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Bacillus anthracis Diverges from Related Clades of the *Bacillus cereus* Group in 16S-23S Ribosomal DNA Intergenic Transcribed Spacers Containing tRNA Genes

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Mung bean nuclease treatment of 16S–23S ribosomal DNA intergenic transcribed spacers (ITS) amplified from several strains of the six species of the *Bacillus cereus* group showed that *B. anthracis* Davis TE702 and *B. mycoides* G2 have other intermediate fragments in addition to the 220- and 550-bp homoduplex fragments typical of the *B. cereus* group. Long and intermediate homoduplex ITS fragments from strains Davis TE702 and G2 and from another 19 strains of the six species were sequenced. Two main types of ITS were found, either with two tRNA genes ($tRNA^{Ile}$ and $tRNA^{Ala}$) or without any at all. Strain Davis TE702 harbors an additional ITS with a single tRNA gene, a hybrid between the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, suggesting that a recombination event rather than a deletion generated the single tDNA-containing ITS. Strain G2 showed an additional ITS of intermediate length with no tDNA and no similarity to other known sequences. Neighbor-joining analysis of tDNA-containing long ITS indicated that *B. cereus* and *B. thuringiensis* represent a single clade. Three signature sequences discriminated *B. anthracis* from *B. cereus* and *B. thuringiensis*, indicating that the anthrax agent started evolving separately from the related clades of the *B. cereus* group. *B. mycoides* and *B. weienstephanensis* were very closely related, while *B. pseudomycoides* appeared the most distant species.

Bacillus anthracis, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihenstephanensis* are six related species, five of which have an important impact on human activity (16, 37, 45, 46, 51): *B. anthracis* is the active agent of anthrax (48); *B. cereus* causes food-borne disease syndromes associated with enterotoxin and emetic toxin (16, 25); *B. thuringiensis* is an insect pathogen (45) and is widely used for the biological control of insects in crop protection; *B. mycoides* has been recognized as a plant growth-promoting bacterium associated with conifer roots (38); *B. weihenstephanensis*, a psychrotolerant species frequently found in pasteurized milk, is a potential cause of spoilage problems (32). The six species are not easily distinguished on the basis of phenotypic or genetic traits (48). Recently, *B. anthracis*, *B. cereus*, and *B. thuringiensis* were found to be very closely related, and it has been proposed that they belong to a single species (24, 33). This proposal has been based on multilocus enzyme electrophoresis data and sequencing of discrete genetic loci (24) and on the presence of an S-layer on the cell surface (33). The model considering *B. anthracis*, *B. cereus*, and *B. thuringiensis* as subspecies of a phylogenetically monomorphic group, differing mainly in characters linked to mobile genetic elements such as plasmids, is supported by very high sequence homology in the conserved molecular chronometers of the ribosomal operons, the 16S and 23S ribosomal DNA (rDNA), and the short intergenic spacer between them (3–5, 7, 21, 30). Considering the dangerousness of *B. anthracis* and the wide in-field application of *B. thuringiensis* as a biological insecticide, it would be opportune to further evaluate the phylogenetic relationship between the different clades of the *B. cereus* group. Whole-genome sequence-based analysis could give a definitive view of the genetic relationship between these species (29). However, an approach that is economically feasible, given current technology, is possible for few strains in a given species (42). Hence, for phylogenetic surveys based on a relatively large number of isolates of each species, permitting an assessment of the amount of variability and overlap within a species, the best means of approach remains the use of highly conserved molecules with no, or a low, horizontal gene transfer rate such as the ribosomal operon.

In the prokaryote genome, the ribosomal operon can be present in multiple copies, up to 15 copies in *Clostridium paradoxum* (41). The 16S–23S rDNA intergenic transcribed spacers (ITS) are the most variable regions of the ribosomal operon, and, apart from interoperonic nucleotide substitutions, insertions, and deletions, such ITS can be differentiated, given the presence of the different numbers and types of tRNA genes (9, 10, 31, 49). Since the ITS have fewer functional constraints than the adjacent ribosomal genes, which undergo concerted evolution (17–19), their sequences can contain traces of ribosomal operon rearrangements and species-specific or even strain-specific traits that are useful for strain typing.

An analysis of ITS homoduplex-heteroduplex polymorphisms has shown that wide variability exists in the strains of the six species of the *B. cereus* group (14), indicating widely different length and sequence polymorphisms among the 8 to 12 ribosomal operons (26). In the present study, we examined the sequence heterogeneity of the ITS of several strains of the six species showing different ITS homoduplex-heteroduplex

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TABLE 1. Signature nucleotides in long ITS containing tDNA typical of the species and clades of the *B. cereus* group

Species or clade[a]	Strains[a,b]	Nucleotide position[c]	Signature sequence
BA	BA: 7700, 663, 282, Davis TE702	75–80	AAAAAG
		121	A
		213–214	AT
BC/BT	BC: 31^T, 345, 626, V65SP	75–76	TC
	BT: 2046^T, HD1, Bt14, BMG1.7, Ht39	117–121	CCTGC
		213–220	TTTTGG
BM/BW	BM: 2048^T, 299, 309	117–121	TCCGC
	BW: 10204^T, 10202, 10208	213–218	TTTTAC
BP	BP: BD10	75–81	AAATAT
		117–121	TCTGC
		213–214	AC

[a] BA, *B. anthracis*; BC, *B. cereus*; BM, *B. mycoides*; BP, *B. pseudomycoides*; BT, *B. thuringiensis*; BW, *B. weihenstephanensis.*
[b] Strains that harbor the signature sequences.
[c] Nucleotide positions based on the sequence coordinates of *B. anthracis* 7700 reported in Fig. 3.

polymorphism haplotypes (14). It has been shown that four different types of ribosomal spacers can be found and that genetic structural variation (22–24) is not restricted to variable species like *B. cereus* and *B. thuringiensis*; in fact, major variation can also be found in the very monomorphic species *B. anthracis* (27). Since no sequences of the tDNA-containing ITS of strains of the *B. cereus* group have been reported until now, we focused our attention on this region, which has been reported to allow the separation of closely related strains of different ecotypes in other species (see, for example, reference 43). On the basis of the tDNA-containing ITS sequences, the phylogenetic relationship between the species was evaluated, and it has been shown that *B. anthracis* represents a phylogenetic clade diverging from *B. cereus* and *B. thuringiensis*.

MATERIALS AND METHODS

Strains, PCR amplifications, and 16S–23S rDNA ITS fingerprinting. The strains used in this study (Table 1) (14) were grown routinely, as previously described (6, 11, 13), and the DNA suitable for amplification was obtained through lysis by boiling (15) or by sodium dodecyl sulfate-proteinase K treatment (44), as already described (2, 6, 11, 13). The ITS homoduplex-heteroduplex polymorphism profiles were obtained by PCR followed by electrophoresis in MDE gel (BioWhittaker Molecular Applications, Milan, Italy) and silver staining using the procedures described previously (14). MDE is a separation matrix made of a polyacrylamide specifically designed to separate nucleic acid fragments on the basis of their secondary structure. It can be used to highlight nucleotidic polymorphisms on the basis of differential single-strand or heteroduplex conformations.

The homoduplex fragments in the ITS homoduplex-heteroduplex polymorphism profiles were highlighted by subjecting the ITS amplified products to mung bean nuclease treatment, which eliminates single strands in heteroduplex products, permitting only homoduplex products to be detected in the gel (14). The mung bean nuclease reaction was performed as previously described (14), the resulting sample was electrophoresed in MDE gel, and the DNA bands were revealed by silver staining.

Cloning and sequencing of the 16S–23S rDNA ITS. The homoduplex fragments separated in the MDE gel after mung bean nuclease treatment were excised from the gel (46), reamplified using the same forward (S-D-Bact-1494-a-S-20 on the 16S rDNA) and reverse (L-D-Bact-0035-a-A-15 on the 23S rDNA) primers (13) used for the generation of the original ITS homoduplex-heteroduplex polymorphism profiles, and sequenced in an ABI Prism 310 sequence analyzer (Applied Biosystems, Milan, Italy). Besides direct sequencing of the PCR product, the long ITS of *B. anthracis* 282 was sequenced after being cloned in the pMOS cloning kit (Amersham Pharmacia Biotech, Milan, Italy), as specified by the supplier. Cloned ITS were sequenced using the T7 and U19 primers on the vector. For all the strains, the sequences were confirmed by sequencing the DNA fragments obtained by amplifying the 5′- and 3′-end stretches of the long ITS from the genomic DNA. The primers targeting 16S rDNA (ITS-A-f, [5′-CCTTGTACACACCGCCCGT-3′]) and 23S rDNA (ITS-B-r [5′-GTGGGT TTCCCCATTCGG-3′]) were designed in a more internal position on the respective gene than were the original primers used to amplify ITS, and they were used in combination with two ITS internal primers (ITS-A-r [5′-AAAATAGC TTTTTGGTGGAG-3′]) and (ITS-B-f [5′-AAATTTGTATGGGCCTATAG-3′]), designed from $tDNA^{Ala}$ and $tDNA^{Ile}$, respectively (see Fig. 2A). The short spacer of *B. anthracis* strain Cepanzo was sequenced after being cloned in the pMOS kit by the same procedure that was used for the long ITS of strain 282.

Phylogenetic relationship in the *B. cereus* group based on sequences of ITS-containing tDNA. The sequences of the long ITS, determined for 21 strains, were used for neighbor-joining analysis to assess the phylogenetic relationship between the species of the *B. cereus* group. The sequences were aligned and the alignment was checked manually. Similarity values and a neighbor-joining tree were determined using Jalview software (http://circinus.ebi.ac.uk:6543/jalview).

RESULTS AND DISCUSSION

ITS length variation in the *B. cereus* group. During the PCR for amplification of the ITS of strains of the *B. cereus* group, heteroduplex cross-hybridization products may be formed between the different ITS (14). The heteroduplex products are visualized in electrophoretic gels as discrete bands and may cause overestimation of the ITS size types in a given strain. To avoid this artifact and to obtain a realistic description of the ITS in a strain, we treated the PCR products with mung bean nuclease, which cleaves the unpaired DNA strands of the heteroduplex products, releasing the original homoduplex fragments and the digestion products. This resulted in two major ITS size classes: one of 220 to 250 bp (short ITS; size measurement including the 16S and 23S rDNA stretches obtained with amplification) and the other of 450 to 550 bp (long ITS). In Fig. 1, the ITS homoduplex-heteroduplex polymorphism profiles after mung bean nuclease treatment are illustrated for some strains of the *B. cereus* group. On comparing the profiles in Fig. 1, it can be seen that *B. anthracis* Davis TE702 and *B. mycoides* G2, besides the short and the long ITS, show additional fragments of 350 and 320 bp (intermediate ITS), respectively.

ITS size classes and organization in the *B. cereus* group. To compare the structure between the different types of ITS in the *B. cereus* group, differently sized ITS, i.e., short, intermediate, and long ITS, were studied. The sequences of the short ITS of *B. anthracis* Cepanzo, intermediate ITS of *B. anthracis* Davis TE702, intermediate ITS of *B. mycoides* G2, and long ITS of *B. anthracis* Cepanzo, determined in this study, were compared.

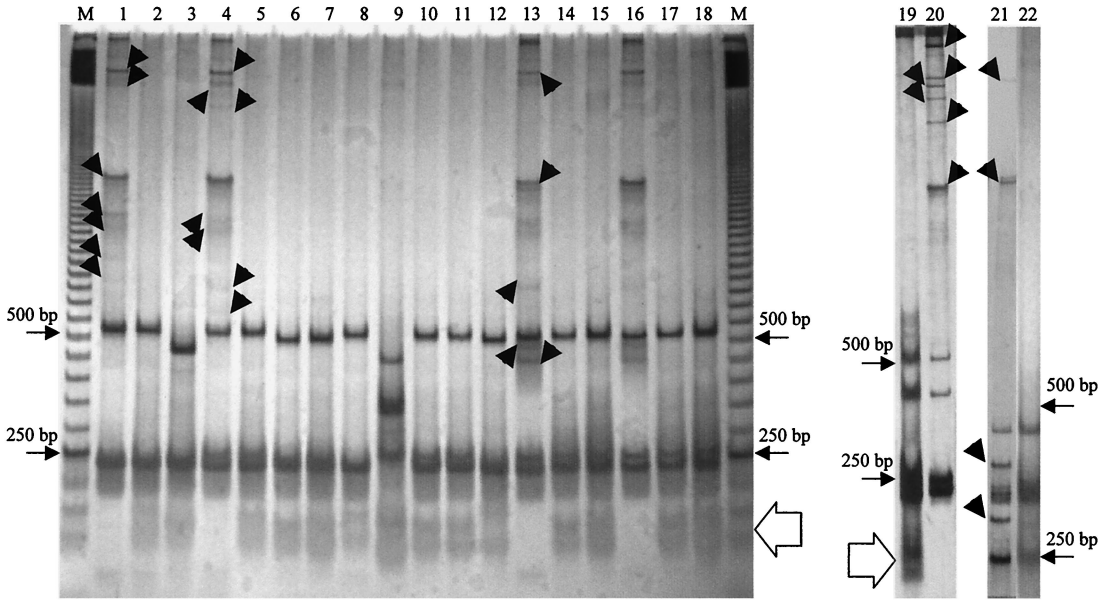

FIG. 1. Identification of the homoduplex bands in the ITS homoduplex-heteroduplex polymorphism patterns separated in a MDE gel after mung bean nuclease digestion of the PCR products. Lanes M contain a 50-bp ladder. Normal ITS homoduplex-heteroduplex polymorphism profiles: lanes 1, 4, 13, 16, 20, and 21, *B. weihenstephanensis* 10204^T, *B. thuringiensis* Ht39, *B. anthracis* 376, *B. anthracis* Cepanzo, *B. anthracis* Davis TE 702, and *B. mycoides* G2. ITS homoduplex-heteroduplex polymorphism profiles after treatment with mung bean nuclease: lanes 2 and 3, *B. weihenstephanensis* 10204^T and *B. pseudomycoides* 617^T; lanes 5 to 12, *B. thuringiensis* Ht39, *B. thuringiensis* 2046^T, *B. cereus* V65SP, *B. mycoides* 2048^T, *B. mycoides* G2, *B. anthracis* 256, *B. anthracis* 282, and *B. cereus* 31^T; lanes 14 and 15, *B. anthracis* 376 and *B. anthracis* 779; lanes 17 and 18, *B. anthracis* Cepanzo and *B. anthracis* 663; lanes 19 and 22, *B. anthracis* Davis TE 702 and *B. mycoides* G2. The 250- and 500-bp bands of the ladder are indicated. Arrowheads indicate the heteroduplex products removed by the mung bean nuclease. The open arrows indicate degradation products of the heteroduplex bands.

Figure 2B depicts the schematic organization of the four ITS types found in the *B. cereus* group. The first type was a classical 144-bp ITS without tDNA genes, already reported for almost all the species of the *B. cereus* group (7, 21, 30). The second main ITS type was 359 to 371 bp long and contained two tRNA genes, tRNA^{Ile} and tRNA^{Ala}, as in *B. subtilis* (29). The third ITS type, typical of *B. anthracis* strain Davis TE702, was 286 bp long and contained single putative tRNA^{Ala} gene. The 5′-end stretch (19 bp) of the tRNA^{Ala} gene could be identified with the corresponding stretch of the tRNA^{Ile} gene (Fig. 2B). This hybrid sequence suggests that a recombination event, rather than a deletion, of the tRNA^{Ile} gene generated the single tDNA-containing ITS.

A fourth type of ITS, 242 bp long, was found in *B. mycoides* strain G2. In this ITS, no tRNA genes were found.

The nucleotide sequence of the end regions flanking both 16S and 23S rDNA was mostly identical in all of the spacers (Fig. 2C). The conserved regions at the 5′ and 3′ ends of the ITS were 67 to 68 and 51 to 56 bp long, respectively. Several other conserved DNA sequence regions were found in the noncoding regions flanked by tRNA genes (regions i, ii, and iii). Regions i (81 bp) and ii (24 bp) were identical in the different tDNA-containing ITS. Region iii (13 bp) was identical in the short ITS without tRNA genes and the intermediate ITS of strain G2.

The 5′-end (67-bp) and 3′-end (51-bp) regions of the intermediate ITS of strain G2 were homologous to the corresponding regions of the short and long ITS in the *B. cereus* group. The remaining sequence of 127 bp did not show significant homology to any sequence in the databases (data not shown).

Signature nucleotides for the *B. cereus* group species in ITS. To evaluate the presence of species-specific signature nucleotides, the sequences of long ITS containing tRNA genes were determined for another 20 strains belonging to the six species of the *B. cereus* group. Seven polymorphic regions were found. Figure 3 shows the sequence alignment of the polymorphic regions of the long ITS of all the strains analyzed. Based on these polymorphic regions, Table 1 shows the signature nucleotides for the different species of the *B. cereus* group as deduced from Fig. 3. By referring to the sequence coordinates of long ITS of *B. anthracis* 7700 (Fig. 3), *B. anthracis* can be discriminated from the other species at positions 75 to 80 (AAAAAG), 121 (A), and 213 to 214 (AT). In particular, the AT positions appear useful discriminants, since they are located in the intergenic region between the two tRNA genes (Fig. 2); this region is absent in the short ITS.

B. cereus and *B. thuringiensis* could not be discriminated from each other, but together they could be discriminated from all the other species at positions 75 to 76 (TC), 117 to 121 (CCTGC), and 213 to 220 (TTTTGG).

B. mycoides and *B. weihenstephanensis* could not be discriminated, confirming the close genotypic and phenotypic relationship already described for these two species (14, 30, 40, 50). *B. mycoides* and *B. weihenstephanensis* could be discriminated from all the other species at positions 117 to 121 (TCCGC) and 213 to 218 (TTTTAC) (Table 1). The only exception was strain G2, which showed nucleotide variations in these sites.

B. pseudomycoides could be discriminated from all the other species at positions 75 to 81 (AAATAT), 117 to 121 (TCTGC), and 213 to 214 (AC) (Table 1).

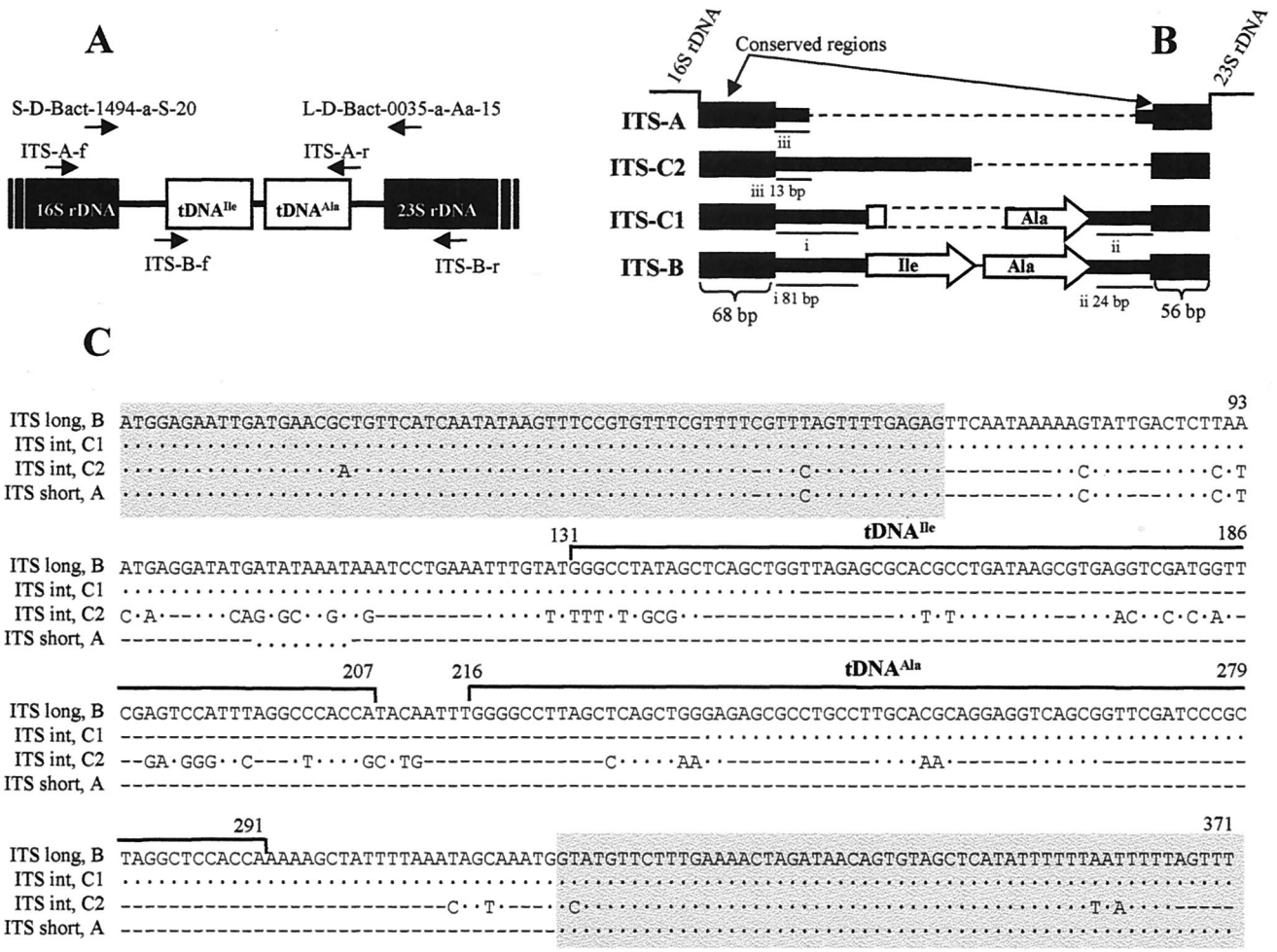

FIG. 2. (A) Position of the tDNA-targeting primers designed for sequencing the long 16S–23S rDNA ITS of *B. cereus* group. S-D-Bact-1494-a-S-20 plus L-D-Bact-0035-a-A-15 (13) is the universal primer set initially used for ITS homoduplex-heteroduplex polymorphism analysis. ITS-A-f plus ITS-A-r and ITS-B-f plus ITS-B-r are primer sets used to amplify and sequence the 5′- and 3′-end regions, respectively, of the long ITS containing tRNA genes. (B) Schematic structure of the 16S–23S rDNA ITS types found in the *B. cereus* group. The genes encoding tRNA are indicated by open arrows. Conserved regions flanking 16S or 23S rDNA are indicated by bold bars. The other conserved regions are underlined and labeled i, ii, and iii. The broken lines are gaps for the alignment. (C) Sequence alignment of different types of 16S–23S rDNA ITS found in the *B. cereus* group. ITS B, long ITS of *B. anthracis* 7700; ITS C1, intermediate ITS of *B. anthracis* Davis TE702; ITS C2, intermediate ITS of *B. mycoides* G2; ITS A, short ITS of *B. anthracis* Cepanzo. Dots indicate nucleotides identical to those of ITS-B. Sequence gaps for the alignment are shown by hyphens. The genes encoding tRNA are indicated by a continuous black line. Conserved regions flanking 16S and 23S rDNA are shaded in grey.

The signatures in Table 1 appear useful for designing species-specific probes or primers for the rapid identification of *B. cereus* group isolates (12).

Phylogenetic relationship in the *B. cereus* group based on sequences of ITS containing tDNA. To evaluate the phylogenetic relationship between the species of the *B. cereus* group on the basis of the long ITS containing tDNA, a phylogenetic tree including all the sequences determined in this study was established by using Jalview software (Fig. 4). The phylogenetic relationship based on the long ITS resulted in the identification of four groups of strains, the first containing *B. cereus* and *B. thuringiensis*, the second containing *B. anthracis*, the third containing *B. mycoides* and *B. weihenstephanensis*, and the fourth containing *B. pseudomycoides.*

B. anthracis was grouped in a branch separated from *B. cereus* and *B. thuringiensis*, indicating that *B. anthracis*, even though related, represents an independent phylogenetic lineage that diverges from *B. cereus* and *B. thuringiensis.* It is probable that the particular niche occupied by *B. anthracis* led to a divergence that also began to show differences at the intraspecies level. These differences can be observed in the ribosomal operon organization (35, 36, 39) or at the level of the ITS, as in strain Davis TE702 (14, 24, 35) or the strains isolated from the Pyrenees and Alps (37) during the 1994 and 1997 outbreaks (14). The few variations observed in *B. anthracis* by ITS homoduplex-heteroduplex polymorphism analysis (14) and sequencing of ITS containing tDNA mirror the whole-genome polymorphisms identified by amplified fragment length polymorphism (27, 28), long-range repetitive elements PCR (8), and, recently, whole-genome sequencing (42).

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            12          27      72          84      90          101
BA 7700     atgaacgctgttcatc     aataaaaagtatt     ttaaatgaggat
BA 663      ................     ............     ............
BA 282      ................     ............     ............
BA TE702    ................     ............     ............
BC 31T      ..a.g.....c.t...     ...tc.--.....     ............
BC 345      ..a.g.....c.t...     ...tc.--..r..     ............
BC 626      ..a.g.....c.t...     ...tc.--.....     ............
BC V65SP    ......c.........     ...tc..--c...     ............
BT 2046T    ................     ...tc..--c...     ............
BT HD1      ..a.g.....c.t...     ...tc.--..g..     ............
BT Bt14     ..a.g.....c.t...     ...tc.--a....     ............
BT BMG1.7   ..a.g.....c.t...     ...tc.--..g..     ............
BT Ht39     ..a.g.....c.t...     ...tc.--a....     ............
BW 10204T   ......a......g..     ....--.-.....     ......ag.a..
BW 10202    ........a.......     ....t..-.....     ......ag.a..
BW 10208    ......a......g..     ....--.-.....     ......ag.a..
BM 2048T    ........a.......     ....t..-.....     ......ag.a..
BM 299      ........a.......     ....t..-.....     ......ag.a..
BM 309      ......a.........     ....t..-.....     ......ag.a..
BM G2       ....g.....c.t...     ..---........     .....a...a..
BP BD10     ....g.....c.t...     ....-..ta.g..     ..t..aa..a..
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            110         125     211    220     301  308     358 364
BA 7700     aataaatcctgaaatt     caattt--gg     tttaaata     ttaattt
BA 663      ................     ......--..     ........     .......
BA 282      ................     ......--..     ........     .......
BA TE702    ................     ......--..     ........     .......
BC 31T      ............c....     ..t...--..     ........     .......
BC 345      ............c....     ..t...--..     ........     .......
BC 626      ............c....     ..t...--..     ........     .......
BC V65SP    ............c....     ..t...--..     ........     .......
BT 2046T    ..c.........c....     ..t...--..     ........     .......
BT HD1      ............c....     ..t...--..     ........     .......
BT Bt14     ............c....     ..t...--..     ........     .......
BT BMG1.7   ............c....     ..t...--..     ........     .......
BT Ht39     ............c....     ..t...--..     ........     .......
BW 10204T   .......t.c.c....     ..t...ac..     ....tg..     ..-.a..
BW 10202    .......t.c.c....     ..t...ac..     ....t...     ..-.a..
BW 10208    .......t.c.c....     ..t...ac..     ....t...     ..t.a..
BM 2048T    .......t.c.c....     ..t...ac..     ....t...     ..-.a..
BM 299      .......t.c.c....     ..t...ac..     ....t...     ..-.a..
BM 309      .......t.c.c....     ..t...ac..     ....t...     ..t.a..
BM G2       ...t.t..t..tgt..     ...a..ac..     ..a.tg..     ..t.a..
BP BD10     ..at...t...c....     ...c..--..     ....t...     ....-..
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FIG. 3. Alignment of DNA traits showing sequence variability in the long 16S–23S rDNA ITS containing tRNA genes of 21 strains of the *B. cereus* group. BA, *B. anthracis*; BC, *B. cereus*; BM, *B. mycoides*; BP, *B. pseudomycoides*; BT, *B. thuringiensis*; BW, *B. weihenstephanensis.*

These data on ITS sequences confirm the observation that *B. cereus* and *B. thuringiensis* cannot be distinguished easily, apart from the entomocidal genotype and phenotype. The sequence similarity of ITS containing tDNA supports the proposal that *B. cereus* and *B. thuringiensis* are monophyletic and represent a single clade (14, 24, 34, 47). From the practical point of view, species separation can be useful in distinguishing *B. thuringiensis* from *B. cereus*, with the former generally being regarded as safe and being used throughout the world for the biological control of insect pests, while the latter is pathogenic. Although genes and proteins toxic to mammals have been found in *B. thuringiensis* (reference 20 and references therein), *B. thuringiensis* strains have been associated with human disease in only a very few cases (reference 20 and references therein; 25). It has been suggested that besides mammalian and insecticidal toxins, other genetic determinants, such as PlcR, a pleiotropic regulator of extracellular virulence, drive the pathogenic activity in *B. cereus* or activate entomocidal patterns in *B. thuringiensis* (1). These determinants could permit a genetic and functional distinction between these two clades in the *B. cereus* group (1).

The close relation of *B. mycoides* to *B. weihenstephanensis* was confirmed by the sequence of the tDNA-containing ITS. It has been proposed that the only characteristic that can distinguish these two species is colony morphology, which shows typical rhizoid growth in *B. mycoides* (30). This characteristic is ambiguous since *B. mycoides* strains lacking the rhizoid phenotype have been isolated from soils (52). On the basis of all the characteristics and genetic markers analyzed until now, the relatedness of these species appears very high, but DNA mark-

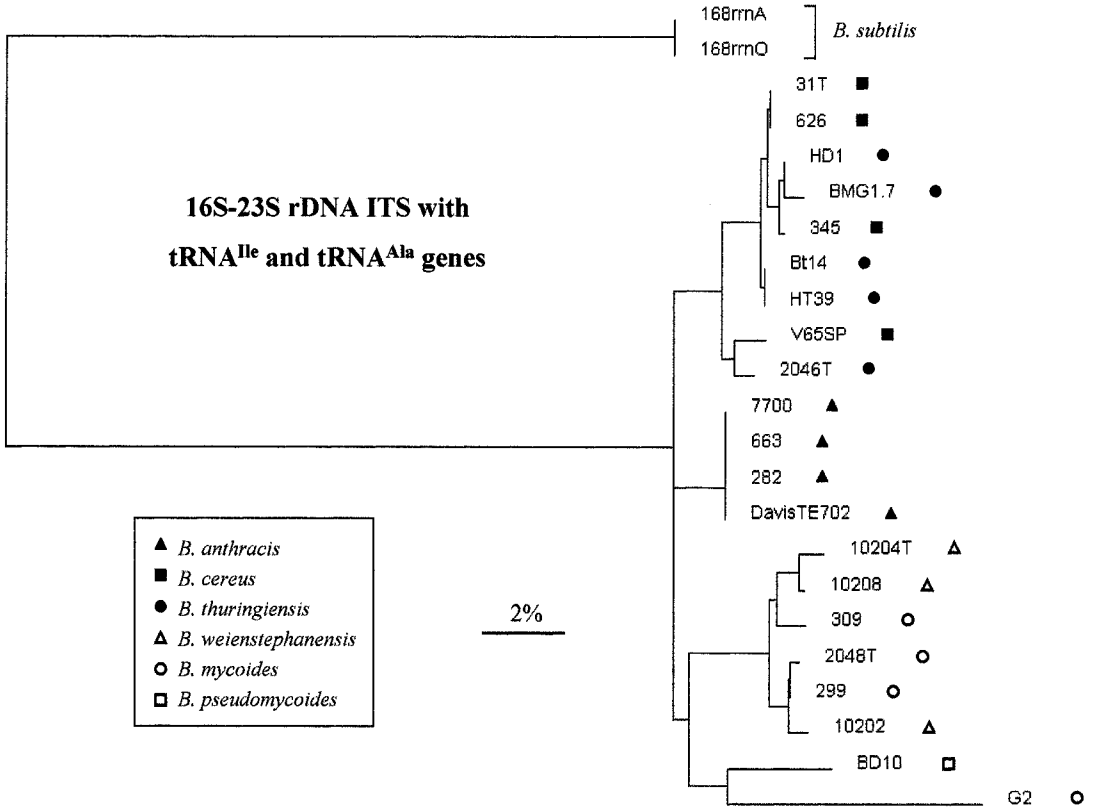

FIG. 4. Phylogenetic relationship between strains of the species of the *B. cereus* group determined by neighbor-joining analysis of the sequences of the long 16S–23S rDNA ITS containing tRNA genes, as determined in this study. Symbols indicate the different species of the *B. cereus* group. Sequences without symbols are from *B. subtilis* strain 168. Bar, 2% of the phylogenetic distance.

ers that clearly discriminate the two species still remain to be identified. (21, 30, 40, 50). Strain G2 harbors a relatively low sequence homology of 91.3% to *B. pseudomycoides*, the closest species. This confirms that wide genetic variability exists in the *B. cereus* group (14).

From the sequences of the ITS containing tDNA, it was confirmed that *B. pseudomycoides* is a clearly separate species from *B. mycoides.* Further studies need to be performed on *B. pseudomycoides* to evaluate its degree of activity in the soil and rhizosphere environment.

Summary. This study reports, for the first time, sequences of ITS containing tDNA from strains of the six species of the *B. cereus* group. It is shown that these sequences are more informative for species discrimination that are those of short ITS, which were shown by Bourque et al. (7) to be highly conserved among the different species of the *B. cereus* group. The use of the long ITS containing tDNA allowed us to discriminate *B. anthracis* from the related clades *B. cereus* and *B. thuringiensis.* From these data, it is proposed that when inferring the genetic relationship between closely related clades on the basis of the sequences of the ITS, the use of ITS containing tDNA is more informative and appropriate than ITS without tDNA for the discrimination of clades of different ecotype.

ITS nucleotide sequence accession numbers. The nucleotide sequences of the short and the tDNA-containing intermediate and long ITS analyzed in this study have been deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the following accession numbers (the corresponding strains are given in parentheses): *B. anthracis* long ITS: AJ420048 (7700), AJ420049 (663), AJ420050 (282) and AJ420051 (Davis TE702); *B. anthracis* intermediate ITS: AJ420069 (Davis TE702); *B. anthracis* short ITS: AJ420071 (Cepanzo); *B. cereus* long ITS: AJ420052 (31^T), AJ420053 (345), AJ420054 (626), and AJ420055 (V65SP); *B. thuringiensis* long ITS: AJ420056 (2046^T), AJ420057 (HD1), AJ420058 (Bt14), AJ420059 (BMG1.7), and AJ420060 (Ht39); *B. weihenstephanensis* long ITS: AJ420061 (10204^T), AJ420062 (10202), and AJ420063 (10208); *B. mycoides* long ITS: AJ420064 (2048^T), AJ420065 (299), AJ420066 (309), and AJ420067 (G2); *B. mycoides* intermediate ITS: AJ420070 (G2); *B. pseudomycoides* long ITS: AJ420068 (BD10).

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