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Structural Stability of *Bacillus thuringiensis* δ-Endotoxin Homolog-Scanning Mutants Determined by Susceptibility to Proteases

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Forty homolog-scanning (double-reciprocal-crossover) mutant proteins of two *Bacillus thuringiensis* δ-endotoxin genes (*cryIAa* and *cryIAc*) were examined for potential structural alterations by a series of proteolytic assays. Three groups of mutants could be identified. Group 1, consisting of 13 mutants, showed no δ-endotoxin present during overexpression conditions in *Escherichia coli* (48 h at 37°C, with a p_{tac} promoter). These mutants produced full-sized δ-endotoxin detectable by polyacrylamide gel electrophoresis with Coomassie blue staining or Western immunoanalysis after 24 h of growth but not after 48 h, suggesting sensitivity to intracellular proteases. Group 2 consisted of 13 mutants that produced stable δ-endotoxins that were completely digested by 2% bovine trypsin. In contrast, native δ-endotoxin produces a 65,000-Da trypsin-resistant peptide, which is the active toxin. Group 3 mutants expressed δ-endotoxin and trypsin-stable toxins, similar to the wild type. In this study, 12 group 3 mutant toxins were compared with wild type toxins by thermolysin digestion at a range of temperatures. The two wild-type toxins exhibited significant differences in thermolysin digestion midpoints. Among the group 3 mutants, most possessed significantly different protein stabilities relative to their parental toxins. Two of the group 3 mutants were observed to have exchanged the thermolysin sensitivity properties of the parental toxins.

Bacillus thuringiensis is a gram-positive bacterium that produces a proteinaceous crystal insecticide during sporulation. When the crystal is ingested by susceptible insect larvae, the high pH and reducing environment of the insect midgut aid in dissolution of the crystal into 130,000-Da protoxin subunits (21). The protoxin is digested by trypsin-like proteases in the insect midgut to form the activated toxin (20). The 65,000-Da activated toxin is resistant to further trypsin digestion and is thus referred to as the trypsin-resistant core (6).

The mode of action of the toxin is theorized to begin with the binding of the activated toxin to specific receptors on the insect midgut (18). Once the toxin is bound, formation of a nonspecific pore by the toxin leads to lysis of the midgut epithelial cells (17). Paralysis results from the midgut cell disruption, and the insect soon dies. The *B. thuringiensis* spores germinate and grow in the midgut of the dead insect (2).

Many different crystal toxin genes exist, and they have been organized into four classes by Höfte and Whiteley (19). The *cry* (crystal insecticide toxin) classification system is based on the range of susceptible insects and the amino acid sequence of the protoxins. CryI, CryII, CryIII, and CryIV are toxic toward Lepidoptera, Lepidoptera and Diptera, Coleoptera, and Diptera insects, respectively. Recently, most research attention has centered on the *cryI* class. This class consists of several subclasses: *cryIA*, *cryIB*, *cryIC*, *cryID* (19), *cryIE* (35), *cryIF* (4), and *cryIG* (13).

The *cryIA* subclass is composed of three closely related genes known as *cryIAa*, *cryIAb*, and *cryIAc*, where *cryIAb* is apparently a natural single crossover of the other two *cryIA* genes. CryIAa and CryIAc protoxins have an overall 82% amino acid homology and share long tracks of extensive homology with regions of hypervariability (11, 19). Bioassay comparison data demonstrate large differences in insect specificity between CryIAa and CryIAc. CryIAa possesses 400-fold more *Bombyx mori* (silkworm) toxicity than does CryIAc (10). CryIAc possesses 10-fold more activity than CryIAa against both *Heliothis virescens* (tobacco budworm) and *Trichoplusia ni* (cabbage looper) (9). CryIAa and CryIAc possess equal *Manduca sexta* (tobacco hornworm) toxicities (32).

One of the more important techniques used to locate specificity-determining regions of the CryIA toxins is the homolog-scanning mutagenesis technique (7). This technique is based on the assumption that reciprocal exchanges of genetic material between highly homologous proteins will exchange functional regions of the protein and that a loss (or gain) of a functional property is not caused by structural alterations in the newly constructed protein. By using the homolog-scanning mutagenesis technique, the CryIAa *B. mori* (10) and the CryIAc *H. virescens* and *T. ni* (9, 32) specificity-determining regions have been identified. The *B. mori* specificity-determining region of CryIAa is located within residues 332 to 450, the region corresponding to the first half of the hypervariable region of the toxin. The *H. virescens* and *T. ni* specificity-determining regions of CryIAc are located within residues 332 to 612 and residues 332 to 450, respectively. Other researchers have also located specificity-determining sequences in homologous regions of other toxins (28, 36). These studies, however, did not examine the mutant δ-endotoxins for potential protein structure alterations or analyze the effect, if any, of these protein structural alterations on the insect toxicities.

In this paper, we describe the structural stability of an extensive set of homolog-scanning mutants constructed by reciprocal exchanges of genetic material between the *cryIAa*

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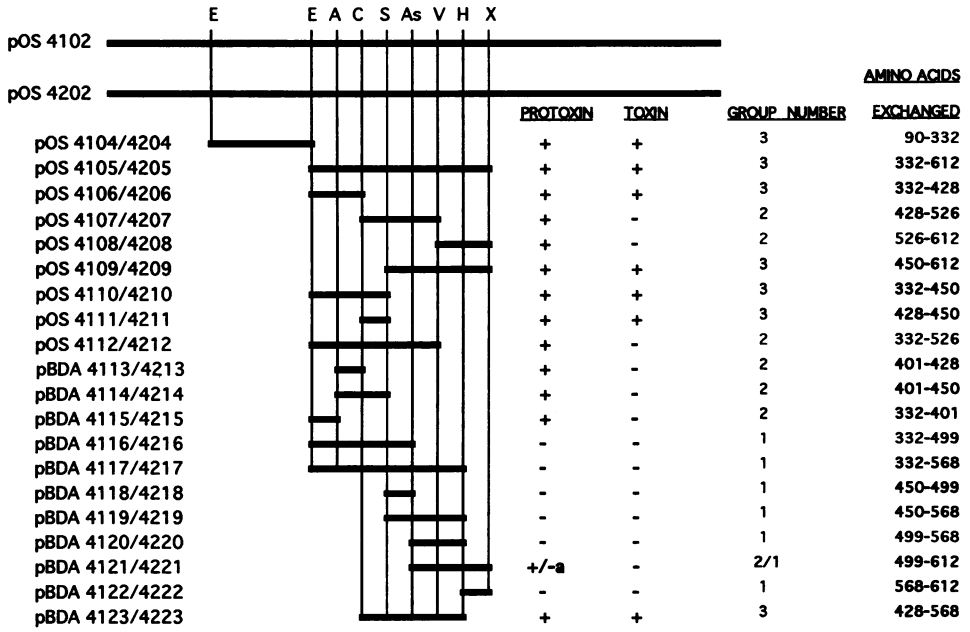

	PROTOXIN	TOXIN	GROUP NUMBER	AMINO ACIDS EXCHANGED
pOS 4104/4204	+	+	3	90-332
pOS 4105/4205	+	+	3	332-612
pOS 4106/4206	+	+	3	332-428
pOS 4107/4207	+	-	2	428-526
pOS 4108/4208	+	-	2	526-612
pOS 4109/4209	+	+	3	450-612
pOS 4110/4210	+	+	3	332-450
pOS 4111/4211	+	+	3	428-450
pOS 4112/4212	+	-	2	332-526
pBDA 4113/4213	+	-	2	401-428
pBDA 4114/4214	+	-	2	401-450
pBDA 4115/4215	+	-	2	332-401
pBDA 4116/4216	-	-	1	332-499
pBDA 4117/4217	-	-	1	332-568
pBDA 4118/4218	-	-	1	450-499
pBDA 4119/4219	-	-	1	450-568
pBDA 4120/4220	-	-	1	499-568
pBDA 4121/4221	+/-a	-	2/1	499-612
pBDA 4122/4222	-	-	1	568-612
pBDA 4123/4223	+	+	3	428-568

a. 4121 formed a protoxin, 4221 did not.

FIG. 1. Restriction enzyme maps of *cryIAa* and *cryIAc* showing the regions exchanged in the homolog-scanning mutant alleles 4102 to 4223. The presence or absence of protoxin in a 48-h culture and toxin after trypsin digestion is indicated by + or −. Abbreviations: E, *Eco*RI; A, *Acc*I; C, *Cla*I; S, *Sac*I; As, *Asu*II; V, *Eco*RV; H, *Hin*dIII; X, *Xho*I.

and *cryIAc* genes. This study includes previously constructed chimeric δ-endotoxin genes (the pOS series) (9, 10) and newly constructed chimeric δ-endotoxin genes (the pBDA series). Susceptibility to two protease assay systems, *Escherichia coli* intracellular proteases (in vivo protease assay) and trypsin (in vitro protease assay), was used to detect protein structural alterations. Differential protease susceptibility, representing degrees of protein structural alterations caused by mutagenesis, allowed for the categorization of the chimeric proteins into three distinct groups. Some of the chimeric proteins were found to be resistant to both protease assay systems (group 3), and therefore to detect alterations in protein stability, we used the members of group 3 in a modified thermolysin digestion protocol. By this protocol, a region (residues 450 to 612) influencing the protein stability of δ-endotoxins was localized. The thermolysin-digestion protocol was found to be an easy and reliable method to detect protein structural alterations in δ-endotoxins.

MATERIALS AND METHODS

Construction of homolog-scanning mutants. Construction of reciprocal switches pOS4102 through pOS4112 and pOS4202 through pOS4212 was described previously (10). In summary, pOS4102 and pOS4202 contain *cryIAa* and *cryIAc* genes, respectively. The genes were cloned into the *E. coli* expression vector pKK223-3 (Pharmacia) and expressed in *E. coli* JM103 cells (8). Reciprocal switches pBDA4113 through pBDA4123 and pBDA4213 through pBDA4223 have been constructed in this work. To begin the reciprocal homolog construction and the site-directed mutagenesis, the *Hin*dIII restriction site was removed from the pUC118 (34) multiple-cloning site. The vector pUC118 was digested with *Hin*dIII, and the cohesive ends were made into blunt ends by use of the end-filling reaction of the Klenow polymerase (Boehringer Mannheim). The resulting vector is referred to as pUC118(-*Hin*dIII). To aid in site-directed mutagenesis, the region of the pOS4102 plasmid from the second *Eco*RI site to the *Sac*I site of the gene (nucleotides 996 to 1350, corresponding to residues 332 to 450 of the protein) was cloned into pUC118(-*Hin*dIII) and the resulting construct was named pUC118(-*Hin*dIII)/4102. One restriction enzyme site, *Acc*I, was added to the *cryIAa* gene at nucleotide 1203, corresponding to an *Acc*I restriction site at this location in the *cryIAc* gene. To assist in the addition of the other two restriction sites into the *cryIAc* gene, the region from *Sac*I to *Xho*I (nucleotides 1350 to 1836, corresponding to residues 450 to 612 of the protein) of *cryIAc* was cloned into pUC118 (-*Hin*dIII), and the resulting construct was named pUC118 (-*Hin*dIII)/4202. Site-directed mutagenesis was performed to add two restriction sites to the *cryIAc* gene. The two new restriction enzyme sites, *Asu*II at nucleotide 1497 and *Hin*dIII at nucleotide 1704, correspond to restriction sites already present at homologous positions in the *cryIAa* gene. Construction of the homolog-scanning mutants proceeded with the restriction sites available. A diagram of the chimeric toxins and restriction sites used for their construction is shown in Fig. 1. The constructs were then transformed into JM103 [gift from J. Messing; *supE thi rpsL* (*strA*) *endA1 sbcB15 hsdR4* Δ(*lac-proAB*) (F′ episome—*traD36 proAB lacI*^{q}ZΔM15)] and subsequently into SG21163 ([gift from S. Gottesman; Δ*lon-510 hptR* Em^{r} Δ*lac supF15 mc4100 bkg*)].

Site-directed mutagenesis. Site-directed mutagenesis was performed with single-stranded pUC118(-*Hin*dIII)/4102 and pUC118(-*Hin*dIII)/4202 by using the single-oligonucleotide method and the Kunkel selection method (24). Mutations were identified by the presence of a new restriction site(s),

subcloned into their respective pOS vectors, and transformed into *E. coli* JM103 for expression of the protoxin.

Crystal purification and protoxin solubilization. The *E. coli* cells expressing the mutant protoxins were grown for 72 h at 37°C in 500 ml of 2× YT medium (16 g of Bacto-tryptone, 10 g of Bacto-yeast extract, and 5 g of NaCl in 1 liter of H_2O) containing 50 μg of ampicillin per ml. To purify the crystals, the cells were pelleted and resuspended in 50 ml of lysing solution (15% sucrose, 50 mM EDTA, 50 mM Tris [pH 8.0], 10 μg of lysozyme per ml). The cells were incubated in lysing solution for 1 h at 37°C and then overnight at 4°C. Lysis of the cells by sonication (sonicator model W-385; Heat Systems-Ultrasonics, Inc.) was monitored by microscopic analysis. After lysis of the cells, the resulting pellet was washed three times with 0.5 M NaCl–2% Triton X-100, six times with 0.5 M NaCl, and once with deionized water. The crystals were solubilized in 50 mM Tris (pH 9.5)–10 mM dithiothreitol for 2 h at 37°C. The protein concentrations of the protoxins were determined by using the Coomassie Protein Determination Kit (Pierce).

Determination of *E. coli* intracellular protease sensitivity. To determine whether the chimeric toxin proteins are sensitive to *E. coli* intracellular proteases, we used the following cell lysate procedure. *E. coli* JM103 and SG21163 containing the pBDA constructs were grown for 24 h at two different temperatures, 20 and 37°C, in 2× YT medium containing 50 μg of ampicillin per ml. After 24 h of growth, 1.5 ml of the cultures was standardized to an optical density at 600 nm of 10.0, centrifuged, and resuspended into 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25) loading dye. To lyse the cells, we boiled the mixture for 10 min. Insoluble protein material was pelleted by centrifugation, and the supernatant fraction was analyzed by SDS-PAGE (7.5% acrylamide). After Coomassie brilliant blue staining, the total protein and 130,000-Da peak intensities were determined by densitometry (Hoefer, Inc.), and the peak area was calculated by using a digitizer (Numonics, Inc.).

Determination of altered protein structure. To determine the intracellular stability of the mutant protoxins, *E. coli* JM103 containing the mutagenized genes was grown at 37°C. After 48 h of growth, 1.5 ml of the cultures was standardized to an optical density at 600 nm of 10.0, centrifuged, resuspended in 200 μl of SDS-PAGE loading dye, and lysed by boiling for 10 min. The insoluble material was pelleted by centrifugation.

Trypsin digestion was used to determine whether the mutant protoxins could form trypsin-resistant toxins. Trypsin (1% [wt/wt]) was added to the protoxin solution (pH 9.5) and incubated at 37°C. After 1 h of incubation, 1% (wt/wt) trypsin was added and the mixture was incubated for another 1 h at 37°C. SDS-PAGE (7.5% acrylamide) was used to monitor the transition from the 130,000-Da protoxin to the 65,000-Da toxin.

A thermolysin digestion protocol modified from the procedure of Gent et al. (12) was used to further analyze structural alterations of mutant protoxins that yielded trypsin-resistant toxin cores. The modifications to the protocol were determined by experimentation to generate consistent data. Toxin (300 μg) was incubated at various temperatures with 2% (wt/wt) thermolysin (Boehringer Mannheim) in 50 mM Tris (pH 9.5)–10 mM $CaCl_2$. Aliquots calculated to contain 30 μg of toxin were withdrawn after a 30-min incubation at 37, 43.5, 50, 52.5, 55, 57.5, 60, 62.5, 65, or 70°C in a Brinkmann RM6 water bath. Thermolysin digestion was terminated by addition of 20 mM EDTA (final concentration). Samples were analyzed by SDS-PAGE (7.5% acrylamide), and the toxin protein bands were detected by Coomassie brilliant blue staining (Fig. 2). A linear correlation between the intensity of Coomassie brilliant blue staining and Cry protein concentration has been established by experimentation (3). To determine the relative amount of toxin remaining after thermolysin digestion at the indicated temperature, the band intensities were scanned with a densitometer (Hoefer, Inc.) and the area of the peaks was determined with a digitizer (Numonics, Inc.). The data were graphed as temperature versus percentage of toxin remaining (Fig. 3). The T_s value is the temperature at which 50% of the toxin remained after a 30-min digestion with thermolysin.

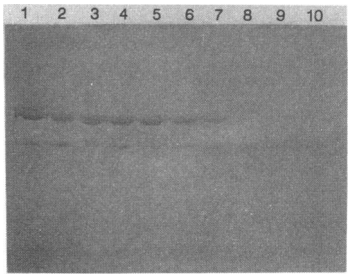

FIG. 2. Thermolysin digestion of P4111 toxin (trypsin-resistant peptide). Lanes: 1, control, no thermolysin digestion; 2, 37°C; 3, 43.5°C; 4, 50°C; 5, 52.5°C; 6, 55°C; 7, 57.5°C; 8, 60°C; 9, 62.5°C; 10, 65°C. The upper band in the gel is trypsin-resistant toxin, and the lower band is the thermolysin protein.

RESULTS

Intracellular stability of protoxin. Figure 1 illustrates the homolog-scanning mutant constructs used in this study. Previous results indicated that *E. coli* strains expressing Cry proteins produce a maximum yield at 48 h (8). The protoxin produced by each of these constructs was evaluated for altered protoxin structure by examination of the whole-cell protein pattern by SDS-PAGE at 48 h of growth. These data are summarized in Fig. 1. Mutants that do not

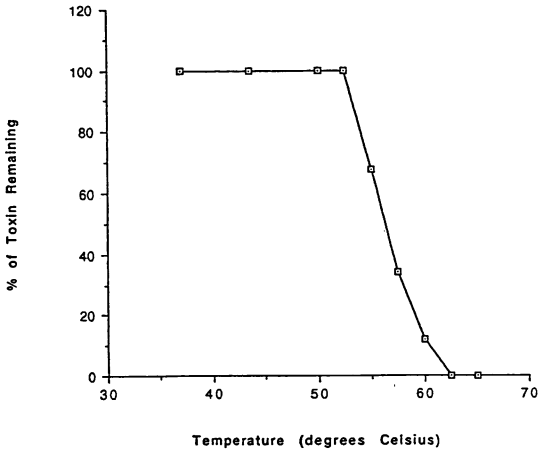

FIG. 3. Thermolysin digestion profile of P4111 toxin (trypsin-resistant peptide). The T_s value corresponds to the temperature at which 50% of the toxin remains. The profile represents one of three independent thermolysin/heat digestions of the P4111 toxin.

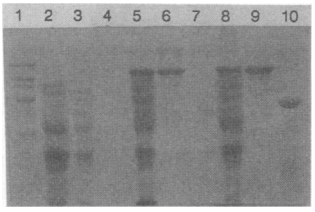

FIG. 4. Protease sensitivity comparison of group 1, 2, and 3 mutant protoxins. Lanes: 1, molecular mass standards (200, 116.25, 97.4, 66.2, and 45 kDa); 2 to 4, pBDA4117; 5 to 7, pBDA4115; 8 to 10, pOS4102. Lanes 2, 5, and 8 are 24-h whole-cell lysates. Lanes 3, 6, and 9 are isolated protoxins. Lanes 4, 7, and 10 represent trypsin digestion of the isolated protoxins found in lanes 3, 6, and 9, respectively.

accumulate protoxin are classified as group 1 mutants: pBDA4116, pBDA4216, pBDA4117, pBDA4217, pBDA 4118, pBDA4218, pBDA4119, pBDA4219, pBDA4120, pBDA4220, pBDA4221, pBDA4122, and pBDA4222, i.e., 13 of the 40 homolog-scanning mutants. An example of cell extracts of one of these mutants, pBDA4117, is shown in Fig. 4, lane 2.

Stability of protoxin—trypsin digestion. The presence of a 65,000-Da trypsin limit product, the toxic core, was determined by SDS-PAGE analysis of trypsin-digested protoxin. Of the 40 homolog-scanning mutants, 13 exhibited trypsin-sensitive toxins (group 2 mutant proteins): P4107, P4207, P4108, P4208, P4112, P4212, P4113, P4213, P4114, P4214, P4115, P4215, and P4121, as illustrated in Fig. 1. An example of one of these mutant proteins, P4115, is shown in Fig. 4 (lanes 5 through 7), compared with a group 1 protein, P4117 (lanes 2 through 4) and the native protein, P4102 (lanes 8 through 10).

Evidence for intracellular protease digestion. An experiment was designed to determine whether the absence of the group 1 protoxins is due to loss of the gene, failure of the gene to express, or susceptibility of the mutant protoxins to intracellular proteolysis. Mutants pOS4102 and pBDA4215 were chosen as representatives of group 3 and group 2, respectively, along with pBDA4116 and pBDA4216, which were chosen as representatives of group 1. Aliquots of *E. coli* JM103 cultures containing pOS4102, pBDA4215, pBDA4116, and pBDA4216 were withdrawn at 24 h and 48 h. The 130,000-Da protoxins can be seen in 24-h cell lysates of pOS4102, pBDA4215, pBDA4116, and pBDA4216 (Fig. 5, lanes 2, 4, 6, and 8). After 48 h of growth at 37°C and no change in the optical density at 600 nm, the cell lysates

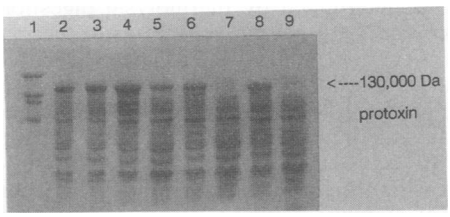

FIG. 5. Comparison of 24- and 48-h whole-cell lysates of pOS4102, pBDA4215, pBDA4116, and pBDA4216. Lanes: 1, molecular mass standards (200, 116.25, 97.4, and 66.2 kDa); 2, pOS4102, 24 h; 3, pOS4102, 48 h; 4, pBDA4215, 24 h; 5, pBDA4215, 48 h; 6, pBDA4116, 24 h; 7, pBDA4116, 48 h; 8, pBDA4216, 24 h; 9, pBDA4216, 48 h.

TABLE 1. *E. coli* 24-h cell lysate data

Construct	Group no.	% of lysate that is 130,000 protoxin in[a]:			
		JM103		SG21163	
		37°C	20°C	37°C	20°C
pOS4202	3	16	10	8	7
pKK223-3	NA[b]	0	0	0	0
pBDA4113	2	17	10	0	9
pBDA4213	2	18	5	0	8
pBDA4115	2	16	11	16	9
pBDA4215	2	5	10	0	0
pBDA4116	1	10	8	0	5
pBDA4216	1	8	0	0	0
pBDA4118	1	0	0	0	0
pBDA4218	1	0	0	0	0

[a] Numbers indicate percentage of total cellular lysate protein that is 130,000-Da protoxin as determined by SDS-PAGE (7.5% acrylamide) and scanning densitometry.
[b] NA, represents not applicable.

showed that protoxins produced by pOS4102 and pBDA4215 were stable (lanes 3 and 5), but no protoxin band was visualized in pBDA4116 and pBDA4216 (lanes 7 and 9). In similar experiments with the remainder of the group 1 mutants, the 130,000-Da band was not detected at 24 or 48 h by SDS-PAGE.

Two different *E. coli* strains, JM103 and SG21163, and two different incubation temperatures, 37 and 20°C, were used to compare the amount of protoxin accumulation for the designated mutants. Overall, the level of protoxin accumulation in JM103 was higher than that of the same construct in SG21163 (Table 1). All constructs in JM103, except pBDA4216, pBDA4118, and pBDA4218, accumulate protoxin at both 37 and 20°C. Only pOS4202 and pBDA4115 were found to accumulate protoxin in SG21163 at 37 and 20°C. pBDA4113, pBDA4213, and pBDA4116 demonstrated protoxin accumulation at 20°C but not at 37°C in SG21163.

Stability of toxin—thermolysin digestion. Homolog-scanning mutants that form a 65,000-Da tryptic resistant core on digestion with trypsin include group 3 mutant proteins: P4104, P4204, P4105, P4205, P4106, P4206, P4109, P4209, P4110, P4210, P4111, P4211, P4123, and P4223. The wild-type toxin proteins, CryIAa (P4102) and CryIAc (P4202), and group 3 mutant toxin proteins were analyzed by thermolysin digestion at a range of temperatures as described in Materials and Methods. The significance of the T_s values was determined by the standard t test ($\alpha = 0.01$). Interestingly, wild-type toxins P4102 and P4202 have significantly different thermolysin T_s values, 61 and 67°C, respectively. When subfractions of the region from nucleotides 996 to 1836 (corresponding to residues 332 to 612) are exchanged, the reciprocal exchange mutants have T_s values that are the same as or lower than those of their parental toxin (Table 2). Thus, the 4200 series has a higher range of T_s values (67 to 59°C) than the range of the 4100 series (61 to 56°C, except for 4105 which has a T_s of 65°C). Chimeric proteins P4105, P4106, and P4111 were found to be significantly different from P4102 (CryIAa control). The following were found to have significantly different T_s values from P4202 (CryIAc control): P4205, P4209, P4210, and P4211 (Table 2).

DISCUSSION

The chimeric protoxin genes whose construction was described in this work (pBDA) and others (pOS) described

TABLE 2. Temperature midpoint for thermolysin sensitivity of wild-type CryIAa (P4102) and CryIAc (P4202) proteins and group 3 mutant derivatives

Toxin	T_s (°C)[a]
P4102	61 ± 1
P4104	58 ± 2
P4105	65 ± 2[b]
P4106	56 ± 2[b]
P4109	61 ± 3
P4110	58 ± 2
P4111	58 ± 1[b]
P4202	67 ± 2[b]
P4204	65 ± 1
P4205	59 ± 2[c]
P4206	63 ± 2
P4209	62 ± 2[c]
P4210	59 ± 2[c]
P4211	61 ± 2[c]

[a] Mean and standard deviation for three independent tests.
[b] t-tested ($\alpha = 0.01$) significant difference from P4102.
[c] t-tested ($\alpha = 0.01$) significant difference from P4202.

elsewhere (10) were originally developed to locate functional regions of the toxin. Their insect specificity (9, 10) and receptor-binding (26) regions were previously analyzed by bioassay and binding studies, respectively. Because of the importance of protein structure for function, these constructs were scrutinized for altered protein structure by examination of their susceptibility to proteases. A total of 40 homolog-scanning mutants were analyzed (Fig. 1). On the basis of differing levels of susceptibility to proteases, three groups of mutant proteins were recognized. Examples from each group and their sensitivity to a variety of proteases are shown in Fig. 4.

Group 1 mutants are mutant protoxins that cannot be isolated as intact 130,000-Da proteins by the standard crystal isolation procedure. Examination of group 1 mutants by SDS-PAGE reveals the presence of multiple protein bands after solubilization of the crystal, but all bands are less than 130,000 Da (Fig. 4, lane 3). In the experiment designed to determine whether the lack of the 130,000-Da protoxin was due to an increased sensitivity to *E. coli* intracellular proteolysis, 24-h whole-cell lysates show the presence of the 130,000-Da protoxin band (Fig. 5, lanes 2, 4, 6, and 8) but after 48 h of incubation the protoxin band is absent in the group 1 mutants (Fig. 5, lanes 7 and 9). The presence of the protoxin band at 24 h and its absence after 48 h of incubation suggest that these chimeric protoxins are susceptible to intracellular proteolysis. Studies have demonstrated that as *E. coli* cells approach starvation conditions, the level of intracellular proteolysis also increases (14). Other studies have shown increased intracellular protease sensitivity to be correlated with alterations in protein stability and/or structure (31).

In another experiment reiterating the susceptibility of these mutants to intracellular proteases, *E. coli* SG21163, which has been determined to have a decreased level of intracellular proteolysis because of the possession of the *lon* mutation (15), was used. Overall, the level of protoxin accumulation for the protoxins is lower in SG21163 than in JM103. At 37°C, the control (pOS4202) in SG21163 accumulates only half the level of protoxin found in JM103. The amount of protoxin accumulation for the mutant pBDA4115 was found to be the same in both strains and at both temperatures. The protoxin accumulation levels for the remainder of the mutants tested were lower in SG21163 than in JM103. Only one of the mutants, pBDA4115, demonstrated protoxin accumulations at both 20 and 37°C in SG21163. In summary, the *lon* mutation of *E. coli* SG21163 offered no protection from intracellular proteolysis for the chosen mutant proteins (Table 1). The reason for the lower level of protoxin accumulation in SG21163 is not known. One possible explanation for higher protoxin accumulation in JM103 than in SG21163 could be that the higher level of protoxin expression allows the protoxins to form aggregates that protect mutant proteins from intracellular degradation (5).

All of the group 1 mutant proteins involve reciprocal exchanges within the carboxyterminal portion of the toxin. This region (residues 450 to 612) contains three of the five highly conserved residue blocks which are theorized to play a significant role in the protein structure of the toxin (27). This disruption of structurally important regions might explain the increased susceptibility of these chimeric proteins to intracellular proteases.

For the homolog-scanning mutants that express a stable protoxin, the stability of the protein structure was examined by several methods. The simplest assay was digestion with trypsin (2% [wt/wt]), which approximates the activation of the protoxin by insect proteases (6). Cell lysates and crystal isolation demonstrate that 130,000-Da protoxins can be isolated from group 2 mutants (Fig. 4, lanes 5 and 6). Group 2 protoxins, however, did not form a 65,000-Da trypsin-resistant toxic core and are therefore referred to as trypsin sensitive (Fig. 4, lane 7). Trypsin sensitivity could be caused by the alteration of the protein structure of the toxins to expose arginines, lysines, and, to a lesser degree, histidines for cleavage by trypsin. Results from Sauer's laboratory have demonstrated that protein structural stability is correlated to intracellular protease susceptibility (29) as well as to exogenous protease treatment (30). Differences in proteolytic digestion measure the stability of a protein since denatured or unfolded proteins are more sensitive to proteases than are native ones (16). This supports the conclusion that group 2 proteins possess a lesser degree of structural alteration than group 1 proteins do. From the perspective of the insecticidal activity of the toxin, the trypsin sensitivity of the group 2 mutant proteins is a selective disadvantage because trypsin-like proteases within the insect midgut would render the toxin inactive. Certain members of group 2, in particular P4107, P4207, P4108, and P4208, have been found to possess large decreases in insecticidal activity (10). This reduction in insecticidal activity is most probably due to the increased trypsin susceptibility of these group 2 mutants.

Group 3 mutants form trypsin-resistant cores (Fig. 4, lane 10), but, as determined by thermolysin digestion profiles, these toxic cores are not equal in terms of thermal stability (Table 2). Thermolysin digestion has been used to determine protein structural differences between homologous proteins (33), changes in protein conformation as a result of external environmental stimuli (23), and proteolysis of renaturation products (22). In the present study, thermolysin combined with increasing temperatures has been used as a comparative measure of stability between native and mutant proteins (12). Thermolysin will digest unfolded or denatured proteins far more readily than folded proteins (16). The differences in the T_s values between mutant and native toxins results from alterations in the protein stability. Alterations in protein stability are a direct result of alterations in the protein

structure of the toxin. By comparing the X-ray crystal structures of wild-type and temperature-sensitive mutant lysozymes, specific differences in their three-dimensional protein structures can be correlated with changes in stability (1).

The thermolysin data generated for the group 3 chimeric toxins (Table 2) indicate a significant difference in T_s values between the parental toxin proteins, CryIAa (61°C) and CryIAc (67°C). These two toxins share extensive homology in the N-terminal region (residues 1 to 331), but they contain a region of hypervariability within the C-terminal region from residues 332 to 612 (11, 19). This would suggest that residues 332 through 612 influence the stability of the protein toxins. Indeed, when these regions are exchanged, as in P4105 and P4205, their thermal stability properties are exchanged (Table 2).

The T_s values generated for the group 3 mutants were compared with previously reported bioassay data (9, 10) to determine whether a correlation between protein stability and insecticidal activity exists. Some group 3 mutant toxins were found to exhibit the same T_s value but possess differences in insect toxicities. For example, P4104, P4110, and P4111 all possess a T_s value of 58°C, but in a *B. mori* bioassay they have 50% lethal concentrations (LC_{50}s) of 0.014, 35.202, and 0.200 μg, respectively. *H. virescens* and *T. ni* bioassay data also demonstrated the same type of results, i.e., the same T_s value but significantly different insect toxicities. P4102, P4109, and P4211 all have T_s values of 61°C, but in a *B. mori* bioassay they have LC_{50}s of 0.002, 0.031, and >7.0 μg, respectively. P4102, P4109, and P4211 also have the same T_s value but have different insecticidal activities against *H. virescens* and *T. ni* (9). In a *B. mori* bioassay, P4205 and P4210 have LC_{50}s of 0.004 and 0.047 μg, respectively. An *H. virescens* bioassay shows that they have LC_{50}s of 4.21 and 0.37 μg, respectively. Both chimeric proteins, however, have T_s values of 59°C. We conclude that these mutants affect function but not structure.

Conversely, some toxins are shown to have relatively the same insecticidal activity but different T_s values. In an *H. virescens* bioassay assay, P4202 and P4210 have LC_{50}s of 0.37 and 0.39 μg, respectively, but P4202 has a T_s value of 67°C whereas P4210 has a T_s value of 59°C. In the same *H. virescens* bioassay, P4204 and P4209 had LC_{50}s of 0.29 and 0.28 μg, respectively. As in the first example, the T_s values for P4204 and P4209 were determined to be different, i.e., of 65 and 62°C, respectively. P4102 and P4106 have relatively the same *H. virescens* bioassay activity, resulting in LC_{50}s of 2.97 and 3.16 μg, respectively. The T_s values for these two proteins are 61 and 56°C, respectively. Chimeric proteins P4105 and P4209 have different T_s values, of 65 and 62°C, respectively. In the *H. virescens* bioassay, P4105 and P4209 demonstrate relatively the same activity, 0.33 and 0.28 μg, respectively. The wild-type proteins, P4102 (CryIAa) and P4202 (CryIAc), have approximately the same *M. sexta* insecticidal activity (32), but the T_s values determined for this paper show that P4202 has a T_s value of 67°C whereas P4102 has a T_s value of 61°C. These examples illustrate that a structurally stable protein may have a range of T_s values. We conclude that in none of the group 3 mutant proteins did a structural alteration cause the loss or alteration of biological activity.

In summary, it was observed that mutations in *B. thuringiensis* δ-endotoxin that led to structural alterations may be degraded in situ by *E. coli* cellular proteases. In vitro degradation by trypsin also identifies structural alterations caused by mutagenesis. The use of thermolysin and heat reveals minor structural alterations, but a direct correlation between lower T_s values and a decrease in insecticidal toxicity could not be found. The thermolysin technique, however, allowed the detection of protein structural differences between native and mutant proteins that were not found by other protease digestion methods. Interestingly, we observed that P4105 and P4205, chimeric proteins in which the hypervariable regions of CryIAa and CryIAc have been exchanged, demonstrate an exchange in their T_s values. However, at this time the importance of the T_s differences in relation to alterations in the CryIA protein structure cannot be determined. The CryIA protein structure has yet to be elucidated; therefore, the absolute structural differences between the mutant and parental proteins cannot be found. The T_s values, in the context of this paper, were determined to be a reliable and uncomplicated measurement of the overall stability of a protein and the effect of mutagenesis on protein stability. We conclude that some group 3 mutants in our study have minor structural alterations but that these were not significant relative to their biological activity.

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