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Expression of Three Genes Coding for 135-Kilodalton Entomocidal Proteins in *Bacillus thuringiensis kurstaki*

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Abstract. The HD-1 strain of *Bacillus thuringiensis* (B.t.) *kurstaki* contains three homologous genes coding for 130–134-kilodalton entomocidal proteins [13]. In the present study, expression levels of these genes in strains of B.t. *kurstaki* were determined. In attempts to isolate a protein coded by a single gene, a number of variants were derived from strains of B.t. *kurstaki,* such as HD-263 and HD-1, by plasmid curing. The entomocidal proteins produced by the parental strains and their plasmid-cured variants were isolated by Sephacryl S-300 column chromatography and peptide-mapped by high performance liquid chromatography (HPLC). The results indicated that HD-263 produced two distinctive proteins, one identical with the protein of HD-73, which contains only a "6.6 kb Hind III class" gene, and the other protein presumably coded by a "4.5 kb Hind III class" gene. HPLC analysis revealed that 70% of the total protein in the HD-263 crystals consisted of the product of the 6.6 kb gene (6.6-kb protein), and the remaining 30% was the 4.5-kb protein. In the case of HD-1, the crystal consisted of at least two different proteins in equal amounts (50% each). The gene coding for one of these proteins was presumed to be a "5.3 kb Hind III class" gene. The remaining 50% of the HD-1 crystal was accounted for by a protein similar to the 4.5-kb protein identified in HD-263. It appeared that the 6.6-kb protein was expressed poorly, if it was indeed expressed, in the HD-1 strain.

Bacillus thuringiensis (B.t.) is pathogenic to a variety of insects, notably to lepidopterous species. The bacillus produces a spore and one or more proteinaceous endotoxins in crystalline form. A large number of strains have been isolated from dead insects, soil samples, etc., and classified into some 20 subspecies, primarily on the basis of flagellar antigens [2]. The HD-1 strain (subsp. *kurstaki*), isolated as a highly potent strain [3], has been commercially produced for many years in the USA. Because of its economic importance, intensive studies have been conducted on the endotoxins of HD-1. The major component of the HD-1 crystals is a protein or proteins having molecular weights of approximately 130,000 [10]. The HD-1 strain also shows a weak but significant toxicity to mosquito larvae, while HD-73, a different strain of subsp. *kurstaki*, does not [8]. The mosquitocidal activity specific to HD-1 led Yamamoto and McLaughlin [23] to discover a 65-kilodalton mosquitocidal protein (termed P-2), which occurred in some *kurstaki* strains such as HD-1 and HD-263. An electron microscopic analysis of the crystals of HD-1 and HD-73 revealed that the HD-1 strain produced two differently shaped crystals, one bipyramidal and the other cuboidal, whereas HD-73 had only bipyramidal crystals [11].

Bacillus thuringiensis strains have been shown to harbor a variety of plasmids, some of which have been implicated in crystal (endotoxin) production [6, 14]. Gonzalez et al. [7] reported that the 50-megadalton plasmid of HD-73 appeared to carry a gene for crystal production and was different in size from a 44-megadalton crystal-coding plasmid of HD-263. When these plasmids were transferred to acrystalliferous strains of B.t. and *B. cereus*, the resulting trancipient strains produced crystal proteins serologically identical with the crystal proteins of the donor strains. Schnepf and Whiteley [18] reported cloning a toxin protein gene from an HD-1 strain in *Escherichia coli*. The HD-1 used in their cloning study was isolated from a commercial B.t. formulation of HD-1 called Dipel; its plasmid pat-

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tern is somewhat different from that of another HD-1 strain currently available from the USDA B.t. culture collection [14]. Therefore, the strain was designated as HD-1-Dipel [18]. Subsequently, several additional genes coding for 130- to 134-kilodalton toxin proteins were cloned from HD-1 and other *kurstaki* strains [1, 9, 12, 15, 20]. These genes were classified into three classes as 6.6, 5.3, and 4.5 kb genes by Hind III digestion and Southern blotting [13]. It was demonstrated that HD-1 contains all three different class genes [13, 21].

In the present study, expression levels of these toxin genes in B.t. *kurstaki* strains were determined by HPLC peptide mapping.

Materials and Methods

Strains. *Bacillus thuringiensis kurstaki* strains HD-1, HD-73, HD-87, HD-255, HD-263, and HD-279 were obtained from the USDA culture collection in Brownsville, Texas. They were reisolated by plating on nutrient agar. Since there is a possibility that the reisolated strains could be different from the original strains, they were renumbered as HD1-1, HD73-1, HD87-1, HD255-1, HD263-1, and HD279-1. Partially cured variants of HD1-1 and HD263-1 were isolated after spontaneous loss of plasmid(s) or after growth at 42°C. HD1-27 was isolated from a commercial B.t. formulation called Thuricide. HD-1-Dipel was kindly provided by Dr. H. R. Whiteley, University of Washington, and used without any further isolation.

Plasmid pattern analysis. The plasmid array of each strain was analyzed on agarose gels by a modification of the slot-lysis method of Eckhardt [4], as previously described [7].

Analysis of crystal production. Crystal production was monitored by phase-contrast microscopy. In addition, production of 130- to 134-kilodalton proteins (in this paper, they are termed 135-kilodalton proteins when described as a group) and the 65-kilodalton mosquitocidal protein (P-2) was confirmed by immunoelectrophoresis of two differently prepared samples. For bacteria grown on nutrient agar at 30°C for 72 h, a loopful of the sample containing spores and crystals, if any, was taken from the agar plate and suspended in 30 μl of 2% 2-mercaptoethanol-NaOH, pH 10.5 (Me-NaOH). After 30-min incubation on ice, 15 μl of 1 *M* Tris-HCl, pH 7, was added to lower the pH to about 8. The neutralized sample (4 μl) was then analyzed by rocket immunoelectrophoresis with rabbit antisera directed against total HD-1 and HD-73 crystal proteins and against P-2 isolated from HD-1 [23]. The details of rocket immunoelectrophoresis were described elsewhere [24]. This method was used to screen a large number of B.t. strains for the expression of a specific protein. Alternatively, crystal proteins were extracted from the crystal sample produced in a peptonized milk-nutrient broth, chromatographed on a Sephacryl S-300 column, and the column eluate was analyzed by fused-rocket immunoelectrophoresis with antiserum against HD-1 crystal proteins as described previously [22].

HPLC peptide mapping. For HPLC peptide mapping of crystal proteins, sample preparation and analysis were done according to the method of Yamamoto [22] with a slight modification as outlined as follows: B.t. strains were cultured in twelve 500-ml flasks, each containing 100 ml of peptonized milk–nutrient broth at 30°C for 72 h. The crystal–spore mixture was extensively washed in 0.5 *M* NaCl to remove contaminating cellular proteinases. The toxin protein was purified by Sephacryl S-300 column chromatography, digested with TPCK-treated trypsin, and the resulting peptides were mapped by HPLC, with a Varian 5020 equipped with a MCH-5-N-CAP reverse phase column. The peptides were eluted from the column with acetonitrile. Beginning at 0.5 min, the concentration of acetonitrile in 0.1% phosphoric acid was increased from 0% to 35% over a 50-min period, and from 35% to 50% in the following 5 min. In order to ensure that no cellular proteinases were contaminating the B.t. crystal proteins, all samples were subjected to the incubation at 37°C without trypsin and analyzed by HPLC for small fragments.

Isolation and characterization of the trypsin-resistant core. The 135-kilodalton protein produced by HD1-2 was isolated by Sephacryl S-300 column chromatography. The isolated protein was dissolved in 100 m*M* Tris-HCl, pH 8, at 10 mg/ml and then digested by trypsin at 0.1 mg/ml at 37°C. During the 4-h digestion, progress of the digestion was monitored by SDS–PAGE. The trypsin-resistant core of 66-kilodalton was then isolated by FPLC (Pharmacia) with a MonoQ HR5/5 column. The isolated core was denatured in 8 *M* urea at 100°C for 10 min, dialyzed in water, and then lyophilized. The core was then digested by trypsin for the second time in 0.1 *M* ammonium bicarbonate until its completion. Peptides produced by the second trypsin digestion were separated by HPLC, and their molecular weights were determined by liquid matrix secondary ion mass spectrometry (VG-ZAB HF).

Results

Plasmid curing. Isolation of spontaneous or heat induced variants of HD-1 and HD-263 yielded a large number of partially cured strains, some of which produced the crystals, while others did not. A representative selection of these strains is listed in Table 1, and their plasmid patterns are shown in Fig. 1. Interestingly, the 44-megadalton toxin plasmid of HD-1 was cured spontaneously with detectable frequency, as in HD1-2 and HD1-10, while the 44-megadalton toxin plasmid of HD-263 was cured only after growth at 42°C, as in HD263-4. Curing of the large toxin plasmids of HD-1 (110 megadalton) and of HD-263 (115 megadalton) required growth at the elevated temperature, as in HD1-9 and HD263-7.

Production of the toxin proteins. Sephacryl column chromatography permitted isolated of 135-kilodalton toxin proteins and the 65-kilodalton (P-2) protein from crystal extracts with reasonable purities for peptide mapping (Fig. 2A). In Fig. 2, two chromatographs of HD263-5 and HD263-7 are shown to illustrate representative P-2-producing

Table 1. Correlations between plasmid patterns and crystal proteins *thuringiensis* subsp. *kurstaki* and their variants

HD No.	1-1	1-2	1-7	1-9	1-10	Dipel	263-1	263-4	263-5	263-7
					Protein production					
135[a]	+	+	+	+	−	+	+	+	+	+
65[a]	+	+	+	−	−	+	+	+	+	−
					Plasmid (megadalton)					
	120	120				120	130	130	130	130
	110	110	110			110	115	115	115	
							60	60	60	60
	52	52	52	52	52	52				
	51	51	51							
	44		44	44			44		44	44
							43	43	43	43
	29	29	29	29	29	29				
	9.3	9.3								
							7.5	7.5	7.5	7.5
	5.4	5.4					5.4	5.4		
	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2
							5.0	5.0		
	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9

[a] Grown in the peptonized milk–nutrient broth, analyzed by immunoelectrophoresis.

and non-producing strains, respectively. The 135-kilodalton protein or proteins eluted at a volume of 210 ml, and P-2, if it was produced by the strain, at 320 ml. After chromatography, the column eluates were examined by fused-rocket immunoelectrophoresis (Fig. 2B). Since the 135-kilodalton proteins are highly homologous, the immunoelectrophoresis could not determine the number of 135-kilodalton proteins in the first peak. In the case of P-2-producing strains, such as HD263-5, the 135-kilodalton protein(s) partially overlapped P-2 at an elution volume of around 260 ml. By discarding these overlapping fractions, pure samples were obtained.

In this study, Sephacryl chromatography was also used to determine whether strains produced P-2. The results were correlated with the presence or absence of specific plasmids, as summarized in Table 1. When HD-1 lost its 110-megadalton plasmid, it ceased producing P-2, and a similar observation was obtained for the 115-megadalton plasmid of HD-263. These results indicate that 110-megadalton and 115-megadalton plasmids encode genes for P-2 production. We confirmed the previous report [24] that HD-73 did not produce P-2.

The situation with the 135-kilodalton proteins was more complicated than for P-2. Kronstad et al. [14] have shown by DNA hybridization that HD-1 contains three plasmids encoding gene(s) for 130-kilodalton (135 kilodaltons) crystal protein. Presumably, two of these three plasmids are those we term 44-megadalton and 110-megadalton plasmids. We have obtained data suggesting that these plasmids participate in the production of the protein. As shown in Table 1, HD-1 ceased producing the 135-kilodalton proteins only when it lost both the 44-megadalton and the 110-megadalton plasmids. Likewise, in HD-263, loss of either the 44-megadalton or the 115-megadalton plasmids did not eliminate the production of these proteins. It has been shown that an additional HD-1 plasmid of either 51-megadalton or 52-megadalton bears a toxin gene [14]. The plasmid curing study indicated that the gene on one of these plasmids was not expressed at a detectable level.

Expression of the 135-kilodalton proteins. Peptide maps of the 135-kilodalton proteins isolated from variants of HD-1 and HD-263 are shown in Fig. 3. The peptide pattern of HD263-7 was obviously different from that of HD263-4. Plasmid analysis had shown that HD263-4 retained the 115-megadalton plasmid but lost the 44-megadalton plasmid, and HD263-7 retained the 44-megadalton plasmid, but lost the 115-megadalton. The peptide patterns of HD263-4 and HD263-7 proteins were different from that of HD263-1, which contains both the 44-megadalton and 115-megadalton plasmids. The peptide mapping further indicated that the protein coded by

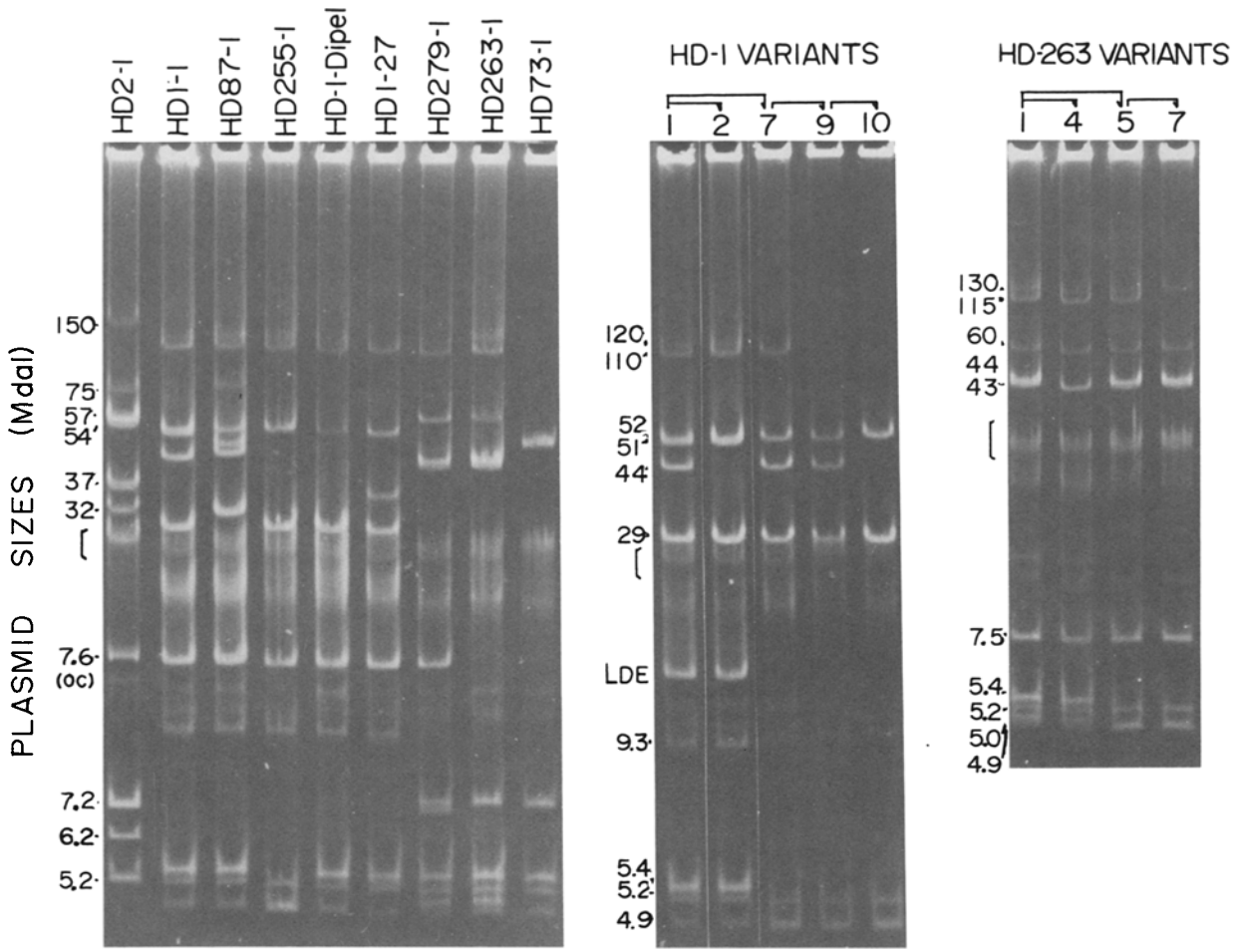

Fig. 1. Modified Eckhardt lysate electrophoresis of plasmids isolated from several strains of subsp. *kurstaki* and their derivatives. Spheroplasts of *Bacillus thuringiensis* cells were lysed on the agarose gel (0.5%) by using a layer of the SDS solution. Electrophoresis was conducted with constant current at 3 mA for the first 60 min, and then 7 mA for the following 30 min, and finally at 28 mA for 3–4 h. After electrophoresis, plasmid bands were visualized with EtBr staining. The strain number of each isolate or derivative is written above the line. The bracket ([) indicates the region where linear fragments of large plasmids and chromosomal DNA are found. Arrows drawn above the strain numbers of HD-1 variants (B) and HD-263 variants (C) indicate the genetic derivations of the variants.

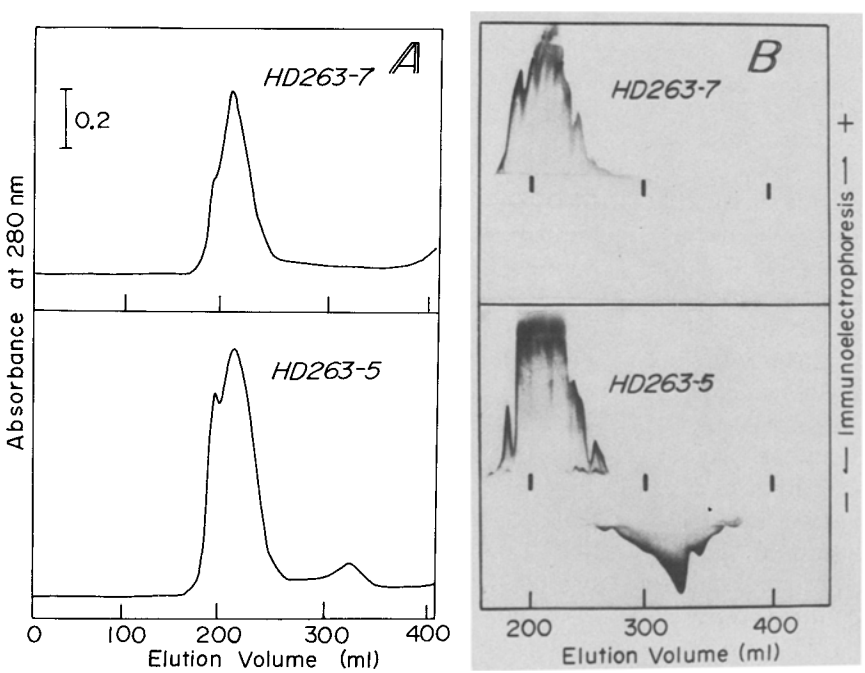

Fig. 2. Separation of P-1 and P-2 toxin proteins by Sephacryl S-300 column chromatography. Crystals (200 mg) from a strain producing only P-1 (HD263-7) or P-1 and P-2 (HD263-5) were solubilized in 4 ml Me-NaOH at pH 10. The solution was clarified by centrifugation at 100,000 *g* for 30 min and the extract (supernatant) was chromatographed on a Sephacryl S-300 column (2.5 × 100 cm), equilibrated and eluted with 50 m*M* Tris-HCl, pH 8, containing 2% 2-mercaptoethanol and 1 m*M* EDTA. Elution was monitored by UV absorption at 280 nm (A) and fused-rocket immunoelectrophoresis with an agarose gel (10 × 10 × 0.1 cm) containing 250 μl anti-HD-1-crystal-protein-serum (B). The antiserum was raised in rabbits with a mixture of P-1 and P-2 isolated from HD-1 crystals. P-1: The 135-kilodalton protein.

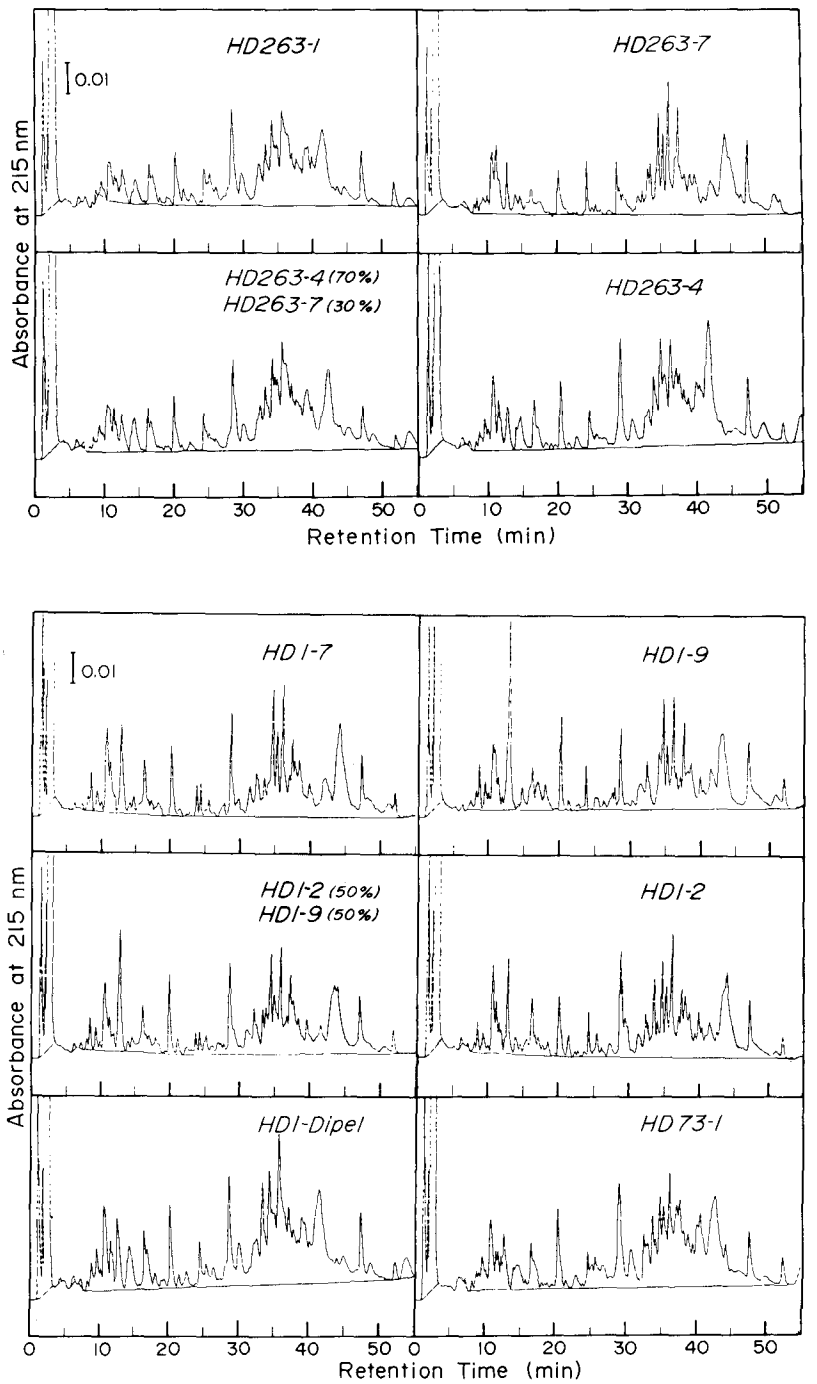

Fig. 3. Peptide maps of P-1 proteins isolated by column chromatography. P-1's were isolated from strains whose numbers appear on the chromatogram by Sephacryl S-300 column chromatography, as shown in Fig. 2. The P-1 proteins were concentrated by precipitation at pH 4.4 (pI of P-1), dissolved in 100 m*M* Tris-HCl, pH 8, at a concentration of 10 mg/ml, and digested with 1/100 volume of TPCK-treated trypsin added twice during a 4-h incubation at 37°C. After the digestion, 100 μl was sampled, mixed with 25 μl acetic acid, and filtered through a 0.45 μ filter. Peptides in the 10-μl filtrate were separated on a reverse phase column (Varian MCH-5-N-CAP, 0.4 × 15 cm) by using a Varian 5020 HPLC. Elution was first with 0.1% phosphoric acid for 0.5 min, followed by gradually increasing acetonitrile from 0% to 35% over a 50-min period, and then to 50% over the last 5 min. The peptides were detected with a UV monitor (Varian UV-5) at 215 nm. The baseline drift due to the absorbance of acetonitrile was computer corrected, by use of data obtained without sample injection. P-1: The 135-kilodalton protein.

the 115-megadalton plasmid of HD263-4 was very similar to, if not the same as, the 135-kilodalton protein of HD-73. When the proteins of HD263-4 and HD263-7 were mixed in different ratios at 10% increments and the mixtures peptide-mapped, a mixture of 70% of HD263-4 and 30% of HD263-7 produced the same peptide pattern as that of HD263-1.

There were minor but significant differences in the patterns of HD1-2 and HD1-9 which had lost either the 44- or 110-megadalton plasmids. The peptide pattern of the 135-kilodalton protein isolated from HD1-9 was unique to this strain. The peptide pattern of HD1-9 suggested that no HD-73 (6.6-kb) protein was expressed in an amount detectable by HPLC. The pattern of HD1-2 was somewhat similar to that of HD263-7, but HPLC indicated that they were not identical proteins. As in the case of HD-263, the proteins of HD1-2 and HD1-9 were mixed and peptide mapped. A mixture which contained

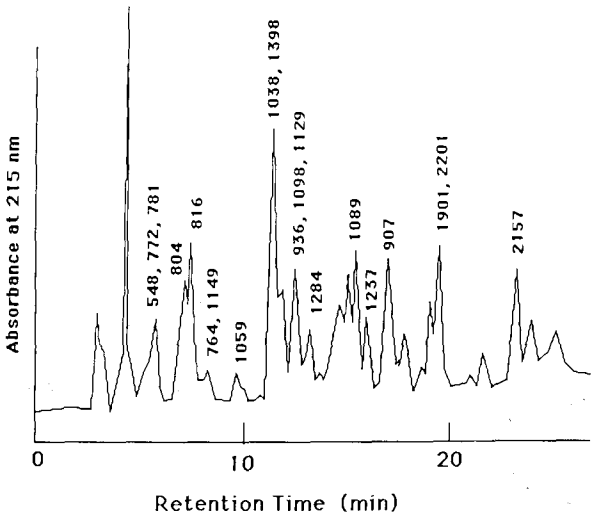

Fig. 4. Peptide mapping of the trypsin-resistant core of the protein from HD1-2. The 135-kilodalton protein produced by HD1-2 was isolated by Sephacryl S-300 column chromatography, as shown in Fig. 2. The isolated protein dissolved in 100 m*M* Tris-HCl, pH 8, at 10 mg/ml was digested by trypsin at 0.1 mg/ml at 37°C for 4 h. The trypsin digests containing the resistant core were then separated by FPLC (Pharmacia) with a MonoQ HR5/5 column. The core isolated by FPLC was denatured in 8 *M* urea, dialyzed in water, and then lyophilized. The denatured core was then digested by trypsin for the second time in 0.1 *M* ammonium bicarbonate until completion. Peptides produces by the second trypsin digestion were separated by HPLC, as in Fig. 3, and their molecular weights were determined by liquid matrix secondary ion mass spectrometry.

Table 2. Molecular weights of tryptic peptides derived from the proteinase-resistant core of the HD1-2 protein

	AA residue no.[a]		
MW[b]	4.5 kb[c]	5.3 kb[c]	6.6 kb[c]
548	255–258	—	255–258
764	88–93	88–93	88–93
772	287–292	—	—
781	193–198	193–198	193–198
804	259–265	259–265	259–265
816	218–224	218–224	218–224
907	174–181	174–181	174–181
936	360–367	—	—
1038	210–217	210–217	210–217
1059	502–511	—	—
1089	490–500	—	—
1098	512–521	514–523	—
1129	449–457	—	—
1149	478–489	—	—
1234	182–192	182–192	182–192
1284	199–209	—	199–209
1398	116–127	116–127	116–127
1901	266–281	266–281	266–281
2157	601–618	603–620	—
2201	458–477	—	—

[a] Number of amino acid residues showing at the start and end of each tryptic peptide.
[b] Molecular weights of tryptic peptides isolated from the core of the HD1-2 protein by HPLC.
[c] Type of the proteins: 4.5 kb, Schnepf et al. [19]; 5.3 kb, Thorne et al. [20]; 6.6 kb, Adang et al. [1].

equal amounts (i.e., 50% each) of the proteins from the two variants showed a peptide pattern identical with that of either HD1-1 or HD1-7. HD1-7 is a partially cured variant of HD1-1, but it retains both the 44- and 110-megadalton plasmids.

HD1-2 appeared to be producing a protein slightly different from the protein coded by the 4.5-kb-class gene of HD263-7. The differences, however, might be due to the second protein of HD1-2. The second protein could be a 6.6-kb protein. This was the case with HD-1-Dipel, which had two proteins. The peptide pattern of HD-1-Dipel was identical with the pattern of a mixture of the HD263-4 (6.6 kb) and the HD263-7 (4.5 kb) proteins. Unlike HD-1-Dipel, mixing two proteins from HD273-4 and HD263-7 failed to reproduce the peptide profile of HD1-2. In order to confirm that HD1-2 is not expressing a significant amount of a 6.6-kb protein, the trypsin-resistant core of the HD1-2 protein was examined by peptide mapping and mass spectrometry. Comparison of the amino acid sequences of three proteins—the 4.5-kb protein of HD-1-Dipel [19], the 6.6-kb protein of HD-73 [1], and the 5.3-kb protein of HD-1 [20]—revealed notable differences, particularly in the domain from residues 280 to 740. Therefore, separation and subsequent analysis of the trypsin-resistant core of the 4.5-kb protein (29 Ile to 608 Arg or 611 Lys; unpublished data of Yamamoto and Ehmann) from HD1-2 should distinguish it from the cores of the other two proteins. Trypsin digestion of the HD1-2 protein produced only one core separable by MonoQ HR column chromatography. Several peaks were observed, but only one of these contained a protein (66 kilodalton) large enough to be the trypsin-resistant core. After urea denaturation, the trypsin-resistant core isolated by MonoQ HR column chromatography was further digested by trypsin. Tryptic peptides were separated by HPLC and analyzed by mass spectrometry (Fig. 4). All molecular weights shown in Fig. 4 indicated that the core protein was derived from the protein coded by the 4.5-kb-class gene of HD-1-Dipel, whose nucleotide sequence had been determined by Schnepf et al. [19] (Table 2). Although there are technical limitations in this method (e.g., if a peptide is too large [>3,000], its accurate

molecular weight cannot be determined), no tryptic peptides having molecular weights specific to tryptic peptides of the 5.3-kb protein of HD-1 or the 6.6-kb protein of HD-73 were observed.

Discussion

The present study has demonstrated that the HD-1-type B.t. strains including HD-1 and HD-263 were indeed expressing at least two genes coding for 135-kilodalton proteins in different relative amounts. These strains also express one 65-kilodalton P-2 protein. Minnich and Aronson [17] reported that the production of the toxin protein in HD1-9 is temperature dependent. Our HD1-9, when cultured in the peptonized milk–nutrient broth, produced the crystals at 25°C as well as at 30°C. Therefore, the toxin synthesis in HD1-9 may be not only temperature dependent but also nutrient dependent. It is possible that the wild-type HD-1 and H-263 strains express their genes at different levels when grown in different conditions. However, our preliminary study (unpublished) in this regard suggests that the expression of a toxin gene is fairly stable relative to the expression of the other gene regardless of the culture conditions.

Recent developments in HPLC instrumentation have made it possible to analyze a very complex peptide mixture [5], and a change of only one amino acid residue in a peptide can be distinguished by HPLC [16], in particular with instruments equipped with a computer-controlled solvent delivery system. Yamamoto [22] has demonstrated that HPLC could be effectively used in characterization of the endotoxins isolated from B.t. crystals. His results indicated that the HPLC peptide patterns were very reproducible when the same column and the same analytical conditions were employed. In the present study, we confirmed Yamamoto's observation that standard deviations of retention times and area counts of peptide peaks eluted in the first 40 min were well within the limits of 1% and 15% respectively. Our conclusion that strains HD-1 and HD-263 express at least two genes coding for the 135-kilodalton proteins is strongly supported by the duplication of the peptide pattern of the protein found in the parental strain by simply mixing two proteins isolated from plasmid-cured variants. Furthermore, we have shown that HPLC can be used to classify the 135-kilodalton proteins produced by different B.t. strains. For example, HD279-1, which was expected to be similar to HD263-1 from their plasmid patterns, had a peptide pattern identical with that of HD263-1. Likewise, HD87-1, similar to HD1-1 in its plasmid pattern, had an HD-1-type pattern.

Approximately 70% of the 135-kilodalton proteins produced by HD-263 appeared to be the same as the protein of HD-73 or HD253-4. Kronstad and Whiteley [13] have reported that HD-263 contains 4.5-kb and 6.6-kb class genes, whereas HD-73 contains only the 6.6-kb gene. The gene coding for the protein comprising 70% of the HD-263 proteins is undoubtedly the same as the 6.6-kb gene of HD-73. The remaining 30% of the HD-263 proteins are unique to HD263-7 and presumed to be coded by a 4.5-kb class gene. In the case of HD-1, expression levels of its genes were different from the case of HD-263. HD-1 reportedly contains a 5.3-kb class gene on the 66-kb plasmid (i.e., 44-megadalton plasmid in our term) in addition to two other genes found in HD-263 [13]. One of our plasmid-cured variants of HD-1, HD1-9, which retained the 44-megadalton plasmid but lost the 51- and 110-megadalton plasmids, produced a unique protein comprising 50% of the HD-1 proteins. Although the HD1-9 protein has not been sequenced, it is certain that the protein is the product of the 5.3-kb gene of HD-1. Table 2 clearly indicates that the HD1-2 protein, if the variant produces one protein, is coded by a 4.5-kb class gene. We could not detect the 6.6-kb protein of HD-73 in HD1-2. Since there are very minor but significant differences in peptide patterns between the proteins of HD263-7 and HD1-2, it is possible that HD1-2 produces a small amount of the second protein. The techniques employed in this study were not sensitive enough to detect the second protein if it is expressed in an amount of less than 10% of the major protein. It is interesting to note that HD1-1 and its variants, including HD1-2 and HD1-7, do not express the 6.6-kb class gene in a detectable amount, but the gene was expressed in HD-1-Dipel.

Previously, Yamamoto et al. [24] characterized the 135-kilodalton proteins isolated from over 20 naturally occurring B.t. subsp. *kurstaki* strains and found that the proteins produced by HD-1-type strains were different from one another in immunochemical and insecticidal characters. However, no such differences were found among the HD-73-type strains. Prior to the discovery of three genes in HD-1 and its related strains, it was difficult to explain why HD-1-type strains were heterogeneous. This heterogeneity can now be explained by our present study demonstrating differences in the expression level of these genes. Differences in expression lev-

els of the genes among HD-1-type strains may be reflected in differences in their insecticidal activity spectra. For example, the crystals of HD-263 were reported to be more active than those of HD-1 on *Heliothis virescens,* whereas the situation was reversed for *trichoplusia ni* [24]. The amount of P-2 produced by both HD-1 and HD-263 is a small fraction of that of the 135-kilodalton proteins, and the activities of P-2 against these insects were reported to be almost equal to or less than that of the 135-kilodalton proteins.

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