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Comparative Analysis of the 16S to 23S Ribosomal Intergenic Spacer Sequences of *Bacillus thuringiensis* Strains and Subspecies and of Closely Related Species

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***Bacillus thuringiensis* spacer regions between the 16S and 23S rRNAs were amplified with conserved primers, designated 19-mer and 23-mer primers. A spacer region of 144 bp was determined for all of 6 *B. thuringiensis* strains, 7 *B. thuringiensis* subspecies, and 11 *B. thuringiensis* field isolates, as well as for the closely related species *Bacillus cereus* and *Bacillus anthracis*. Computer analysis and alignment of nucleotide sequences identified three mutations and one deletion in the intergenic spacer region (ISR) of *B. thuringiensis* subsp. *kurstaki* HD-1 when compared with ISR sequences from other subspecies. The same differences were identified between the ISR of *B. thuringiensis* strains and the ISR of *B. cereus* and *B. anthracis*. These minor differences do not seem to be sufficient to allow the design of a species-specific oligonucleotide probe.**

Bacillus thuringiensis is currently used to control a variety of foliage lepidopterans such as moths and budworms, dipterans such as mosquitoes and blackflies, and coleopterans such as Colorado potato beetles (8, 12, 17). The biological insecticide *B. thuringiensis* serovar H3a3b (subsp. *kurstaki*) is the only one used in Quebec, Canada, to repress the spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae) (25, 30). Usually, the commercial products contain different *B. thuringiensis* subsp. *kurstaki* strains with differences in pathogenic potency (1, 2, 31).

Many methods of identifying *B. thuringiensis* subspecies have been suggested (4, 15, 18–20). Currently, *B. thuringiensis* strains are classified on the basis of flagellar antigens into more than 34 serotypes (9, 10). Isolates within serotypes differ in their general biochemical characteristics, plasmid patterns, shape, stability, and host range activity of the entomocidal crystals. Some of the serotypes are divided into subserotypes, which can now be differentiated by PCR (6, 7).

Similarities among *Bacillus* species are naturally reflected at the nucleic acid level. *B. anthracis*, *B. cereus*, and *B. thuringiensis* are grouped together on the basis of whole-genome hybridization (16, 23, 28). Variable regions in the 16S rRNA and DNA sequences have been frequently used as targets for DNA probes to identify microorganisms. In some situations, there is very little sequence variation between the 16S rRNA genes of closely related microorganisms (3). Barry et al. (5) have established a general method to obtain species-specific probes by using the intergenic spacer region (ISR) between the 16S and 23S rRNA genes. They argued that the ISR should be under minimal selective pressure during evolution and therefore should vary more extensively than sequences within genes that have functional roles.

In this study, we have adapted the method described by Barry et al. (5) and attempted to differentiate *B. thuringiensis* subspecies from *B. cereus* and *B. anthracis*. Furthermore, we have examined the ability of this method to discriminate among commercial *B. thuringiensis* strains and field isolates.

Bacterial strains. A complete list of the bacterial strains used is given in Table 1. All bacterial strains were maintained in tryptic soy agar (Difco Laboratories, Detroit, Mich.) enriched with 0.3% (wt/vol) yeast extract (Difco). Field isolates of *B. thuringiensis* were collected between 1987 and 1991 from residual populations of spruce budworm from *B. thuringiensis*-treated forests in Quebec, Canada.

Enzymes. Enzymes were purchased from Pharmacia-LKB, Baie-d'Urfé, Quebec, Canada. All enzymes and buffers were used as recommended by the manufacturer.

Preparation of DNA templates for PCR. DNA templates were prepared from a 16- to 18-h culture in tryptic soy broth (Difco) enriched with 0.3% (wt/vol) yeast extract (Difco). A 100-μl sample of the bacterial culture (2×10^6 bacteria per ml) was centrifuged at 17,000 rpm (34,800 × *g*) for 10 min, the supernatants were removed, and the bacterial pellets were resuspended in 200 μl of sterile deionized water. The cell pellets were frozen in dry ice for 5 min, boiled for 5 min, frozen, and then boiled again (29). Finally, bacterial debris was removed by centrifugation, and the DNAs were resuspended in 40 μl of sterile deionized water.

PCR amplification of the 16S to 23S rDNA ISR. PCR amplifications were routinely carried out in a 50-μl reaction volume that contained 2 μl of template; primers A1-2 (5′-AGTC GTAACAAGGTAGCCG-3′) and B1-2 (5′-CCGCTGCCAA GGCATCCACCTAT-3′) at 0.2 μM each; 50 mM KCl; 10 mM Tris-chloride (pH 8.3); 4.0 mM $MgCl_2$; dATP, dCTP, dGTP, and dTTP (Pharmacia) at 200 μM each; and 1.25 U of *Taq* DNA polymerase (Pharmacia). The reaction mixture was covered with 50 μl of mineral oil and heated to 94°C for 5 min. Samples of reaction mixtures were amplified in the DNA thermal cycler (Perkin-Elmer Cetus, Montreal, Quebec, Canada), which was programmed for 35 cycles of amplification. Parameters for the amplification cycles consisted of 45 s at 94°C (denaturation), 45 s at 55°C (primer annealing), and 45 s at 72°C (primer extension-polymerization). An aliquot of the reaction mixture (10 μl) was analyzed by agarose gel electro-

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TABLE 1. Bacterial strains used for the 16S to 23S rDNA intergenic spacer sequence analysis

Strain	Source
B. anthracis HER 1140	M. Sterne
B. cereus ATCC 27877	ATCC[a]
B. thuringiensis subsp. *kurstaki* HD-1	H. T. Dulmage
B. thuringiensis subsp. *kurstaki* HD-73	H. T. Dulmage
B. thuringiensis subsp. *kurstaki* NRD-12	N. R. Dubois
B. thuringiensis subsp. *kurstaki* Dipel	Abbott Co.
B. thuringiensis subsp. *kurstaki* Thuricide	Sandoz Co.
B. thuringiensis subsp. *kurstaki* Futura XLV	Solvay Co.
B. thuringiensis subsp. *kurstaki* Novo	Novo Co.
B. thuringiensis subsp. *galleriae*	O. I. Schvetsova
B. thuringiensis subsp. *israelensis*	Sandoz Co.
B. thuringiensis subsp. *tenebrionis*	A. Krieg
B. thuringiensis subsp. *entomocidus*	H. de Barjac
B. thuringiensis subsp. *kenyae*	P. Fast
B. thuringiensis subsp. *alesti*	H. de Barjac
B. thuringiensis subsp. *kurstaki* coll. 1.8.87[b]	This study
B. thuringiensis subsp. *kurstaki* coll. 3.61.87	This study
B. thuringiensis subsp. *kurstaki* coll. 1.17.88	This study
B. thuringiensis subsp. *kurstaki* coll. 2.40.88	This study
B. thuringiensis subsp. *kurstaki* coll. 1.24.89	This study
B. thuringiensis subsp. *kurstaki* coll. 1.9.90	This study
B. thuringiensis subsp. *kurstaki* coll. 1.14.90	This study
B. thuringiensis subsp. *kurstaki* coll. 3.52.90	This study
B. thuringiensis subsp. *kurstaki* coll. 1.6.91	This study
B. thuringiensis subsp. *kurstaki* coll. 1.11.91	This study
B. thuringiensis subsp. *kurstaki* coll. 1.20.91	This study

[a] ATCC, American Type Culture Collection, Rockville, Md.
[b] coll., collection strains. The subspecies of all 11 *B. thuringiensis* subsp. *kurstaki* collection strains were identified by biochemical tests.

phoresis (1.5% agarose), and the products were stained with ethidium bromide and visualized under UV light (21).

The 234-bp DNA fragment obtained as a PCR product was excised from the agarose gel with a sterile scalpel, and a second PCR amplification was carried out in a 100-μl reaction volume as described above, except that the template used was a small slice of agarose (~1 mm thick) and 2.5 U of *Taq* DNA polymerase was used.

The reaction mixture was purified by using the Magic DNA Clean-up System (Promega Corp., Madison, Wis.), and an aliquot (4 μl) was tested for purity by electrophoresis on 1.5% agarose gels.

Cloning of PCR products. The 234-bp PCR product was modified to make DNA ends flush for blunt-end cloning. The reaction was performed with approximately 2 μg of DNA, 10 μl of 10× polymerase I buffer (0.5 M Tris [pH 7.5], 0.1 M $MgCl_2$, 10 mM dithiothreitol, 0.5 mg of bovine serum albumin per ml, 200 μM deoxynucleoside triphosphate), ATP to 1 mM, and about 10 U each of T4 polynucleotide kinase and DNA polymerase I in a final volume of 100 μl. The modified DNA preparations were incubated at 37°C for 1 h, and the reaction was stopped by adding 1 μl of 0.5 M EDTA (pH 8.0).

The preparations were pooled and ligated to *Sma*I-digested pGEM-3zf(+) plasmid vector (Promega) previously treated with alkaline phosphatase. Vector and DNA inserts (1:3 mol/mol) were mixed at a concentration of 20 μg/ml in 20 μl and ligated with 8 Weiss units of T4 DNA ligase for 18 h at 14°C. Recombinant molecules were transformed into *Escherichia coli* DH11S (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with 5 ng of DNA per 20 μl of competent cells, as recommended by the manufacturer. The transformants were selected on Luria broth plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Promega), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Promega), and 100 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml. DNA from selected transformants was purified with Magic Minipreps DNA Purification System (Promega) and visualized on a 1.0% agarose gel for the presence of the expected DNA fragment.

Sequence analysis. Single-stranded DNA was prepared by standard procedures and recovered from transformants with the helper phage M13K07 (Promega). DNA sequencing was performed by the dideoxynucleotide T7 polymerase chain termination method (22) with the universal primer and the T7 sequencing kit (Pharmacia). The DNA sequence was analyzed with the software package developed by the University of Wisconsin Genetics Computer Group (11).

Amplifications of the DNA spacer regions between the 16S and 23S rDNA genetic loci were carried out with genomic DNA from 13 strains of *B. thuringiensis* representing seven subspecies and 6 strains of *B. thuringiensis* subsp. *kurstaki*. Genomic DNA from two other *Bacillus* species (*B. cereus* and *B. anthracis*) closely related to *B. thuringiensis* was also included. For comparative analysis, 11 *B. thuringiensis* field isolates were also tested.

The PCR amplification product for each genomic DNA preparation tested gave a fragment in the size range of 220 to 250 bp. The product is consistent in DNA fragment, and only one band was amplified for each *Bacillus* species genomic DNA tested. The exact size of the DNA fragment was determined from sequencing as 234 bp. The nucleotide sequence of the ISR fragment is shown in Fig. 1. This region of 144 bp represents the 16S to 23S ribosomal spacer region of the bacterial strains used for this study.

DNA was prepared for two transformants selected for each *B. thuringiensis* subspecies (Fig. 1). Computer analysis and alignment of all nucleotide sequences revealed single-nucleotide substitutions. Each substitution or set of substitutions was unique to a single PCR product, indicating that such sequence alterations were random events and are presumably due to PCR amplification or random mutations. For example, at position 31, the C nucleotide (Fig. 1) was present in all the ISR *B. thuringiensis* subspecies except for *B. thuringiensis* subsp. *entomocidus* (E5), which contained a T. We think that the changes observed may be due to the PCR amplifications, because these two transformants had those differences. However, other differences seem representative of subspecies. At position 62, there is an A nucleotide in *B. thuringiensis* subsp. *kurstaki* HD-1 whereas all other subspecies have a G. Similar changes occur at position 90; for *B. thuringiensis* subsp. *kurstaki* HD-1, there is a T whereas all other subspecies had a C. Another difference was noted at position 83, where a T nucleotide was present or absent between subspecies.

Similar analyses were done with the ISR sequences from *B. thuringiensis* strains and two other species (Fig. 1). The DNA of one transformant was tested for each strain, and the nucleotide sequences are highly homologous when the sequences from *B. thuringiensis* subsp. *kurstaki* HD-1 were compared with the ISR sequences from other strains and species. The same differences in nucleotides were observed between ISR sequences from *B. thuringiensis* subsp. *kurstaki* HD-1 and other subspecies. Analysis of ISR sequences from *B. cereus* and *B. anthracis* and comparisons with *B. thuringiensis* subsp. *kurstaki* HD-1 did not reveal significant differences.

Also, each ISR sequence of 11 *B. thuringiensis* field isolates was compared with the ISRs from *B. thuringiensis* subsp. *kurstaki* HD-1 Futura and Dipel (Fig. 1). In general, the *B. thuringiensis* isolates obtained were confirmed to originate from the commercial preparations Futura or Dipel. However,

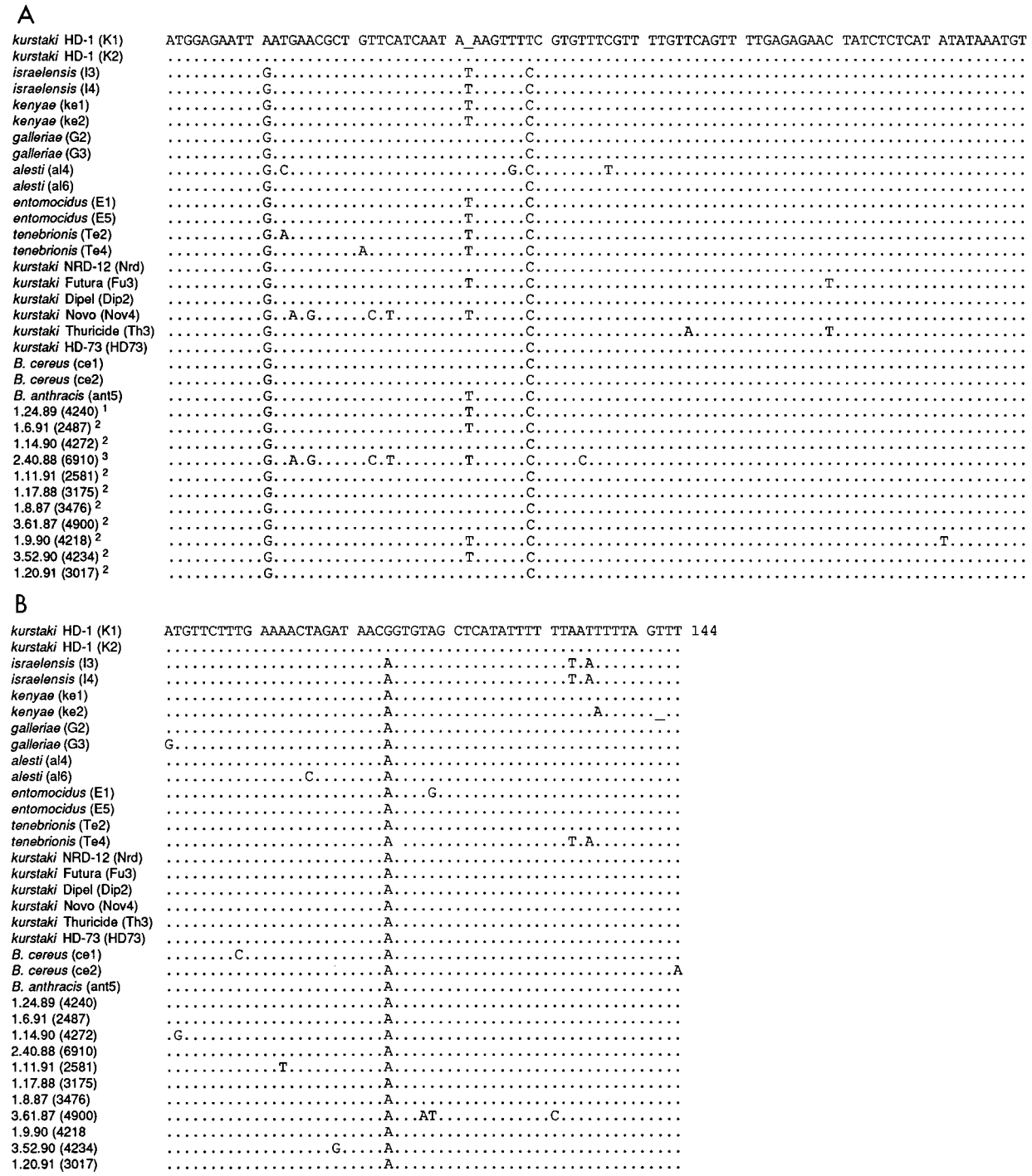

FIG. 1. Comparative sequence analysis of the rDNA ISRs from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* strains and subspecies, other *Bacillus* species, and *B. thuringiensis* field isolates (commercial formulations Dipel,[1] Futura,[2] and unknown[3]). (A) Sequence from positions 1 to 90; (B) sequence from positions 91 to 144.

the ISR sequences of the field isolates did not seem to correspond to those of *B. thuringiensis* subsp. *kurstaki* Futura or Dipel. In position 83, the T substitution is not constant for isolates identified as Futura and Dipel. It was noted that the ISR sequence of *B. thuringiensis* subsp. *kurstaki* HD-1 seems different from the other ISR sequences.

The nucleotide sequences of the 144-bp ISR DNA fragment have high overall sequence similarity (97 to 99%) for all the *B. thuringiensis* strains tested. The relationship between the ISR of *B. thuringiensis* subsp. *kurstaki* HD-1 and the ISR of the other subspecies is reflected by the fact that only point substitutions are observed in their sequences: three mutations at

positions 62, 90, and 165, and one deletion at position 83. The same differences were found between the ISRs of *B. thuringiensis* strains and related species.

The species *B. anthracis*, *B. cereus*, and *B. thuringiensis* share many phenotypic properties, and several workers have questioned their status as separate species (13, 26, 27). Previous chromosomal DNA-DNA hybridization investigations indicated that these species are closely related and probably represent a single species (23, 28). Ash et al. (3) have demonstrated, with their 16S rRNA sequence data, that these species form a genealogically well-defined group of microorganisms. The high level of sequence similarity does not permit construction of specific sequence probes to be used in identification.

Moreover, analysis of sequences from *B. thuringiensis* field isolates confirmed this observation. All isolates were identified by PCR (6), and they corresponded to *B. thuringiensis* subsp. *kurstaki* Futura or *B. thuringiensis* subsp. *kurstaki* Dipel. The sequence alterations detected were random events and are due to PCR amplifications or random mutations under no revolutionary pressure.

By a technique described as single-strand conformation polymorphism, it has been shown that small variations in sequence composition of single-stranded DNA can result in measurable differences in electrophoretic mobility (14, 24). Because the double-stranded *B. thuringiensis* PCR products have similar sizes, slight differences in electrophoretic mobilities of the single-stranded products make it possible to differentiate between *B. thuringiensis* strains. However, the results were not reproducible because of the sequence alteration present.

Furthermore, we can test the random amplified polymorphic DNA technique to obtain specific and reproducible amplicons. The technique can be refined by sequencing diagnostic DNA fragments and synthesizing specific probes.

Nucleotide sequence accession number. The sequences were submitted to GenBank and assigned no. U20789.

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