid sequences of the pAC2 clone. Differences from the *B. thuringiensis* subsp. *berliner* CryIA(b) protoxin (25) [the deduced sequence of the CryIA(b) protoxin from *B. thuringiensis* subsp. *kurstaki* HD1 differs by only two amino acids (11)] are indicated below each line. The two *Hin*dIII sites which resulted in the 1.4-kb fragment are shown below the sequence. Regions of complementarity to the oligonucleotide primers 282B (5′-CCTAGCGAATTCTTCTATCTTTGGTTAAT) and CE509 (5′-TCTTCTATATAAAG) used for sequencing are also indicated. Dashed lines beneath the nucleotide sequence represent regions deleted in other *cryIA*(b) genes (78 bp) or *cryIA*(a) and *cryIA*(c) genes (12 bp). Subclones and deletion(s) generated with exonuclease III (Erase-a-Base kit from Promega) were sequenced with Sequenase.

TABLE 1. Toxicity of soluble protoxins from inclusions and *E. coli* clones

Protoxin source	LD_{50}[a] (μg of protein cm^{-2})		*H. vir*/*T. ni* (SD)
	H. virescens	*T. ni*	
B. thuringiensis subsp. *alesti* inclusion extracts	0.04 (0.01–0.10)	0.10 (0.04–0.12)	0.40 (0.20)
B. thuringiensis subsp. *kurstaki* HD1-9 inclusion extracts	0.19[b] (0.05–0.40)	0.25[b] (0.08–0.40)	0.76 (0.15)
Clone pAC2 (*B. thuringiensis* subsp. *alesti*)[c]	0.35 (0.10–1.00)	2.50 (0.90–4.00)	0.14 (0.11)
Clone BP10 (*B. thuringiensis* subsp. *kurstaki*)[d]	1.60 (0.80–2.40)	2.00 (1.6–4.00)	0.80 (0.16)

[a] Values are averages of four experiments (<10% lethality in controls) with ranges in parentheses. The *E. coli* extract preparation procedure resulted in a 10- to 20-fold enrichment for protoxin.
[b] Relatively high values (11) due to the instability of the CryIA(b) protoxin (7) and to the more extensive contamination of these inclusion preparations with cell debris.
[c] Results with *E. coli*/pAC16 extracts were very similar.
[d] Results with extracts from a clone of the *B. thuringiensis* subsp. *berliner cryIA*(b) protoxin gene were very similar.

the alteration in pAC6 is not due to a cloning artifact. Cryptic protoxin genes have also been reported for *B. thuringiensis* subsp. *entomocidus* 601 (20) and subsp. *aizawai* HD133 (2).

The selectivity of the pAC2 clone for *H. virescens* larvae (Table 1) is similar to that found with *B. thuringiensis* subsp. *kurstaki* HD73 (13) which contains only a *cryIA*(c) gene (11) or with the cloned CryIA(c) toxin (3). This toxin differs most extensively from CryIA(b) toxins in the second half of the variable region (7, 25), whereas the pAC2 protoxin is most different from other CryIA(b) protoxins in the first half of the variable region (Fig. 2). Therefore, the basis for the selectivity for *H. virescens* larvae of the CryIA(c) and pAC2 CryIA(b) toxins is apparently not the same. It should be noted that competitive binding experiments indicate that there is probably more than one toxin receptor present on *H. virescens* larval midgut cells (24).

The 26-amino-acid deletion found in several CryIA(b) protoxins (7, 11, 15) but in neither CryIA(a) nor CryIA(c) protoxins is responsible for the instability of this protoxin in cells grown at 30°C or higher (6). Cells producing only this protoxin are thus acrystalliferous when grown at 30°C (19). If, as appears likely, *B. thuringiensis* subsp. *alesti* produces only CryIA(b) protoxins, then the formation of inclusions at 30°C in this strain is probably attributable to the absence of this deletion.

Nucleotide sequence accession number. The sequence of the *cryIA*(b) gene reported in this paper has been assigned accession no. M65252.

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REFERENCES

1. **Aronson, A. I., N. Angelo, and S. C. Holt.** 1971. Regulation of extracellular protease production in *Bacillus cereus* T: characterization of mutants producing altered amounts of protease. J. Bacteriol. **106:**1016–1025.
2. **Aronson, A. I., E.-S. Han, W. McGaughey, and D. Johnson.** 1990. The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. Appl. Environ. Microbiol. **57:**981–986.
3. **Arvidson, H., P. E. Dunn, S. Strnad, and A. I. Aronson.** 1989. Specificity of *Bacillus thuringiensis* for Lepidopteran larvae: factors involved *in vivo* and in the structure of a purified protoxin. Mol. Microbiol. **3:**1533–1543.
4. **Chestukhina, G. G., S. A. Tyurin, A. L. Osterman, O. P. Khodova, and V. M. Stepanov.** 1986. Amino acid sequences from the N-terminal domain of *Bacillus thuringiensis*, subspecies *alesti*, δ-endotoxin. FEBS Lett. **198:**283–286.
5. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
6. **Geiser, M. (Ciba Geigy, Basel, Switzerland).** 1990. Personal communication.
7. **Geiser, M., S. Schweitzer, and C. Grimm.** 1986. The hypervariable region in the genes coding for entomopathogenic crystal protein of *Bacillus thuringiensis*: nucleotide sequence of the kurhdl gene of subspecies *kurstaki* HD1. Gene **48:**109–118.
8. **Haider, M. Z., and D. J. Ellar.** 1989. Functional mapping of an entomocidal delta-endotoxin: single amino acid changes produced by site-directed mutagenesis influence toxicity and specificity of the protein. J. Mol. Biol. **208:**183–194.
9. **Haider, M. Z., B. H. Knowles, and D. J. Ellar.** 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal delta-endotoxin by differential processing of the protoxin by larval gut proteases. Eur. J. Biochem. **156:**531–540.
10. **Höfte, H., J. Van Rie, S. Jansens, A. V. Houtven, H. Vanderbruggen, and M. Vaeck.** 1988. Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis*. Appl. Environ. Microbiol. **54:**2010–2017.
11. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
12. **Kronstad, J. W., and H. R. Whiteley.** 1986. Three classes of homologous *Bacillus thuringiensis* crystal protein genes. Gene **43:**29–40.
13. **Krywienczyk, J., H. T. Dulmage, and P. G. Fast.** 1978. Occurrence of two serologically distinct groups within *Bacillus thuringiensis* serotype 3ab var. *kurstaki*. J. Invertebr. Pathol. **31:**372–375.
14. **Lee, C.-S., and A. I. Aronson.** Unpublished data.
15. **Lereclus, D., C. Bourgouin, M. M. Lecadet, A. Klier, and G. Rapoport.** 1989. Role, structure and molecular organization of the genes coding for the parasporal δ-endotoxins of *Bacillus thuringiensis*, p. 255–276. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of prokaryotic development. American Society for Microbiology, Washington, D.C.
16. **Lieberman, H. R.** 1983. Estimating LD_{50} using probit technique: a basic computer program. Drug Chem. Toxicol. **6:**111–116.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
19. **Minnich, S. A., and A. I. Aronson.** 1984. Regulation of protoxin synthesis in *Bacillus thuringiensis*. J. Bacteriol. **158:**447–454.
20. **Sanchis, V., D. Lereclus, G. Menou, J. Chaufaux, S. Guo, and M.-M. Lecadet.** 1989. Nucleotide sequence and analysis of the N-terminal coding region of the Spodoptera-active δ-endotoxin gene of *Bacillus thuringiensis aizawai* 7.29. Mol. Microbiol. **3:**229–238.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
22. **Sharpe, E. S., K. W. Nickerson, L. A. Bulla, Jr., and J. N. Aronson.** 1975. Separation of spores and parasporal crystals of *Bacillus thuringiensis* in gradients of certain X-ray contrasting

agents. Appl. Microbiol. **30**:1052–1053.

23. **Shibano, Y., A. Yamagata, N. Nakamura, T. Iizuka, H. Sugisaki, and M. Takanami.** 1985. Nucleotide sequence coding for the insecticidal fragment of the *Bacillus thuringiensis* crystal protein gene. Gene **34**:243–251.
24. **VanRie, J., S. Jansens, H. Höfte, D. Depheele, and H. VanMellaert.** 1989. Specificity of *Bacillus thuringiensis* δ-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insect. Eur. J. Biochem. **186**:239–247.
25. **Wabiko, H., K. C. Raymond, and L. A. Bulla, Jr.** 1986. *Bacillus thuringiensis* entomocidal protoxin gene sequence and gene product analysis. DNA **5**:305–314.
26. **Wu, D.** Unpublished data.
27. **Young, I. E., and P. C. FitzJames.** 1959. Chemical and morphological studies of bacterial spore formation. J. Biophys. Biochem. Cytol. **6**:483–498.