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Use of Oligonucleotide Probes To Study the Relatedness of Delta-Endotoxin Genes among *Bacillus thuringiensis* Subspecies and Strains†

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Fifteen *Bacillus thuringiensis* strains representing 13 serotypes were screened with five oligodeoxyribonucleotide probes specific for certain regions of two published sequences and one unpublished sequence of *B. thuringiensis* delta-endotoxin genes. Of the 15 cultures, 14 hybridized with at least one probe; the *B. thuringiensis* subsp. *thompsoni* strain alone did not hybridize. Two *B. thuringiensis* subsp. *kurstaki* strains of commercial interest, HD-1 and NRD-12, were found to be so closely related as to be indistinguishable with this technique; the same situation was found with strains from *B. thuringiensis* subspp. *dendrolimus* and *sotto*. Five strains were identified as probably containing only one endotoxin gene. A probe specific for the gene from the *B. thuringiensis* subsp. *kurstaki* HD-73 strain hybridized to only 3 of the 15 cultures tested. The hybridization data suggest that the DNA sequences coding for the C-terminal region of the endotoxin protein are as well conserved as those coding for the N-terminal toxic portion.

Much has been accomplished recently to elucidate the molecular genetics of the crystal endotoxin from *Bacillus thuringiensis*, an industrially important insect pathogen, yet much remains to be learned on the subject (for a recent review, see reference 2). It is now generally accepted that the endotoxin genes are present on plasmids (5, 17) in most *B. thuringiensis* subspecies, although several reports (2) of chromosomal location have also been published. It was shown recently by Debro et al. (11) that in *B. thuringiensis* subsp. *finitimus*, both chromosomal and plasmid-borne genes are present and functional. Debro et al. also demonstrated that chromosomal and plasmid-borne genes of *B. thuringiensis* code for peptides distinct in both their antigenicity and crystal location; the chromosomal gene encodes a protein found in a free inclusion body (outside the exosporium), while the protein coded by the plasmid-borne gene is found within the exosporium.

The great variety of plasmid arrays found within related *B. thuringiensis* subspecies can now be better understood in terms of the conjugationlike (6) plasmid exchange mechanism. Demonstration of the presence of transposonlike inverted repeats flanking the endotoxin genes (18, 20) also helps in understanding how there can be so much variation in the molecular weights of the plasmids shown to contain the endotoxin genes.

It has been known for some time that certain *B. thuringiensis* subspecies contain more than one endotoxin gene and that more than one gene can be expressed in a given subspecies (11, 17, 19, 29). Clearly it will be very difficult to establish structure-activity relationships among the toxins produced by various *B. thuringiensis* subspecies if some of these subspecies express three or more endotoxin genes to produce the parasporal crystal. One can foresee two ways to resolve this problem: either by performing toxicity tests on individual genes cloned and expressed in *Escherichia coli* or *Bacillus subtilis* or by using *B. thuringiensis* subspecies which contain only one gene.

The large degree of homology shown among the published *B. thuringiensis* endotoxin DNA and protein sequences (1, 7, 14–16, 22, 25–28) prompts the question of how many distinct types of endotoxin genes there are from a toxicological viewpoint. A definitive answer to this question would require complete DNA or protein sequencing for all endotoxin genes in the more than 20 serotypes thus far characterized (10), together with the corresponding toxicity testing. Considering the cost of both DNA sequencing and insect bioassays, a preliminary genotype classification of isolates based on data obtained from DNA probes or endotoxin antibodies would be very useful.

This paper explores the use of oligonucleotide probes in comparing the number and type of endotoxin genes among various *B. thuringiensis* cultures and identifying potential single-gene subspecies of *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial strains. The 15 *B. thuringiensis* cultures tested were from a collection maintained by one of us (P.F.) at the Canadian Forestry Service. Strains HD-1 and HD-73 were obtained from H. Dulmage (Brownsville, Tex.); strain NRD-12 was obtained from N. Dubois (U.S. Forest Service, Hamden, Conn.). All other strains were originally obtained from the Pasteur Institute in 1974 as the presumed type cultures for each strain. The lyophilized cultures were subcultured on nutrient agar slants and thereafter maintained as slants at 5°C.

Computer analysis. All homology searches and restriction enzyme searches were performed with the Pustell programs

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Positioning of the oligonucleotide probes. Table 1 summarizes the exact positions, DNA sequences, and literature references for the five probes used in this work. The exact positions of the probes were chosen to limit homology with other regions of the published gene sequences to less than 70%.

Synthesis and labeling of the oligonucleotide probes. Oligonucleotides were synthesized with the solid-phase phosphoramidite chemistry on commercial DNA synthesizers (Genetic Design model 6500 and Applied Biosystems model 380A). The deblocking and initial purification on C-18 Sep-Pak cartridges (Waters Associates) were performed as described by Sanchez-Pescador and Urdea (23). The oligonucleotides were checked for purity and size on analytical 20% polyacrylamide–8 M urea gels and further purified, if necessary, on preparative gels of the same type. Oligonucleotides were labeled at their 5′ end by reaction with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and T4 polynucleotide kinase. A typical reaction used 20 pmol of oligonucleotide, 80 μCi of [γ-^{32}P]ATP, and 1 U of enzyme for 1 h at 37°C. The kinase mixture was used directly in the hybridization step.

Enzymes. Restriction enzymes were purchased from GIBCO Laboratories and Boehringer-Mannheim (Canada) Ltd. Polynucleotide kinase (T4) was from GIBCO.

DNA preparation. Total and plasmid DNAs were prepared as described by Kronstad et al. (17). In contrast to those authors (17), we could find no significant difference in the yield of the higher-molecular-weight plasmids when DNA was harvested from cells grown on rich medium (half-strength tryptic soy broth) versus poor medium (Spizizen). Total DNA cultures were grown at 30°C instead of 37°C on the idea that plasmid stability in *B. thuringiensis* is improved at the lower temperature. We found no significant differences in hybridization patterns between plasmid DNA prepared from strains HD-1, HD-73, and NRD-12 grown at both temperatures.

Restriction enzyme digestions. *Hin*dIII restriction digests were performed on 75 μg of DNA with 150 U of *Hin*dIII enzyme for 18 h at 37°C in the presence of 4 mM spermidine to assist the digestion (3). The digest with *Pvu*II was performed on a smaller scale (10 μg) in an otherwise similar fashion.

Electrophoresis. Digested DNA (10 μg) from each strain or subspecies was electrophoresed on 1% agarose gels (containing ethidium bromide [0.5 μg/ml]) in Tris-acetate-EDTA buffer, pH 8.1 (0.04 M Tris base, 0.002 M sodium acetate, 1 mM disodium EDTA). Agarose LE was from SeaKem. The 15 *B. thuringiensis* cultures were arranged in alphabetical order. Gels were run at 4 V/cm until the bromphenol blue marker had migrated an average of 18 cm. The gels were photographed under UV light and dried in vacuo in a commercial (BioRad Laboratories) gel drier (without heating).

The following molecular weight markers were used as the leftmost lane on every gel: lambda phage DNA digested with *Hin*dIII, giving restriction fragments of 23.1, 9.4, 6.5, 2.3, and 2.0 kilobases (kb).

Hybridization controls were made from *Hin*dIII or *Pvu*II digests of plasmid DNA from the HD-1, HD-73, and NRD-12 strains and from a *Hin*dIII or *Pvu*II digest of plasmid pBP8, a pUC8 derivative containing a cloned endotoxin gene from NRD-12 (M. A. Hefford, R. Brousseau, G. Prefontaine, Z. Hanna, J. Condie, and P. C. K. Lau, manuscript in preparation). These were loaded as the four rightmost samples of every gel.

Hybridization. A hybridization protocol (21) with dried agarose gels was followed. This protocol obviates the problems encountered with Southern transfers when dealing with large-sized DNA fragments. Gels were prehybridized for 2 h at 65°C in hybridization buffer (21); the labeled probe was then added and allowed to hybridize overnight at 30°C. A first exposure was taken to obtain the hybridization data under nonstringent conditions. The gels were then washed (four 15-min washes) at the stringent temperature (generally T_m − 3°C) and exposed again. T_m values were calculated from an empirical method (9) by using the equation T_m = (number of A:T pairs × 2°C) + (number of G:C pairs × 4°C). Exposure was at −80°C for 2 to 7 days with Du Pont Cronex 4 film and Kodak intensifying screens.

RESULTS

Probe design. Using a gene-specific probe, Kronstad and co-workers (17, 19) showed that the HD-1 strain of *B. thuringiensis* contains three toxin genes which are characterized by hybridizing *Hin*dIII restriction fragments of 4.5, 5.3, and 6.6 kb. We positioned three probes within the published (25) "4.5 gene" sequence (Fig. 1). The N-terminal probe RB-9 was positioned at amino acids 60 to 65; this region is predicted, according to calculations made by the method of Chou and Fasman (8), to be part of a β-hairpin structure and therefore was expected to be well conserved. The C-terminal probe RB-18L was positioned so as to allow identification of restriction fragments containing an essentially complete gene when this probe was used on conjunction with RB-9. The central-region probe RB-23 was positioned in the region of maximum divergence between the HD-1 4.5 and the HD-73 "6.6 gene" sequences (1).

The fourth probe, RB-19, was designed to be 100% homologous to part of the central region (nucleotides 1780 to 1800) of the HD-73 6.6 gene.

The fifth probe, U3-18, was a C-terminal probe chosen to identify specifically the sequence of one of the genes from the NRD-12 strain (Hefford et al., manuscript in preparation). This oligonucleotide sequence was subsequently found to be present also in the "5.3 gene" sequences published by several other groups (see below).

TABLE 1. Summary of oligonucleotide probes used

Probe	Gene sequence derivation (reference)	DNA sequence (length)	Positions[a]
RB-9	4.5 gene from HD-1 (25)	5′-CTAGTTGATATAATATGG (18-mer)	178–195
RB-23	4.5 gene from HD-1 (25)	5′-GTCTTCAATTCAGGCAATGAA (21-mer)	1765–1785
RB-18L	4.5 gene from HD-1 (25)	5′-AATACTTCCCAGAAACCGATA (21-mer)	3434–3454
RB-19	6.6 gene from HD-73 (1)	5′-GGGACTGCAGGAGTGATAATA (21-mer)	1780–1800
U3-18	5.3 gene from NRD-12 (Hefford et al., manuscript in preparation)	5′-GAGCCTATGAAAGCAATT (18-mer)	3212–3229

[a] The A of the first ATG is numbered 1.

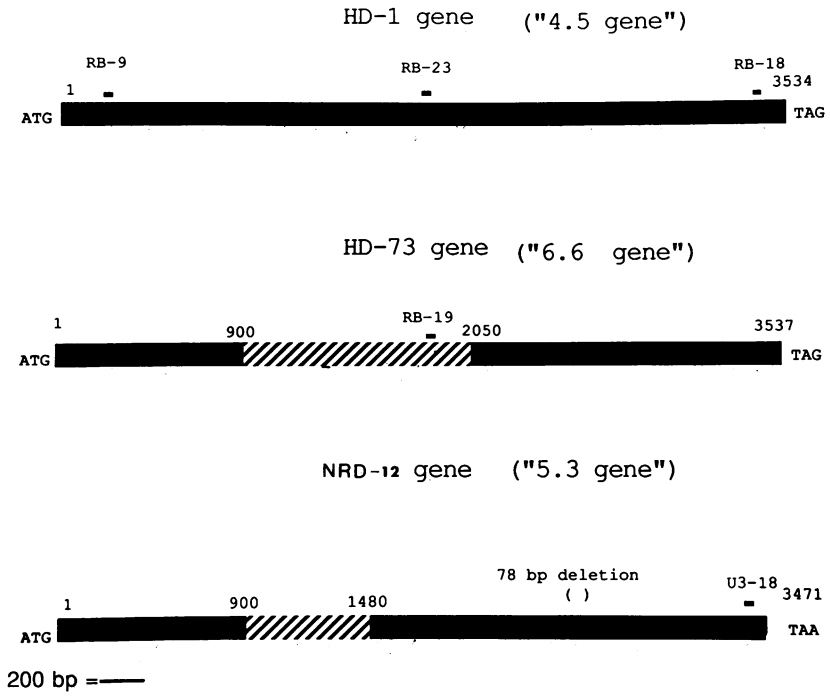

FIG. 1. Positions of the five oligonucleotides probes in relation to the known *B. thuringiensis* endotoxin gene sequences. The 4.5 sequence from Schnepf et al. (25) was used as a reference (solid black line). The regions where the 6.6 and 5.3 genes diverge from the 4.5 sequence are shown as hatched lines.

Results from the amino-terminal probe RB-9. Of the 15 cultures tested, 10 hybridized with the amino-terminal probe in one or more locations (Fig. 2). The result obtained for strain HD-1 confirmed the previous finding (17), obtained with a much longer DNA probe, of three hybridizing *Hin*dIII fragments at 6.6, 5.3, and 4.5 kb. Especially noteworthy was the NRD-12 strain, which also showed three distinct bands indistinguishable from those of HD-1. Strain NRD-12 is of particular interest in the North American forestry context because of the report (12) of a faster kill against the gypsy moth (*Lymantria dispar* L.) and the eastern spruce budworm (*Choristoneura fumiferana*). The amino-terminal probe results indicated a close relationship between HD-1 and NRD-12, and this was supported by the results with our other probes; this opens the possibility that differences in toxicity might result from relatively small changes in the endotoxin genes.

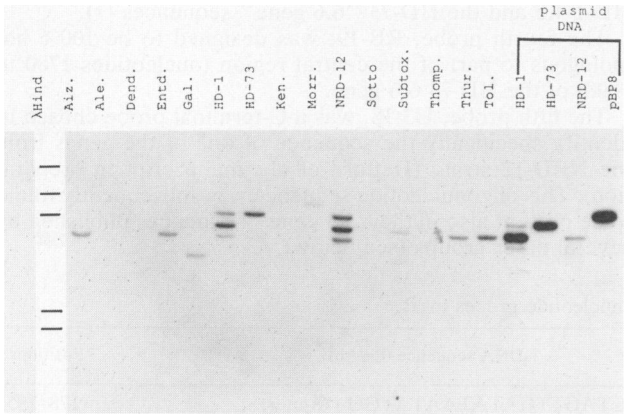

FIG. 2. Autoradiogram of a dried agarose gel containing *Hin*dIII digests of total DNA from *B. thuringiensis* cultures hybridized with the ^{32}P-labeled N-terminal probe RB-9. Molecular weight markers are as discussed in the Materials and Methods section. Strains are listed in the same order as in Table 2. Lines at the left indicate the positions of *Hin*dIII-digested lambda markers (see text).

The result obtained for the strain HD-73 was worth noting in that two bands were observed for the total DNA, a predominant band at 6.6 kb and a much fainter one at 4.5 kb. This 4.5-kb band was only visible in total DNA; it was not visible in the plasmid preparation (Fig. 2, third lane from right) despite a much higher intensity for the 6.6-kb band. A possible explanation would be the presence of a distinct chromosomal gene present in a much lesser amount (<5%) than the major gene; alternatively, there may be fortuitously high homology between the RB-9 probe and a gene unrelated to the delta-endotoxin family.

Also noteworthy was the failure of *B. thuringiensis* subsp. *sotto* to hybridize with the amino-terminal probe despite the perfect identity between the published sequence (26) and the probe. The culture we have is crystalliferous, and its DNA hybridized to some of our other probes (following sections), so it is not that our isolate has lost the toxin-producing gene. We can only conclude that our strain was significantly different from the one studied by Shibano et al. with regard to delta-endotoxin genes.

The 4.5-type probe (RB-23). This central-region probe could not be used on DNA digested with *Hin*dIII, as the small *Hin*dIII fragments present in this region of the genes were lost from the gel after it was washed. However, we obtained good results with *Pvu*II digests (Fig. 3). Perhaps the most interesting observation from the 4.5 probe was the identity of patterns between *B. thuringiensis* subspp.

dendrolimus and *sotto*. This identity held true for our other probes, which were unable to distinguish between these two subspecies. This concurs with the data of Dulmage et al. (13), who stated that these two subspecies must be distinguished on the basis of electrophoretic pattern of esterases, as they have the same 4a4b H serotype. In contrast, our probes distinguished easily between *B. thuringiensis* subspp. *subtoxicus* and *entomocidus*, which were also reported (13) to be similar in both H serotypes and esterase patterns.

The 6.6-type probe. Results with the 6.6 probe are shown in Fig. 4. This probe gave at least a partial answer to the question of whether the 4.5- and 6.6-type central regions were equally prevalent in *B. thuringiensis* isolates. The data showed a much greater prevalence of the 4.5-type central region (10 of 15 cultures hybridized to that probe, Fig. 3) over the 6.6 central region (3 of 15). The 6.6 gene can therefore be considered a somewhat rare, nontypical gene in terms of its central region, at least in the precise region where our probe binds. Furthermore, the cultures which lit up with the 6.6 probe (HD-1, HD-73, and NRD-12) were already very closely related; indeed, one can envision HD-73 as an HD-1 isolate having lost two of its three genes, the ones corresponding to the 4.5- and 5.3-kb *Hin*dIII fragments.

Common C-terminal probe RB-18L. Figure 5 indicates that 11 of 15 of the subspecies tested had one or more bands homologous with this probe. This indicates a high degree of conservation for this region of the gene, which has been shown by others (24) to be nonessential for toxicity. An argument can therefore be made that this region may have an important role for other functions of the protein.

The 5.3-type probe U3-18. Several *B. thuringiensis* endotoxin genes have been isolated and sequenced recently. An interesting aspect of the sequence data is the seeming predominance of the 5.3 type of endotoxin gene. This type of gene has been found by Wabiko et al. (28) and also by Hofte et al. (15) in strain 1715. Oeda et al. (22) found a very similar sequence in a *B. thuringiensis* subsp. *aizawai* strain. The 5.3 genes from the commercially important HD-1 strain have been cloned by Thorne et al. (27), Geiser et al. (14), Kondo et al. (16), and Adang et al. (M. J. Adang, K. F. Idler, and T. A. Rocheleau, *in* K. Maramorosch, ed., *Biotechnology Advances in Invertebrate Pathology and Cell Culture*, in press). It was therefore of interest to design a probe specific

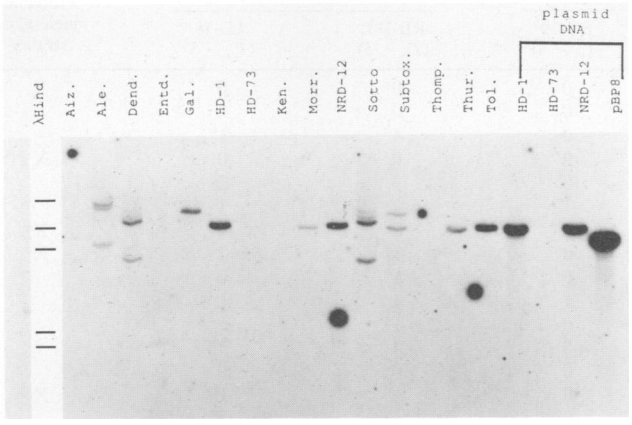

FIG. 3. Autoradiogram of a dried agarose gel containing *Pvu*II digests of total DNA from *B. thuringiensis* cultures hybridized with the labeled central-region probe RB-23. The round spots in this and subsequent figures are artifacts and should be ignored. See Fig. 2 legend for details.

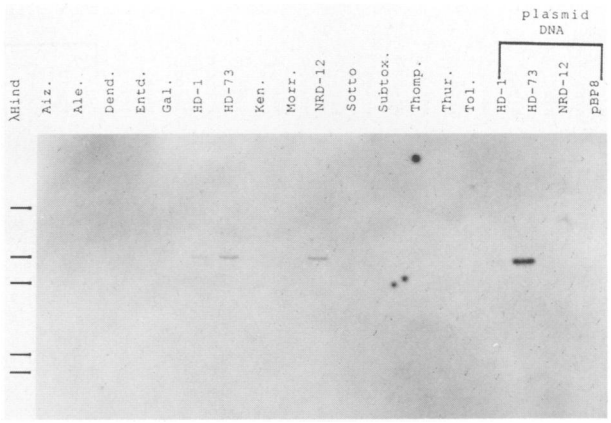

FIG. 4. Autoradiogram of a dried agarose gel containing *Hin*dIII digests of total DNA from *B. thuringiensis* cultures hybridized with the labeled central-region probe RB-19. See Fig. 2 legend for details.

for this type of gene. We found that probe U3-18, positioned over a 12-base-pair (bp) region found only in the 5.3 type of gene, hybridized with 9 of 15 strains (Fig. 6); this confirmed the wide distribution of 5.3-type genes. We also found that our *B. thuringiensis* subsp. *aizawai* strain did not hybridize with this probe despite the perfect homology between the reported sequence (22) and our probe. As with the *B. thuringiensis* subsp. *sotto* isolate with respect to the carboxy-terminal probe RB-9, we conclude that strains of a given serotype may well harbor significantly different endotoxin genes.

The fact that endotoxin gene sequences from many different strains hybridized with the same carboxy-terminal probes strengthens the hypothesis that this region is well conserved; indeed, comparison of the published sequences for the 5.3 genes shows that every one of them possesses the characteristic four-codon insertion which was chosen by us to locate the U3-18 probe. Such a high degree of conservation indicates that the carboxy region of this protein plays an important role in the life cycle of *B. thuringiensis*.

The U3-18 and RB-18L probes allowed us to test the accuracy of our procedures with HD-73 as a test strain. HD-73 has been shown by others (1) to be a single-gene strain, and therefore any hybridization signal coming from it

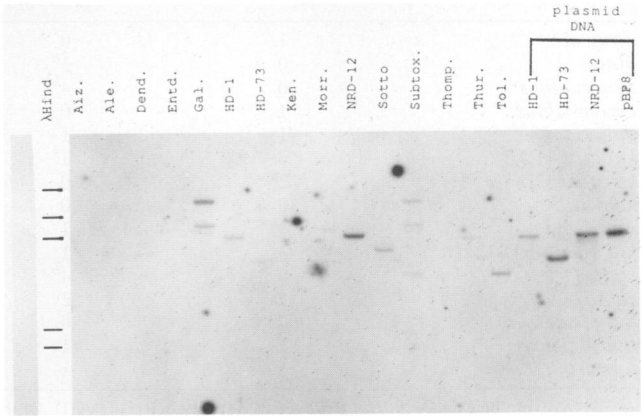

FIG. 5. Autoradiogram of a dried agarose gel containing *Hin*dIII digests of total DNA from *B. thuringiensis* cultures hybridized with the C-terminal probe RB-18L. See Fig. 2 legend for details.

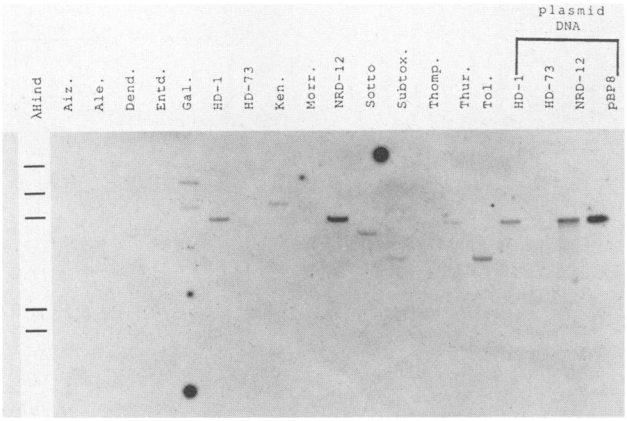

FIG. 6. Autoradiogram of a dried agarose gel containing *Hin*dIII digests of total DNA from *B. thuringiensis* cultures hybridized with the C-terminal probe U3-18. See Fig. 2 legend for details.

can be attributed to the crystal gene itself. U3-18 was positioned to have a maximum of 66% homology with the published HD-73 sequences; RB-18L was identical with the corresponding region of the HD-73 gene. We noted with pleasure that the U3-18 probe gave no significant hybridization to HD-73 DNA even before the stringent washing (data not shown); in contrast, the RB-18L probe still hybridized at $T_m - 5$°C. We can therefore feel reasonably secure that our technique will distinguish even between closely related genes, provided that the probes are properly chosen.

Correlation between the probes. Table 2 shows the relationship between the number of bands we obtained with the various probes at the stringent hybridization temperature and the identification of possible single-gene subspecies or strains.

DISCUSSION

Our data are best summarized as follows. The correlation between serotype and endotoxin genotype seems rather weak. The NRD-12, HD-73, and HD-1 strains differed in the number of genes present as well as in their insect toxicity, yet all three fell within the *B. thuringiensis* subsp. *kurstaki* serotype. In the case of the *B. thuringiensis* subsp. *sotto* serotype, there was obviously a significant difference in the N-terminal region of the endotoxin gene(s) between our isolate and the one studied by Shibano et al. (26). The same situation holds for the *B. thuringiensis* subsp. *aizawai* strain studied by us as opposed to the one studied by Oeda et al. (22). The supposedly distinct *B. thuringiensis* subspp. *dendrolimus* and *sotto* gave very similar or identical hybridization patterns with five different probes. Finally, our probes readily distinguished between our *B. thuringiensis* subspp. *subtoxicus* and *entomocidus* isolates, whereas these serotypes are reported to have the same surface antigens and the same esterase patterns (13). These preliminary results lead us to plan further work aimed at studying hybridization patterns for different isolates possessing the same serotype.

The data we obtained lead us to consider the *B. thuringiensis* subspp. *aizawai*, *entomocidus*, *kenya*, and *thuringiensis* isolates we studied as potential single-gene subspecies. In contrast, the situation for the HD-73 strain, generally thought to be a single-strain species, was not so clear, as we obtained a faint additional hybridization which would correspond to a small amount of 4.5-type gene. Further work is needed to resolve this issue.

The 5′ and 3′ termini of the endotoxin genes seem to be equally conserved. The scores were quite similar for the N-terminal probe (10 of 15 cultures hybridized) and for the two C-terminal probes (11 of 15 and 9 of 15). The significance of the conserved nature of the carboxy terminus remains to be ascertained, as this region, not being necessary to toxicity, might have been expected to be more divergent.

The data we have do not allow us to draw firm conclusions on the exact number of delta-endotoxin genes present in multiple-gene species. The reasons for this are both technical and theoretical.

From a practical viewpoint, weak bands pose an interpretation problem; they can represent either weak hybridization to a distantly related sequence, a small amount of incompletely digested high-molecular-weight DNA, or a gene

TABLE 2. Summary of hybridization data: number of bands seen for the various strains at the stringent temperature[a]

B. thuringiensis subsp. and strain	Amino-terminal probe RB-9 ($T_m - 3$)	Central-region probes		Carboxy-terminal probes		Probable single-gene strains
		RB-23 ($T_m - 3$)	RB-19 ($T_m - 3$)	RB-18L ($T_m - 5$)	U3-18 ($T_m - 5$)	
aizawai	1	0	0	0	0	X
alesti	0	3[b]	0	0	0	
dendrolimus	0	4[b,c]	0	1[b]	1[b]	
entomocidus	1	0	0	0	0	X
galleriae	1	1	0	3	2	
kurstaki HD-1	3	1	1	1	1	
kurstaki HD-73	2[b]	0	1	1[b]	0	X
kenyae	0	0	0	1[d]	1	X
morrisoni	1[b]	1	0	1[b]	0	
kurstaki NRD-12	3	1	1	1	1	
sotto	0	4[b,c]	0	1	1	
subtoxicus	2	2	0	4	3[b]	
thompsoni	0	0	0	0	0	
thuringiensis	1	1	0	1[b]	1[b]	X
tolworthi	1	1	0	1	1	

[a] DNAs were digested with *Hin*dIII except for probe RB-23 (*Pvu*II). Of the 15 strains tested, 10, 10, 3, 11, and 9 hybridized with probes RB-9, RB-23, RB-19, RB-18L, and U3-18, respectively.

[b] One or more faint bands were readily visible on the light box but may not show well on photographs.

[c] Multiple band pattern was not affected by a second digestion with *Pvu*II.

[d] Band is partly hidden by spot on autoradiogram.

present in a much lower copy number relative to other genes. We digested the DNA extensively with a large excess of restriction enzyme to avoid the partial digestion problem; nevertheless, it cannot be assumed to be solved for every sample. The fact that in some cases weak bands appeared after very long exposure on the autoradiogram brings an element of subjectivity to the data evaluation.

From a theoretical viewpoint, DNA probes can only look for known DNA sequences; there might be whole sets of different toxin genes in *B. thuringiensis* that remain to be discovered. It is also theoretically possible that two delta-endotoxin genes present in a strain might differ by only a single base over their 3.5-kb coding region. Assuming that the point mutation falls in a critical region, such genes could well code for different toxicities, yet they would be indistinguishable by any technique except extremely careful and complete DNA sequencing. The recent finding by Thorne et al. (27) of structural similarity between the lepidoptera- and diptera-specific endotoxin genes of *B. thuringiensis* subspp. *kurstaki* and *israelensis* confirms the possibility that the same probe could hybridize to genes encoding proteins of quite different toxicities. To speak of a single-strain gene can therefore only be justified in terms of known, substantially different sequences.

A conceptually similar approach to the classification of *B. thuringiensis* isolates has been published by Calabrese et al. (4). In their system, the molecular weights of the crystal proteins are compared on denaturing polyacrylamide-sodium dodecyl sulfate gels. It is instructive to compare their technical limitations with ours. Their system is critically dependent on the prevention of proteolytic conversion prior to analysis. Furthermore, it is now known that many different delta-endotoxin genes code for proteins of the same molecular weight, either 130,000 or 133,000. As an example, the HD-1 strain possesses two genes coding for 133-kilodalton proteins, in addition to a third gene coding for a 130-kilodalton protein. The three genes are readily distinguished with DNA or oligonucleotide probes, in contrast to the protein gels, where only two bands can be seen. Finally, while the protein gel only indicates size, the DNA data contain both size and sequence information in terms of homology to a given sequence. Oligonucleotides probes readily indicated that one of the three HD-1 genes was related to HD-73; this information could not be inferred solely from protein gels.

In conclusion, oligonucleotide probes provide a powerful means of identifying closely related *B. thuringiensis* strains by their delta-endotoxin genotype. While they suffer technical limitations, their ease of preparation makes them a potentially very interesting tool to establish a *B. thuringiensis* classification scheme more closely correlated with insect toxicity than the current serotype classification.

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