

Homoduplex and Heteroduplex Polymorphisms of the Amplified Ribosomal 16S-23S Internal Transcribed Spacers Describe Genetic Relationships in the “*Bacillus cereus* Group”

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Bacillus anthracis, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycooides*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis* are closely related in phenotype and genotype, and their genetic relationship is still open to debate. The present work uses amplified 16S-23S internal transcribed spacers (ITS) to discriminate between the strains and species and to describe the genetic relationships within the “*B. cereus* group,” advantage being taken of homoduplex-heteroduplex polymorphisms (HHP) resolved by polyacrylamide gel electrophoresis and silver staining. One hundred forty-one strains belonging to the six species were investigated, and 73 ITS-HHP pattern types were distinguished by MDE, a polyacrylamide matrix specifically designed to resolve heteroduplex and single-strand conformation polymorphisms. The discriminating bands were confirmed as ITS by Southern hybridization, and the homoduplex or heteroduplex nature was identified by single-stranded DNA mung bean nuclease digestion. Several of the ITS-HHP types corresponded to specific phenotypes such as *B. anthracis* or serotypes of *B. thuringiensis*. Unweighted pair group method arithmetic average cluster analysis revealed two main groups. One included *B. mycoides*, *B. weihenstephanensis*, and *B. pseudomycooides*. The second included *B. cereus* and *B. thuringiensis*, *B. anthracis* appeared as a lineage of *B. cereus*.

The “*Bacillus cereus* group,” one of the most homogeneous groups of the genus *Bacillus*, encompasses six validly described species, *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycooides*, *B. thuringiensis*, and *B. weihenstephanensis*, all of which have an important impact on human activity (18, 39, 44).

These species are known to be strictly related phylogenetically, as has been shown by DNA-DNA hybridization studies (35, 39, 42–44, 57) and the sequencing of the ribosomal RNA genes (2–4, 39). On the other hand, a marked variability is always observed when large collections of strains are examined by DNA fingerprinting methods that target the whole genome (8–13, 17, 25–29, 36, 58) and/or discrete genes (15, 16, 38, 54, 55, 61). Hence, the phylogenetic and taxonomic relationship among these species is still open to debate. It has been proposed previously that *B. anthracis*, *B. cereus*, and *B. thuringiensis* represent a single species, this conclusion having been reached through genome sizing and mapping (9–13) and, very recently, by multilocus enzyme electrophoresis and the sequencing of nine different DNA loci (27).

The ribosomal operon is a classic molecular marker used to trace genetic relationships and to identify strains rapidly (1). Of all the different regions of the ribosomal operon, the internal transcribed spacers (ITS) between 16S and 23S ribosomal DNA are frequently used as molecular markers to identify microbial species and analyze the phylogenetic relationship between strains (see, for example, references 14, 22, and 24). ITS are generally found in multiple copies in most bacterial genomes (24, 33). Since ITS are hypervariable with respect to adjacent genes, due to a higher mutation rate, they can differentiate between multiple operons in the cell (22–24, 31, 41). Agarose gel ITS-PCR fingerprinting patterns are identical for

the six species of the *B. cereus* group (14, 17) and are thus not suitable for the rapid identification of the species. Since the *B. cereus* group strains have from 8 to 12 ribosomal operons (34, 39, 49) that can differ in ITS sequence, it might be possible to detect interspecies and intraspecies differences through ITS homoduplex-heteroduplex polymorphisms (ITS-HHP) (6, 31–33). The principle of strain discrimination by ITS-HHP analysis is based on the detection of sequence polymorphisms in the form of heteroduplex DNA bands that are generated during PCR when fragments of different length, which contain homologous regions at the 3' and 5' ends, cross-hybridize to form heteroduplex DNA structures. The electrophoretic mobility of these heteroduplex structures is significantly reduced with respect to homoduplex DNA fragments, depending on the amount of single-stranded DNA present in the heteroduplex product and the degree of secondary structures formed within the single-stranded regions (6, 31–33).

The aim of this study was to evaluate whether ITS-HHP can be used advantageously to highlight molecular diversity within the *B. cereus* group and to address the identification of the species themselves or particular phenotypic groups. Thus, the ITS-PCR products from 141 strains of the six species being investigated (Table 1) were subjected to high-resolution polyacrylamide gel electrophoresis, and it was found that ITS-HHP is a further tool toward a greater understanding of the genetic relationship among the species of the *B. cereus* group and for addressing the strain identification.

Total DNA was extracted from the washed cells by sodium dodecyl sulfate-proteinase K treatment (1a, 15). Amplification of the ITS was performed using the already-described primers S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 (17). For each strain, the products of at least two separate amplifications were analyzed individually by electrophoresis.

The ITS-PCR amplification patterns were electrophoresed on standard 2% agarose gels in 0.5× Tris-borate-EDTA buffer and stained for 30 min in an 0.5-mg/liter solution of ethidium

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bromide (53). Separation in 160- by 200- by 0.75-mm polyacrylamide gels was performed in a Protean II apparatus (Bio-Rad, Milan, Italy) in 1× Tris-borate-EDTA buffer for 10 to 14 h at 100 V. To enhance the heteroduplex separation, the samples were run in an 0.6× MDE gel under the same conditions used for standard polyacrylamide gel electrophoresis. All the polyacrylamide gels were cast vertically over a Gel Bond PAG film (FMC Bioproducts, Milan, Italy) to facilitate manipulation for the subsequent staining procedure. After the run, the gels were stained either with ethidium bromide (53) or by silver staining according to a known procedure (5). After staining, the gels were rinsed for 10 min in a 25% (vol/vol) ethanol–10% (vol/vol) glycerol solution, then covered with an extra cellophane sheet (Sigma, Milan, Italy), and left to dry at room temperature for 48 h.

An agarose gel electrophoresis examination of the ITS-PCR products from 50 strains selected randomly from the collection revealed typical *B. cereus* group ITS-PCR patterns with three bands of about 240, 460, and 530 bp, respectively (14) (Fig. 1A). Only strains of *B. anthracis* showed different patterns that could be differentiated into three groups (Fig. 1A).

The band pattern resolution using two other matrices, polyacrylamide and MDE (a particular polyacrylamide specifically designed to enhance the separation of heteroduplex and single-stranded fragments), confirmed the pattern differences in

B. anthracis and showed a marked migration shift of several bands (Fig. 1B and C). By comparison with the bands of the 50-bp ladder, only the 240- and 530-bp fragments of the ITS-PCR patterns observed in the agarose gel showed a similar migration. The polyacrylamide gel revealed additional bands, several of which migrated further when the analysis was carried out on the MDE matrix. This behavior was also observed when the ITS-PCR products obtained from independent amplifications were analyzed (data not shown). The differential migration of several bands could be explained by assuming that the separation of these fragments was based not only on molecular size but also on the formation of secondary structures that slow down the fragment migration and that are typical of heteroduplex and single-stranded fragments (21, 31–33).

To verify that all the bands in the pattern, including the putative heteroduplex–single-stranded products, were ITS fragments and not aspecific PCR products, the bands from a polyacrylamide gel were blotted over a nylon membrane (53) and hybridized with a digoxigenin-labeled (14, 16, 17; Boehringer Mannheim user's guide, Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) short ITS fragment from the *B. anthracis* strain Capanzo, identified as an ITS by cloning and sequencing (data not shown). The Southern hybridization confirmed that the bands showing migration shifts with respect to

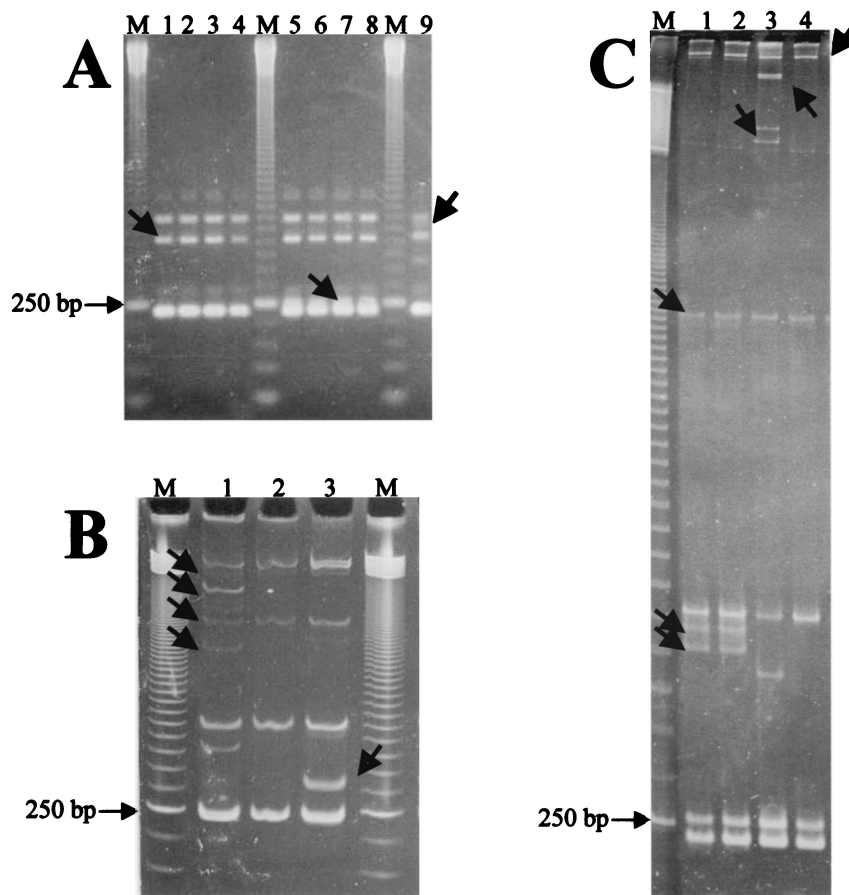


FIG. 1. Example of ITS-PCR fingerprinting patterns of *B. anthracis* strains resolved by agarose (A), polyacrylamide (B), and MDE (C) gel electrophoresis, showing the migration shifts of some amplified products. Lanes M, 50-bp ladder. (A) Lanes 1 to 9, *B. anthracis* strains 256, 282, 582, 846, 7700, 7702, Capanzo, 4229, and Davis TE702, respectively. (B) Lanes 1 to 3, *B. anthracis* strains Davis TE702, 282, and Capanzo, respectively. (C) Lanes 1 to 4, *B. anthracis* strains 7700, 7702, Davis TE702, and 256, respectively. The 250-bp band of the ladder is indicated. Arrows indicate bands that showed different mobilities among agarose, polyacrylamide, and MDE gel electrophoresis assays.

TABLE 1. Strains analyzed in this study, ITS-HHP pattern type number, and relevant characteristics

Species ^a (no. of strains)	H ^b	Strain	Relevant characteristic(s) of strain (reference[s])
BA (27)	30	Davis TE702 ^c	pXO1 ⁻ /2 ⁺ (51)
	31	256 ^c , 282 ^c , 582 ^c , 846 ^c , 6445/RA3 ^c , 6445/RA3R ^c , 6687/4896/L ^c , 6769 ^c , 7611 ^c , 7611R ^c , 9240 ^f	All the strains were pXO1 ⁺ /2 ⁺ except strains 6445/RA3R and 7611R (pXO1 ⁺ /2 ⁻) (48, 51). Strains 256, 282, 582, and 846 were isolated in the French Pyrenees in 1994 (Guy Patra, personal communication). All the other strains were isolated in France, in the Pyrenees and Alps, in 1997 (48).
	32	170 ^f , 227 ^c , 300 ^c , 376 ^c , 663 ^c , 779 ^c , 832 ^c , 957 ^c , 4229 ^c , 6602 ^c , 7700 ^c , 7702 ^c , Capanzo ^c , 2522 ^d , antiMi ^d	All the strains were pXO1 ⁺ /2 ⁺ except 7700 (pXO1 ⁻ /2 ⁻); 957, 7702, and Capanzo (pXO1 ⁺ /2 ⁻); and 4229 and 6602 (pXO1 ⁻ /2 ⁺) (51). Strains 170, 376, 663, 779, and 832 were isolated in France (Guy Patra, personal communication).
BC (23)	13	cer3 ^e	Isolated from candies (17)
	14	po1 ^e	Isolated from ultrahigh-temperature milk (17)
	25	360 ^f	
	26	cer1 ^e	
	27	cer6 ^e	Isolated from tomato sauce (17)
	28	co1 ^e	Isolated from pasteurized milk (17)
	29	2896 ^f	
	33	bc2 ^c	
	34	345 ^f	
	35	cer5 ^e	Isolated from rice (17)
	37	46321 ^f	
	38	487 ^f	
	39	be1 ^e	Isolated from marble of Ca' d'Oro façade, Venice, Italy (17)
	40	31 ^{Te}	<i>B. cereus</i> type strain
	41	cer4 ^e	Isolated from rice (17)
	42	myd ^e	
	43	351 ^f	
	44	my1 ^e	Isolated from ultrahigh-temperature milk (17)
BM (21)	45	co2 ^c	
	47	6127 ^f	
	49	626 ^f	
	50	5148 ^g	Same as strain Bc7, produces the bacteriocin cerein 7 (45)
	58	IO200 ^h	
	3	G2 ^e	Isolated from garden soil, Italy
	15	309 ^f , 384 ^f , bmF ^e	384 strain growth at 7°C (15)
	16	NRS273 ^{Ti}	<i>B. mycooides</i> type strain
	17	2048 ^{Tj} , 229 ^f	2048 ^{Tj} , <i>B. mycooides</i> type strain; growth at 7°C (15)
	18	B615 ⁱ	Isolated from soil (43)
	19	B14828 ⁱ	
	20	BmMed ^e	Isolated from alkaline soil, Italy
	21	BmS ^e	
	22	Ndr ^e , BiF ^e	Isolated from soil, Italy
23	NRS306 ⁱ , NRS319 ⁱ	Isolated from soil (43)	
24	G1 ^e , Nov1 ^e	G1, isolated from garden soil, Italy; Nov1, isolated from maize rhizosphere, Italy	
36	A81 ^e , TP2 ^e	Isolated from soil, Italy	
46	MycH ^e		
48	Nov2 ^c	Isolated from maize rhizosphere, Italy	
BP (8)	7	BD10 ⁱ , BD14 ⁱ	Isolated from soil (43)
	8	A82 ^e	Isolated from soil, Italy
	9	B346 ^j	Isolated from soil (43)
	10	CA ^c	Isolated from soil, Italy
	11	B617 ^{Ti} , B618 ⁱ	B617 ^{Ti} , <i>B. pseudomycooides</i> type strain (44); isolated from soil (43)
	12	TP1 ^e	Isolated from soil, Italy
BT (58)	51	BMG1.6 ^h	Isolated from soil, Tunisia
	52	2046 ^{Tj}	<i>B. thuringiensis</i> type strain, subsp. <i>thuringiensis</i>
	53	Bt10 ^h	<i>B. thuringiensis</i> subsp. <i>darmstadiensis</i>
	54	BUMP30 ^j	Same as strain BST30, <i>B. thuringiensis</i> subsp. <i>sotto</i> (30)
	55	5424 ^f	<i>B. thuringiensis</i> subsp. <i>israelensis</i>
	56	HD868 ^k	<i>B. thuringiensis</i> subsp. <i>tochigiensis</i> ; produces the bacteriocin tochicin (46)
	57	BUMP33 ^j	Isolated from soil, Tunisia
	59	Bt5 ^e	
	60	Bt14 ^h , Bt44 ^h	Bt14, <i>B. thuringiensis</i> subsp. <i>israelensis</i> ; Bt44, <i>B. thuringiensis</i> subsp. <i>dendrolimus</i>
	61	Ht39 ^j	Displays entomocidal activity against <i>Culex</i> sp. larvae
	62	Hc15 ⁱ , Hc24 ⁱ , Hc32 ⁱ , Hc35 ⁱ	Display entomocidal activity against <i>Culex</i> sp. larvae
	63	Hc17 ⁱ	Displays entomocidal activity against <i>Culex</i> sp. larvae
	64	Hc16 ⁱ	Displays entomocidal activity against <i>Culex</i> sp. larvae
	65	Hc13 ⁱ	Displays entomocidal activity against <i>Culex</i> sp. larvae
	66	BMG1.3 ^h , BMG1.4 ^h , BMG1.5 ^h	Isolated from surface water, Tunisia
	67	Ht51 ⁱ	Displays entomocidal activity against <i>Culex</i> sp. larvae
68	Hc45 ⁱ	Displays entomocidal activity against <i>Culex</i> sp. larvae	
69	Hc36 ⁱ	Displays entomocidal activity against <i>Culex</i> sp. larvae	
70	BX1 ^h , BX3 ^h , BX8 ^h , BX9 ^h , BX11 ^h , BX12 ^h , BX16 ^h , BX19 ^h , BX20 ^h , BX21 ^h , BX22 ^h , BG7 ^h	BX strains were isolated from hypersaline soils in Tunisia and display antifungal activity against <i>Fusarium</i> sp.	
71	BMG1.2 ^h , BMG1.7 ^h , BMG1.8 ^h , BMG1.9 ^h , BUMP24 ^j , BUMP25 ^j , BUMP26 ^j , BUMP28 ^j , BUMP29 ^j	Isolated from soil, Tunisia; strain BMG1.7 produces the bacteriocin thuricin 7 (Cherif et al., unpublished)	

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TABLE 1—Continued

Species ^a (no. of strains)	H ^b	Strain	Relevant characteristic(s) of strain (reference[s])
	72	BTX ^c , PTB ^e , BTK ^e , HD2 ^k	Strain HD2, <i>B. thuringiensis</i> subsp. <i>thuringiensis</i> , produces the bacteriocin thuricin (19)
	73	Bt1 ^h , Bt7 ^h , Bt9 ^h , Bt33 ^h , Bt55 ^h , HD1 ^m , Al ^f	Bt1, <i>B. thuringiensis</i> subsp. <i>thuringiensis</i> ; Bt7, <i>B. thuringiensis</i> subsp. <i>aizawai</i> ; Bt9, <i>B. thuringiensis</i> subsp. <i>tolworthii</i> ; Bt33 and HD1, <i>B. thuringiensis</i> subsp. <i>kurstaki</i> ; Bt55, <i>B. thuringiensis</i> subsp. <i>galleriae</i>
	74	BMG1.1 ^h	Isolated from soil, Tunisia
	75	Bt13 ^h	Bt13, <i>B. thuringiensis</i> subsp. <i>pakistani</i>
BW (4)	4	10201 ⁿ	Growth at 7°C (39, 50); isolated from milk (39)
	5	10208 ⁿ	Growth at 7°C (39, 50); isolated from milk (39)
	6	10204 ^{ln} , 10202 ⁿ	10204 ^l , <i>B. weihenstephanensis</i> type strain; growth at 7°C (39, 50); isolated from milk (39)
BL (1)	1	BI32	
PL (1)	2	BX7	

^a Abbreviations used to indicate the *B. cereus* group species; BA, *B. anthracis*; BC, *B. cereus*; BM, *B. mycoides*; BP, *B. pseudomycoloides*; BT, *B. thuringiensis*; BW, *B. weihenstephanensis*. The outgroup species were *B. licheniformis* (BL) and *Paenibacillus lentimorbus* (PL).

^b H, ITS-HHP type number (Fig. 4).

^c Institut Pasteur, Paris, France. Total DNA of the *B. anthracis* strain was kindly provided by M. Mock.

^d Istituto Zooprofilattico, Milan, Italy. Total DNA of the *B. anthracis* strains was kindly provided by M. Luini.

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^f Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

^g Spanish Type Culture Collection. *B. cereus* strain 5148 was kindly provided by Federico Uruburu.

^h Laboratoire de Microbiologie de Tunis. BX strains were isolated and kindly provided by N. Sadfi. The reference strains of *B. thuringiensis* subspecies Bt1, Bt7, Bt9, Bt10, Bt13, Bt14, Bt33, and Bt55 were kindly provided by A. Boudabous.

ⁱ Agricultural Research Service Culture Collection, Peoria, Ill. Strains were kindly provided by L. K. Nakamura.

^j Centre de Biotechnologie de Sfax. BUMP strains were kindly provided by S. Jaoua.

^k *Bacillus* Genetic Stock Center. *B. thuringiensis* strains HD2 and HD868 were kindly provided by D. R. Zeigler.

^l Strains kindly provided by Hala Khyami-Horani, Department of Biological Sciences, Amman, Jordan.

^m Strain derived from commercial *B. thuringiensis* based bioinsecticides.

ⁿ Weihenstephan *Bacillus* Collection, Weihenstephan, Germany. *B. weihenstephanensis* strains were kindly provided by S. Scherer.

the bands observed in the agarose gel were all homologous to the ITS (data not shown).

The nature of the polymorphic bands was investigated by treating the ITS-PCR products with mung bean nuclease. This treatment eliminates the single strands in the heteroduplex products, permitting only homoduplex products to be detected in the gel (31–33). Fifty microliters of the ITS-HHP product was purified through a QIAquick column (Qiagen) and eluted with 80 µl of resuspension buffer (6 mM Tris-HCl [pH 7.5], 6 mM NaCl, and 0.2 mM EDTA). Thirty-eight microliters of the eluted DNA solution, with an added 10 to 15 U of mung bean endonuclease (Pharmacia Biotech, Milan, Italy) diluted 1/10 (vol/vol) just before use in the dilution buffer (10 mM sodium acetate [pH 5], 0.1 mM zinc acetate, 1 mM cysteine, 0.1% [vol/vol] Triton X-100, and 50% [vol/vol] glycerol), was incubated for 30 min at 30°C. The reaction was performed in a final volume of 50 µl containing 10 µl of 5× reaction buffer made of 150 mM sodium acetate (pH 5.0), 250 mM NaCl, 5 mM ZnCl₂, and 25% glycerol. To stop the reaction, 100 µl of 0.2% (wt/vol) sodium dodecyl sulfate–74 mM Tris-HCl (pH 9.5)–1.2 M LiCl solution was added; the enzyme was removed by phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]) treatment. The aqueous phase was extracted with 1 volume of diethyl ether, and the DNA was precipitated with ethanol and resuspended in 10 µl of Tris-EDTA, pH 7.5. The sample was electrophoresed in an MDE gel, and the DNA bands were revealed by silver staining.

Figure 2 shows the results of these experiments on some strains of *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. thuringiensis*. In all the cases, treatment resulted in the elimination of several bands, especially slowly migrating bands (over 500 to 600 bp), but in the case of the *B. anthracis* strain Cepanzo, also two bands with an apparent molecular size of 450 and 470 bp were eliminated, suggesting that these bands are heteroduplex fragments whose endonuclease digestion products (of less than 200 bp in molecular size) could be detected in the gel (Fig. 2). All the other bands were homoduplex products, since they could not be digested by the enzymatic treatment.

The mung bean nuclease experiments showed that the frag-

ments in the pattern around 250 and 500 bp are indeed the main homoduplex fragments and display several length polymorphisms. Some strains, like Davis TE702, showed additional homoduplex bands of intermediate size (ca. 420 bp in the case of Davis TE702) between short (ca. 250 bp) and long (ca. 510 bp) ITS. The size difference, i.e., less than 100 bp, roughly corresponds to the length of a tRNA gene and could be the result of tDNA gene deletion due to ITS rearrangement.

The entire collection of 141 strains of the six species of the *B. cereus* group plus two outgroup strains (Table 1) was analyzed for ITS-HHP on MDE gels to evaluate the intraspecific and interspecific diversity. To minimize migration shift due to the gel preparation procedure, the strains were loaded on gels prepared from the same MDE master mix and run simultaneously in a four-gel electrophoresis apparatus. The runs were repeated at least three times for each strain even from different PCRs, and the electrophoretic patterns were found to be constant and reproducible. The ITS-HHP patterns were shown to be very discriminative and allowed us to identify 75 pattern types, an unexpected variability in the extremely homogeneous *B. cereus* group (36) (Table 1). Representative ITS-HHP pattern types are shown in Fig. 3. A total of 72 polymorphic bands was detected. The number of bands per profile varied from 3 to 16, and the apparent molecular size ranged from 200 to more than 2,000 bp.

Computer-assisted analysis of the ITS-HHP was performed using the Diversity Database fingerprinting software (Bio-Rad). The banding patterns were acquired from the dried silver-stained gels with the Gel Doc2000 image system (Bio-Rad), using the white-light transilluminator of the device, and stored on disk as TIFF files. The rolling-disk background subtraction method was applied to each gel, and a database containing all the gel images was created. The bands from all the gels were automatically detected and normalized using the 50-bp DNA ladder (Amersham Pharmacia Biotech) as the molecular size marker. A band set including all the polymorphic fragments was created, and each band in each lane was compared with the band set. The similarity between strains was

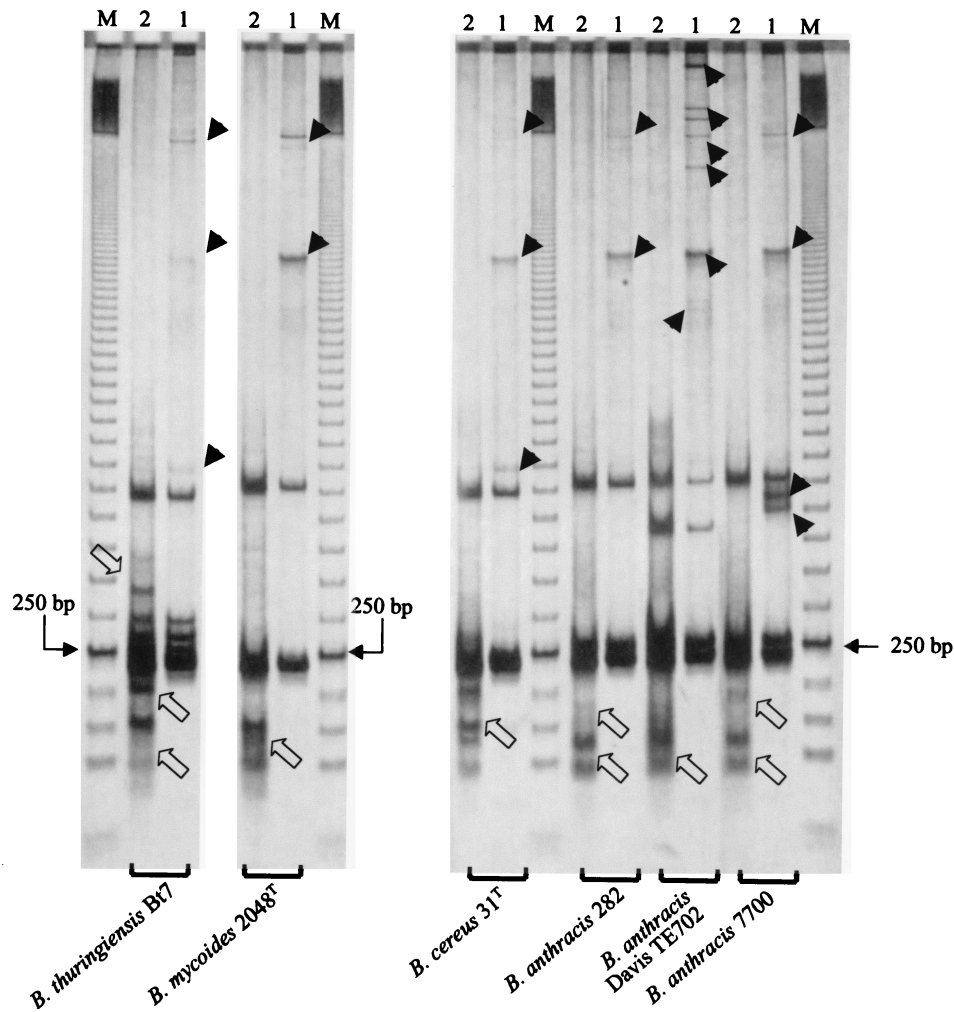


FIG. 2. Identification of the heteroduplex bands in the ITS-HHP patterns separated in an MDE gel after mung bean nuclease digestion of the PCR products. The MDE runs for *B. anthracis* 7700, Davis TE702, and 282; *B. cereus* 31^T; *B. mycooides* 2048^T; and *B. thuringiensis* Bt7 are shown. Lanes M, 50-bp ladder. Lanes 1, normal ITS-HHP profiles. Lanes 2, the same products of lanes 1 after treatment with mung bean nuclease. The 250-bp band of the ladder is indicated. Arrowheads indicate the heteroduplex products removed by the mung bean nuclease. Open arrows show degradation products of the heteroduplex bands.

determined by the band-sharing coefficient calculated by the formula of Jaccard (56), and strain clustering was performed by the unweighted pair group method with arithmetic averages (UPGMA) (56).

The dendrogram generated by UPGMA cluster analysis is shown in Fig. 4. Seven major subclusters (A1, A2, A3, B1, B2, B3, and B4) could be identified, with an early separation between clusters A and B. Cluster A grouped the strains of *B. weihenstephanensis* (subcluster A1), *B. pseudomycooides* (subcluster A2), and *B. mycooides* (subcluster A3).

Cluster B was subdivided into four major subclusters. Subcluster B1 included *B. mycooides* and several strains of *B. cereus*. Subcluster B2 included mainly *B. cereus* strains and two soil isolates of *B. mycooides* that yielded the same ITS-HHP pattern type. In branch B2.a of subcluster B2 (Fig. 4), there was the grouping of the 27 strains of *B. anthracis*, confirming it to be a relatively uniform and clonal species. The subcluster B3 included several *B. cereus* strains as well as two strains of *B. mycooides*. Subcluster B4 included all 58 strains of *B. thuringiensis* plus three strains of *B. cereus*. The subcluster B4 was divided into two main groups, B4.a and B4.b.

The 27 *B. anthracis* strains were grouped in the same three groups observed by agarose gel electrophoresis (Fig. 3 and 4).

The simplest profile was one from strains isolated from the recent anthrax outbreaks that occurred in the French Pyrenees and Alps in 1994 and 1997. These strains could be differentiated from the other species of the *B. cereus* group by the differential migration of the 250-bp bands and/or the 500-bp band. The second group of *B. anthracis* included the classic strains used for the development of vaccines and several strains isolated from France that showed two additional typical signature bands around 450 bp. Strain Davis TE702 had the most diverse band pattern, with a signature band of about 420 bp and six additional bands in the upper part of the gel.

Our ITS-HHP analysis data indicate that it is easy to discriminate *B. anthracis* from related species on chromosomally located sequences. The identification of three lineages in the species suggests that ITS-HHP could be of value in tracing the spread of this pathogen and its genomic differentiation. For example, the strains isolated from the anthrax outbreaks in the French Pyrenees and Alps in 1994 and 1997 (48) are different from all the other strains isolated in France, and it can be hypothesized that (i) they are traces of the introduction of a *B. anthracis* strain quite different from the typical strains found in France, or (ii) they derive from other French strains, differing from them due to the loss of ribosomal operons or to a

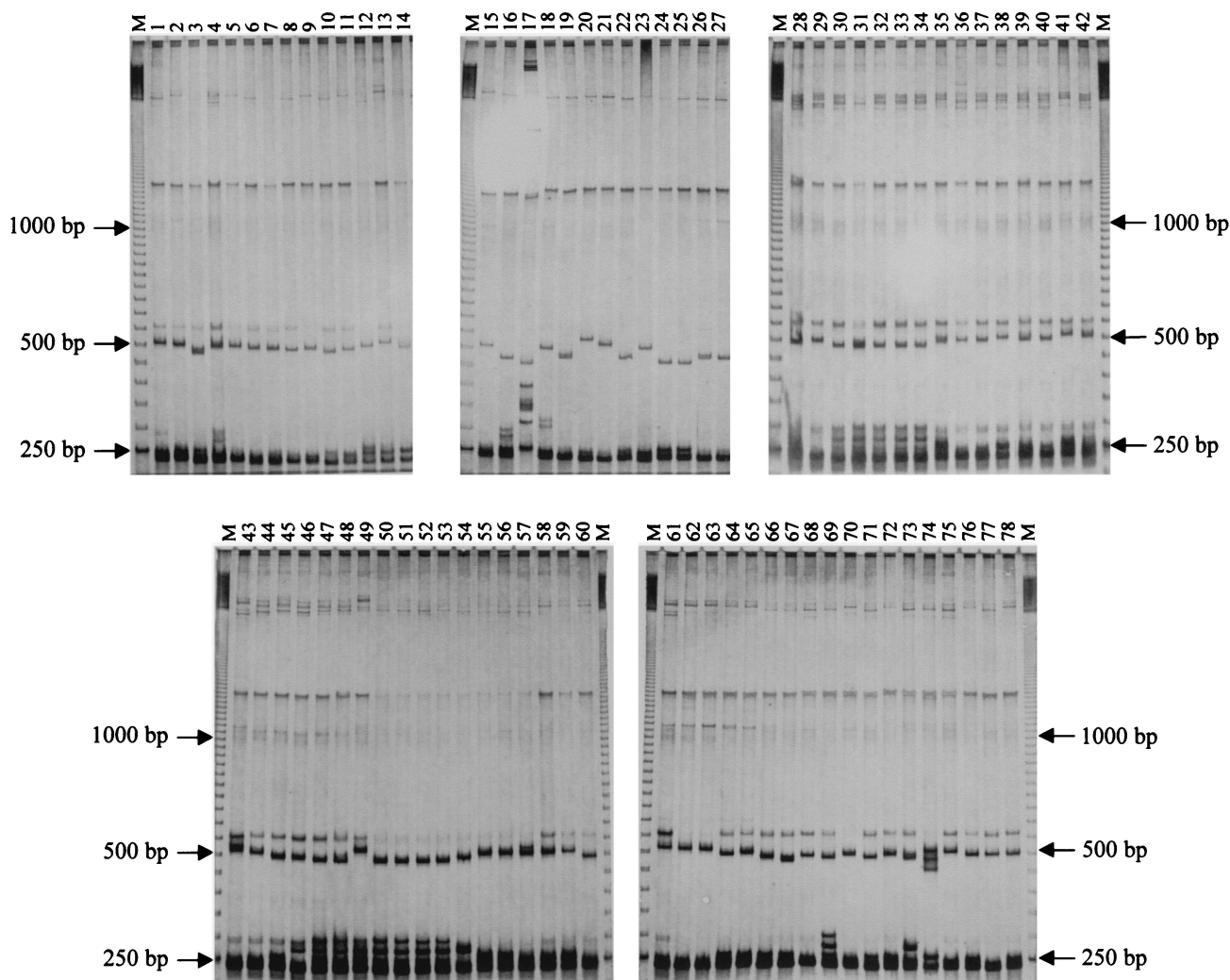


FIG. 3. Examples of the ITS-HHP pattern variability observed in the collection examined. For each strain, the ITS-HHP type (H), (Table 1 and Fig. 4) is marked in parentheses below. Lanes M, 50-bp ladder. Lanes 1 to 14, *B. cereus* 351 (ITS-HHP type H43), myd (H42), co2 (H45), 6127 (H47), 46321 (H37), 2896 (H29), 31^T (H40), co1 (H28), cer4 (H41), cer3 (H13), 360 (H25), 487 (H38), cer6 (H27), and bc1 (H39), respectively. Lanes 15 to 18, *B. mycooides* mych (H46), nov2 (H48), G2 (H3), and NRS273 (H16), respectively. Lane 19, *B. pseudomycooides* TP1 (H12). Lanes 20 and 21, *B. mycooides* 309 (H15) and 2048^T (H17), respectively. Lane 22, *B. pseudomycooides* CA (H10). Lane 23, *B. mycooides* B615 (H18). Lanes 24 to 27, *B. pseudomycooides* BD14 (H7), BD10 (H7), B617^T (H11), and B346 (H9), respectively. Lane 28, *B. cereus* 5148 (H50). Lanes 29 to 60, *B. thuringiensis* HD868 (H56), HD2 (H72), HD1 (H73), BTK (H72), PTB (H72), BTX (H72), Ht51 (H67), Ht39 (H61), Hc45 (H68), Hc36 (H69), Hc17 (H63), Hc16 (H64), Hc15 (H62), Hc13 (H65), BMG1.6 (H51), BUMP30 (H54), BUMP33 (H57), BMG1.1 (H74), BX16 (H70), BMG1.7 (H71), BMG1.3 (H66), Bt33 (H73), Bt9 (H73), Bt7 (H73), Bt1 (H73), Bt13 (H75), Bt44 (H60), Bt14 (H60), Bt10 (H53), Bt5 (H59), 5424 (H55), and 2046^T (H52), respectively. Lanes 61 to 64, *B. weihenstephanensis* 10201 (H4), 10202 (H6), 10204^T (H6), and 10208 (H5), respectively. Lanes 65 to 73, *B. cereus* myd (H42), my1 (H44), co2 (H45), co1 (H28), cer5 (H35), cer4 (H41), cer3 (H13), cer1 (H26), and 345 (H34), respectively. Lane 74, *B. anthracis* 7700 (H32). Lanes 75 to 78, *B. cereus* 351 (H43), bc2 (H33), 2896 (H29), and 360 (H25), respectively. The 250-, 500-, and 1,000-bp bands of the ladder are indicated.

rearrangement of the ITS sequence. This latter possibility could, perhaps, be clarified through a survey of ITS-HHP on a wider collection of *B. anthracis* strains. In any case, ITS-HHP analysis provides a tool in addition to amplified fragment length polymorphism and the multiple-locus variable-number tandem repeat analysis applied by Keim et al. (36, 37) to analyze the polymorphism of this species.

The 23 *B. cereus* strains all had different band patterns characterized by one or two bands around 250 bp, one band around 500 bp (480 to 520 bp), and several other bands of different sizes (Fig. 3). The main differences in the band patterns were due to the presence or absence of the second band at 250 bp and differences in the migration of the 500-bp band.

Most of the 58 *B. thuringiensis* strains were, in general, characterized by the presence of several bands in the lower part of the profile (below 300 bp), allowing us to distinguish 24 ITS-

HHP types (Table 1 and Fig. 3 and 4). Some important *B. thuringiensis* subspecies known to be genetically related were grouped together by ITS-HHP. For example, strains belonging to the *B. thuringiensis* subspecies *aizawai*, *galleriae*, *kurstaki*, and *tolworthi* showed the same ITS-HHP patterns, in agreement with the ribotyping data of Priest et al. (49), the M13 DNA fingerprinting data of Miteva et al. (40), and the DNA-DNA hybridization data of Nakamura (42). The fact that the ITS-HHP patterns of these subspecies were identical reflected their close DNA relatedness (90%) (42), while the *B. thuringiensis* subspecies *darmstadiensis*, *israelensis*, and *sotto*, which have relatively low DNA-DNA hybridization levels (below 70%) (42), showed different ITS-HHP types and were assigned to a cluster different from that of *B. thuringiensis* subsp. *kurstaki* and its related subspecies. The different strains of the *B. thuringiensis* subspecies *thuringiensis* were grouped in differ-

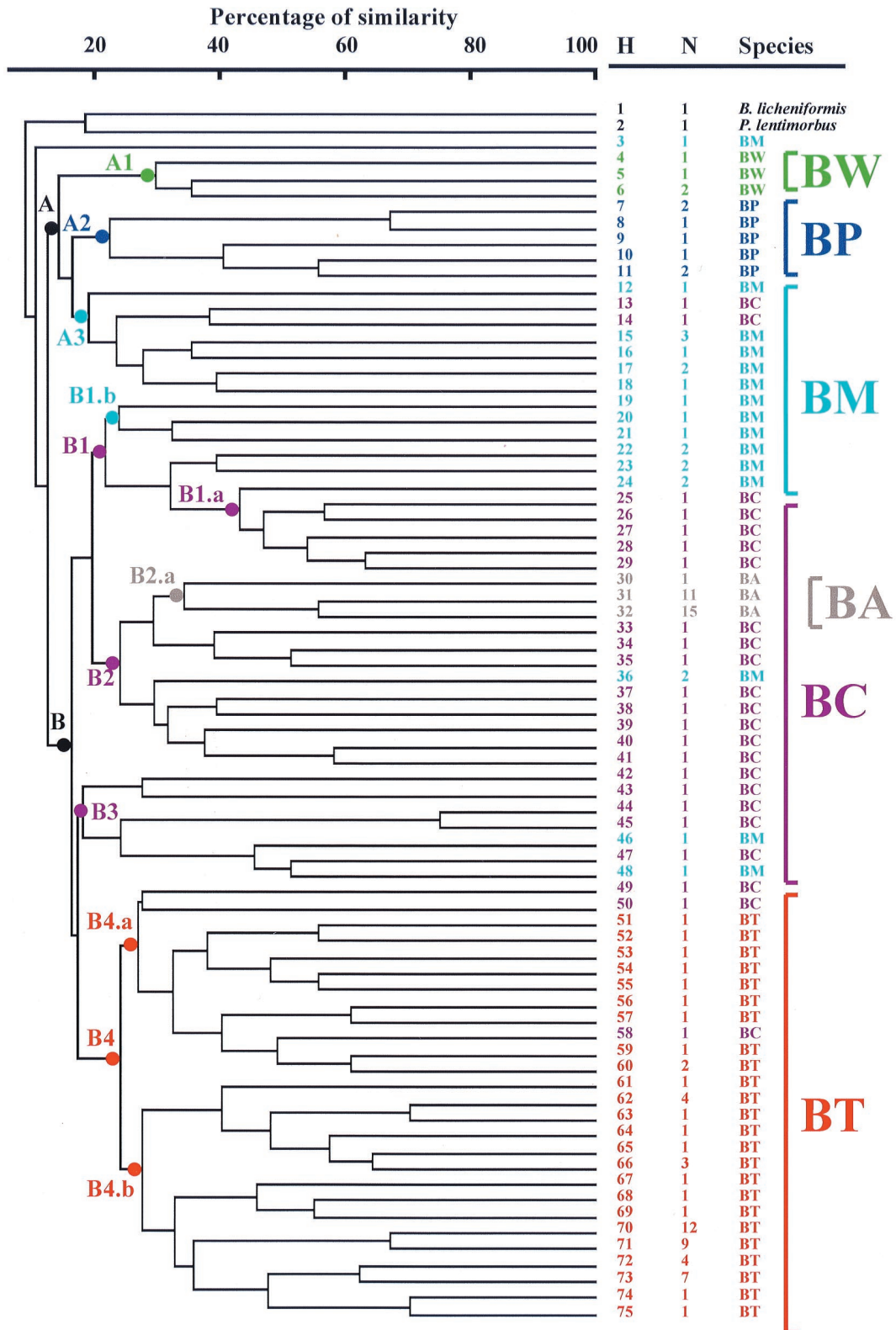


FIG. 4. Genetic relationship among *B. cereus* group strains as described by the UPGMA cluster analysis of the ITS-HHP patterns. The percentages of similarity among the ITS-HHP patterns were calculated using the Jaccard coefficient. H, ITS-HHP type number (Table 1); N, number of isolates for each ITS-HHP type (Table 1). Abbreviations (and colors) used to indicate the *B. cereus* group species are as follows: BA (grey), *B. anthracis*; BC (purple), *B. cereus*; BM (pale blue), *B. mycoides*; BP (blue), *B. pseudomycooides*; BT (red), *B. thuringiensis*; and BW (green), *B. weihenstephanensis*. The dots indicated with letter-number designations (see text) and marked with the colors identifying the six *B. cereus* group species were drawn on the dendrogram nodes where separated clusters and subclusters are evident.

ent branches of the dendrogram, confirming the genetic variability revealed by Priest et al. by ribotyping analysis (49). Interestingly, strains with relevant phenotypic characteristics such as antifungal or antibacterial activity were found to be clustered differently: strains displaying antibacterial activity due to the production of bacteriocins such as *B. thuringiensis* strain HD868 (46), strain HD2 (19), and strain BMG1.7 (A. Cherif, H. Ouzari, D. Daffonchio, M. Gtari, A. Hassen, S. Jaoua, and A. Boudabous, unpublished data) and *B. cereus* strain 5148 (45) appeared far apart, while BX strains with anti-*Fusarium* activity (Sadfi Najla, personal communication) were clustered together. This could be a reflection of the restricted area (Tunisia) from which the anti-*Fusarium* strains were isolated. In contrast, there was marked variation in the 11 *B. thuringiensis* strains isolated in Jordan; all displayed anti-*Culex* activity (Hala Khyami-Horani, personal communication) but were clustered in eight different ITS-HHP types (types 61 to 65 and 67 to 69, Table 1). Probably the bacteriocinogenic and anti-*Culex* activity of these strains in different genetic backgrounds is due to horizontal gene transfer of the genetic determinants for activity, such as *cry* genes, that are frequently carried on plasmids and are hence potentially transferable to other strains (19, 20).

The strains of *B. mycoides* and *B. pseudomycooides* showed a wide migration shift in the 530-bp band that ranged from 450 to 530 bp (Fig. 3). In the rest of the ITS-HHP pattern, the strains were relatively similar. Strain G2 showed a very different ITS-HHP pattern type with several additional bands in the upper and lower parts of the gel. For the 21 strains of *B. mycoides* tested, 14 different ITS-HHP types were detected. Six ITS-HHP pattern types were observed among the eight *B. pseudomycooides* strains examined. Different ITS-HHP types were found for the *B. mycoides* type strains deriving from different culture collections (strain NRS273^T from the Agricultural Research Service Culture Collection and strain 2048^T from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Table 1). This could be due to the different manipulations that the two strains had undergone for maintenance in the different collections. *B. pseudomycooides* was related to *B. mycoides*, having phenotypic characteristics of rhizoid growth in common. The few strains of *B. pseudomycooides* analyzed in this study were separated from *B. mycoides* and grouped in a separate branch of the dendrogram (Fig. 4). In agreement with the DNA-DNA hybridization results of Nakamura and Jackson (43, 44), who found two main groups of strains sharing 75% DNA relatedness, the *B. pseudomycooides* strains analyzed here were clustered by ITS-HHP analysis in two well-separated groups, the first including strains BD10 and BD14 and the second including the type strain B617^T (Table 1 and Fig. 4).

The four strains of *B. weihenstephanensis* were revealed to have three different ITS-HHP patterns (Table 1 and Fig. 3 and 4).

The study has shown that for strain typing the ITS-HHP approach is advantageous with respect to direct sequence comparison in that it takes into consideration, and highlights, sequence polymorphism among all the amplified operons of the genome. With the ITS-HHP analysis, the ribosomal operon diversity in the *B. cereus* group appeared greater than previously estimated. ITS-HHP analysis can also be used to address the identification of uncharacterized isolates. The method permits the rapid identification of *B. anthracis* isolates on the basis of chromosomal traits and independent of the virulence determinants located on the pXO plasmids that may be lost by the bacteria (7, 20, 47, 51, 52, 59, 60). In agreement with recent data (27), the genetic relationships within the *B. cereus* group profiled by the ITS-HHP analysis further support the idea that *B. cereus*, *B. anthracis*, and *B. thuringiensis* are members of a

single species (*B. cereus sensu lato*) that has achieved specialization by acquiring particular plasmid-encoded phenotypes such as pathogenicity. *B. weihenstephanensis*, *B. mycoides*, and *B. pseudomycooides* are the most divergent species (36).

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