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Use of 16S rRNA, 23S rRNA, and *gyrB* Gene Sequence Analysis To Determine Phylogenetic Relationships of *Bacillus cereus* Group Microorganisms

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In order to determine if variations in rRNA sequence could be used for discrimination of the members of the *Bacillus cereus* group, we analyzed 183 16S rRNA and 74 23S rRNA sequences for all species in the *B. cereus* group. We also analyzed 30 *gyrB* sequences for *B. cereus* group strains with published 16S rRNA sequences. Our findings indicated that the three most common species of the *B. cereus* group, *B. cereus, Bacillus thuringiensis*, and *Bacillus mycoides*, were each heterogeneous in all three gene sequences, while all analyzed strains of *Bacillus anthracis* were found to be homogeneous. Based on analysis of 16S and 23S rRNA sequence variations, the microorganisms within the *B. cereus* group were divided into seven subgroups, Anthracis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B, and these seven subgroups were further organized into two distinct clusters. This classification of the *B. cereus* group conflicts with current taxonomic groupings, which are based on phenotypic traits. The presence of *B. cereus* strains in six of the seven subgroups and the presence of *B. thuringiensis* strains in three of the subgroups do not support the proposed unification of *B. cereus* and *B. thuringiensis* into one species. Analysis of the available phenotypic data for the strains included in this study revealed phenotypic traits that may be characteristic of several of the subgroups. Finally, our results demonstrated that rRNA and *gyrB* sequences may be used for discriminating *B. anthracis* from other microorganisms in the *B. cereus* group.

Analysis of 16S rRNA sequences is a simple, commonly used method for the identification of microorganisms (1, 47, 53). However, early studies performed with a limited number of isolates from the *Bacillus cereus* group revealed that the 16S rRNA sequences of species in this group had as high as a 99 to 100% similarity and, thus, suggested that rRNA sequences might not be useful for discrimination of members of that group (6). However, that study examined only five isolates from the *B. cereus* group. The *B. cereus* group contains seven closely related species: *Bacillus anthracis, B. cereus, Bacillus thuringiensis, Bacillus mycoides* (16, 45, 51), *Bacillus pseudomycoides* (38), *Bacillus weihenstephanensis* (33), and *Bacillus medusa* (15). To date, identification and discrimination of these species has been based on analysis of morphological, biochemical, and immunological characteristics.

Although conserved in sequence overall, the 16S rRNAs actually exhibit great variation in some regions. These differences in 16S rRNA sequence provide the basis for the design of nucleic acid probes of various specificities, ranging from probes targeting all living organisms to group-specific and species-specific probes. Another advantage of using the rRNAs as a target is the fact that these molecules are naturally amplified within the cell. In general, rRNA represents about 80% of total nucleic acids in microbial cells and, thus, is present in many hundreds of thousands of copies per cell. This natural amplification allows for direct detection of rRNA sequences without the need for intermediate amplification via PCR (1).

16S and 23S rRNA are currently considered the most useful molecules for the determination of prokaryotic phylogeny. Analysis of these rRNA sequences has resulted in a tremendous expansion in our knowledge of prokaryotic diversity and has demonstrated the limitations of the existing prokaryotic taxonomy, which is based primarily on the analysis of phenotypic traits (35). Attempts have been made recently to address conflicts between molecular and phenotypic data, such as the work on the phylogenetically heterogeneous genus *Pseudomonas* (29). Here we have conducted a similar analysis for the *B. cereus* group. We have investigated the molecular phylogeny of the *B. cereus* group by extensively analyzing a set of *B. cereus* group sequences in order to determine if the rRNA sequences contained enough variation to discriminate *B. anthracis* from other members of the *B. cereus* group.

Previous work has demonstrated that *gyrB* gene sequences may also be useful for discrimination of *B. cereus* group organisms (57). Therefore, in addition to the rRNA analyses, we have also analyzed *gyrB* sequences for members of the *B. cereus* group and compared rRNA-based and *gyrB*-based phylogenies.

MATERIALS AND METHODS

Bacterial strains. Twelve strains belonging to the *B. cereus* group were used for sequencing: *B. anthracis* strain Ames ANR, *B. anthracis* strain Delta Ames-1, *B. anthracis* strain Sterne, *B. anthracis* strain 1, *B. anthracis* strain 2, *B. thurin-*

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TABLE 1. Primers used for PCR and for sequencing of 16S and 23S rRNA genes of *B. cereus* group bacteria[a]

Name	Sequence	Location
P1	5′-GTT TGA TCC TGG CTC AG	11–27 (16S rRNA)
P10	5′-CCA GTC TTA TGG GCA GGT TAC	136–116 (16S rRNA)
P11	5′-TCC ATA AGT GAC AGC CGA AGC	226–206 (16S rRNA)
P5	5′-CTA CGG GAG GCA GCA GTG GG	340–360 (16S rRNA)
P3	5′-GWA TTA CCG CGG CKG CTG	535–517 (16S rRNA)
P2	5′-GGA TTA GAT ACC CTG GTA GT	784–803 (16S rRNA)
P6	5′-CCG TCA ATT CCT TTR AGT TT	926–907 (16S rRNA)
P8	5′-TTC GGG AGC AGA GTG ACA GGT	1029–1049 (16S rRNA)
P9	5′-TAC ACA CCG CCC GTC ACA CCA	1392–1412 (16S rRNA)
P4	5′-RGT GAG CTR TTA CGC	1513–1492 (16S rRNA)
Pr1	5′-CCG AAT GGG GVA ACC C	114–129 (23S rRNA)
Pr13	5′-CCG TTT CGC TCG CCG CTA CTC	262–242 (23S rRNA)
PB1	5′-TAG TGA TCG ATA GTG AAC CAG	485–505 (23S rRNA)
Pr2	5′-CAT TMT ACA AAA GGY ACG C	621–603 (23S rRNA)
Pr3	5′-GCG TRC CTT TTG TAK AAT G	603–621 (23S rRNA)
PB2	5′-TAG TGA TCG ATA GTG AAC CAG	755–736 (23S rRNA)
PB3	5′-TAG TGA TCG ATA GTG AAC CAG	969–990 (23S rRNA)
Pr4	5′-RGT GAG CTR TTA CGC	1151–1137 (23S rRNA)
Pr5	5′-WGC GTA AYA GCT CAC	1136–1150 (23S rRNA)
PB4	5′-CAT ACC GGC ATT CTC ACT TC	1308–1289 (23S rRNA)
PB5	5′-ACA GGC GTA GGC GAT GGA C	1408–1426 (23S rRNA)
PB8	5′-AAC CTT TGG GCG CCT CC	1679–1661 (23S rRNA)
Pr6	5′-CYA CCT GTG WCG GTT T	1673–1659 (23S rRNA)
Pr7	5′-AAA CCG WCA CAG GTR G	1659–1673 (23S rRNA)
Pr8	5′-CAY GGG GTC TTT RCG TC	2092–2076 (23S rRNA)
Pr9	5′-GAC GYA AAG ACC CCR TG	2076–2092 (23S rRNA)
Pr10	5′-GAG YCG ACA TCG AGG	2535–2521 (23S rRNA)
Pr11	5′-CCT CGA TGT CGR CTC	2521–2535 (23S rRNA)
Pr12	5′-GYT TAG ATG CYT TC	2783–2770 (23S rRNA)
R1	5′-GGC GGC GTC CTA CTC TCA C	112–95 (5S rRNA)

[a] Primers P1 to P4, Pr1 to Pr7, and R1 were used for DNA amplification. All other primers were utilized for sequencing. Primers P8, P9, P10, and P11 were selected de novo; other primers were described earlier (for details, see reference 32).

giensis strain B8, *B. cereus* strain NCTC 9620, *B. cereus* strain T, *B. thuringiensis* strain 4Q281, *B. medusa* strain ATCC 25621, *B. mycoides* strain ATCC 6462m, and *B. mycoides* strain ATCC 10206 (obtained as a generous gift from John Ezzell, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.) (see Table 2). Two of the *B. mycoides* strains were isolated as an occasional admixture from a culture previously identified as *B. mycoides* strain ATCC 6462. These strains revealed different colony morphologies and were assigned different strain numbers: *B. mycoides* ATCC 6462m and *B. mycoides* ATCC 10206. *B. thuringiensis* strains 4R1, 4D1, 4F1, 4T1, 4W1, 4J4, 4A1, 4A7, 4Q1, 4Q2, and 4M1 were received as a generous gift from Dan Zeigler of the Bacillus Genetic Stock Center. *B. cereus* HER 1414 was acquired from the National Collection of Type Cultures (NCTC).

Sequencing of 16S and 23S rRNA genes. Total DNA was isolated from frozen cell pellets using the guanidine extraction method as described previously (11). 16S rDNA was amplified from total genomic DNA for 12 strains. The 23S rDNA was amplified for 10 of the 12 strains (*B. anthracis* strains 1 and 2 were excluded). For each amplification reaction mixture, 0.1 μg of bacterial DNA was subjected to PCR in a total volume of 100 μl, with 2.5 U of *Taq* polymerase (Perkin-Elmer, Boston, Mass.), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, a 200 μM concentration of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), and a 6 μM concentration of each of two primers. The primers used for 16S rDNA and 23S rDNA amplification are listed in Table 1. The thermal profile included denaturation at 94°C for 2 min, primer annealing at 45°C for 2 min, extension at 72°C for 2 min, and then 35 cycles of denaturation at 94°C for 15 s, primer annealing at 45°C for 15 s, and extension at 72°C for 4 min. DNA was purified using a QIAquick PCR purification kit (QIAGEN Inc., Valencia, Calif.), and purified PCR products were directly sequenced by the cycle sequencing method using Ampli*Taq* DNA polymerase FS (Perkin-Elmer), fluorescently labeled dye terminators, and 373A fluorescent sequencer (ABI, Perkin-Elmer). Sequencing primers are shown in Table 1. Both strands of studied DNA fragments were sequenced twice for each strand.

Development of expanded sequence databases. We retrieved 16S rRNA, 23S rRNA, and *gyrB* sequences for members of the *B. cereus* group from GenBank (www.ncbi.nlm.nih.gov). Twelve 16S rRNA and 10 23SrRNA sequences were determined in this study, including one *B. anthracis* strain (Sterne) resequenced by our laboratory (Table 1). In this article, numbers are shown in all sequences in accordance with numbering in the *B. anthracis* Ames genome.

Creation of phylogenetic tree. The 16S, 23S rRNA, and *gyrB* sequence databases were used to create phylogenetic trees. Sequences of isolates with different names but representing the same strain, incomplete sequences, sequences containing a large number of mistakes, strain-specific variations, or undetermined nucleotides (particularly at the sites of subgroup-specific signatures) were excluded from consideration. For an accurate determination of species similarities, all 5′ and 3′ ends were cut to identical positions along the 16S rRNA, 23S rRNA, and *gyrB* genes at *B. anthracis* Ames bp 49 to 1462, 24 to 2779, and 381 to 1499, respectively. The sequences were aligned using the multiprocessor (version 1.81) of CLUSTAL W (http:www.cmbi.kun.nl/bioinf/tools/clustalw.shtml). Aligned sequences were analyzed using the Molecular Evolutionary Genetics Analysis package version 2.1 (31; http://www.megasoftware.net). Rooted and unrooted phylogenetic trees were built with minimum evolution, neighbor-joining, unweighted pair group method with averages (UPGMA), and maximum parsimony methods. During analysis of alignments with the minimum evolution method, gaps were considered missing data points, genetic distances were estimated using nucleotide/Jukes-Cantor (for rRNA) or nucleotide/p-distance (for *gyrB*) models, where all substitutions were included in pairwise distance calculations. A close-neighbor-interchange search was performed to examine the neighborhood of the neighbor-joining tree to find a potential minimum evolution tree. Bootstrap confidence values were generated using 1,000 permutations of the data set for 16S rRNA and *gyrB* and 100 permutations for 23S rRNA to derive the nucleotide sequence similarities. In the maximum parsimony method for 16S rRNA and *gyrB*, gaps in the analysis were treated as missing data points; for 23S rRNA, where insertion G(1218-1219) was considered as a subgroup-specific signature,

all sites were included for consideration. We utilized the close-neighbor-interchange algorithm to find the maximum parsimony tree. The random addition option with 10 replicates was used to produce the initial trees. In calculations of tree length, relative weights for different types of changes were specified uniformly using the standard parsimony method. In the both the neighbor-joining and UPGMA methods for *gyrB*, gaps were treated with the "complete deletion" option, genetic distances were estimated using the nucleotide/p-distance method in neighbor-joining analysis and nucleotide/Jukes-Cantor method for UPGMA, and bootstrap confidence values were generated using 1,000 permutations for both the neighbor-joining and UPGMA methods.

RESULTS

Sequencing of 16S and 23S rRNA genes of *B. cereus* group microorganisms. In this study we sequenced 12 16S rRNA and 10 23S rRNA genes (Tables 2 and 3). There are published sequences available (6, 7) for two of the microorganisms that we sequenced, *B. medusa* NCIMB10437 (ATCC 25621) and *B. anthracis* Sterne. We found a number of discrepancies between our sequences and these previously published sequences. The published 16S rRNA sequences of *B. anthracis* Sterne (6; GenBank accession no. X55059) and *B. medusa* NCIMB10437 (7; GenBank accession no. X60628) have a deletion of a C (position 942) in comparison with other strains of *B. anthracis* and *B. medusa* that were later sequenced (Table 2). We did not find this deletion in our resequencing of the 16S rRNA genes of *B. anthracis* Sterne (GenBank accession no. AF176321) and *B. medusa* ATCC 25621 (GenBank accession no. AF155958), or in the recent resequencing of this gene in *B. anthracis* Sterne by Ticknor et al. (50; GenBank accession no. AF290552). We also did not find this deletion in ours or The Institute for Genomic Research's sequencing of *B. anthracis* Ames (GenBank accession numbers AF267734, AE017024 to AE017026, and AE017039). We believe that the reported deletion was a compression artifact of sequencing this GC-rich region (56), i.e., GGGGCCG instead of GGGGCCCG. We suggest that the same compression artifact at the same site may also have compromised the 16S rRNA sequences of *B. cereus* NCDO1771, *B. cereus* NCTC 11143, *B. mycoides* DSM2048T, and *B. thuringiensis* NCIMB9134 (6, 7; GenBank accession numbers X55060 to X55063). For this reason, these deletions were not included in Table 2.

In addition, our resequencing of the 16S rRNA gene for *B. medusa* ATCC 25621 did not reveal the C-to-T transition at position 192 (presence of T instead of C found in *B. anthracis*) or the A-to-G transversion at position 1383 previously reported for *B. medusa* NCIMB10437 16S rRNA (Table 2).

We did not include in Table 2 the 108 inconsistencies found in the six 16S rRNA sequences mentioned above, which had been deposited in GenBank by Ash et al. (6, 7). These were not supported by the resequencing of *B. anthracis* Sterne and *B. medusa* or in any other 16S rRNA sequences (Table 2) and probably represented systematic errors in sequencing which occurred when the base following T was incorrectly read as A, generally, instead of T or G.

We also found differences between the previously published 23S rRNA sequence of *B. anthracis* Sterne (5; GenBank accession no. S43426) and our resequencing of this isolate (GenBank accession no. AT267877). The differences that we found were the following: T instead of C at position 491, deletion of CG (1413, 1414), and T instead of C at position 2651. We also did not find these changes in any other 23S rRNA sequence in the *B. cereus* group, including *B. anthracis* Ames and *B. anthracis* Delta Ames (Table 3). Therefore, we suggest that these differences in *B. anthracis* Sterne and also the same differences in *B. cereus* NCTC 11143 (GenBank accession no. X64646) are due to errors in the previously reported sequences (5).

We did not include in Table 3 the substitutions of A for G, T, and C reported earlier for the 23S rRNA sequences of *B. anthracis* Sterne and *B. cereus* NCTC 11143 (5) that were not confirmed by our resequencing of the same gene in *B. anthracis* Sterne. These inconsistencies with our results, C/A(1037), T/A(1127), G/A(1411), G/A(1827), G/A(1834), C/A(2079), G/A(2182), G/A(2278), and C/A(2391), previously reported in combination with several Ns were not observed in any other isolates (Table 3).

Comparison of 16S and 23S rRNA sequences in the *B. cereus* group. Our analyses indicated that in terms of known 16S and 23S rRNA sequences, *B. anthracis* was the most homogeneous species within the *B. cereus* group (Tables 2 and 3). This finding confirms PCR fingerprinting studies that demonstrated almost complete homogeneity of bulk DNA recovered from different strains of *B. anthracis* (2, 9, 14, 20, 21, 24–27, 30, 34, 39, 40, 43, 44, 48, 54). Because of this homogeneity, we have used the *B. anthracis* 16S and 23S rRNA sequences as a reference for reporting differences among closely related bacteria within the *B. cereus* group (Fig. 1 and 2; Tables 2 and 3).

Analysis of our 16S rRNA sequences and sequences found in GenBank for the other *B. cereus* group organisms identified six characteristic regions which contained the majority of the positional sequence differences: position(s) 77 to 92, 133, 182 to 208, 286, 1015 to 1045, and 1462 (Fig. 1 and Table 2). Because sequence variation in these regions can be used to divide the *B. cereus* group organisms into several large subgroups, we have termed the differences located within these regions subgroup-specific differences. The most common were C/A (1015) and C/T (192). A set of subgroup-specific differences that characterized a subgroup were called subgroup-specific signatures (Table 2). In addition, a number of other differences were observed, which we have termed strain-specific differences (Tables 2 and 3). Most of the strain-specific differences were unique to each strain and were located randomly along the 16S rRNA molecule, i.e., they did not occur within the same sites as the subgroup-specific differences. Based on our resequencing experience, it is possible that some of the strain-specific variants represented mistakes in sequencing.

It is necessary to stress that all of the different subgroup-specific differences were not equally important for subgroup identification. For example, 16S rRNA subgroup-specific differences C/T (182) and T/A (1462) were found in all microorganisms from the Cereus A subgroup. However, both of these alterations, as well as C/Y (192), sometimes appeared in Thuringiensis B isolates (*B. thuringiensis* 82347, *Bacillus* sp. strain AH540, *Bacillus* sp. strain AH533, *Bacillus* sp. strain Termite isolate bac, bromate-reducing bacterium B6, glacial ice bacterium SB100-8-1, unidentified bacterium V, and *B. cereus* AH527) (Table 2). Alterations C/T (182) and C/Y (182) also were found in some Thuringiensis A microorganisms (*Bacillus* sp. strain KPU-0013, *B. cereus* ATCC 43881) (Table 2). These findings suggested close relationships for the subgroups Thuringiensis A, Thuringiensis B, and Mycoides A.

TABLE 2. Classification of bacteria in the *B. cereus* group according to 16S rRNA sequences

Subgroup name	Subgroup-specific signature(s) (position)[a]	Start and end of sequence	Organism[r]	GenBank accession no.	Position(s) of strain-specific variations
Anthracis	Consensus[a]	11–1554	*B. anthracis* Sterne[b]	AF176321	—[s]
		1–1451	*B. anthracis* Sterne[c]	X55059	—
		18–1499	*B. anthracis* Sterne	AF290552	A/W(1147)
		11–1554	*B. anthracis* Ames ANR[b]	AF155950	—
		1–1554	*B. anthracis* Ames (TIGR)[d]	AE017024-26, AE017039	A/W(1146)
		11–1554	*B. anthracis* Delta Ames-1[b]	AF155951	—
		33–1517	*B. anthracis* (wastewater isolate)[e]	AY043083	A/T(1146)
		18–1499	*B. anthracis* Vollum	AF290553	A/W(1147)
		12–1424	*B. anthracis* W21	AF390088	C/A(19), T/A(967), A/T(969), TT/GA(1095,1096), T/G(1100), C/A(1113), ins A(1123-1124), A/T(1146), C/T(1200), A/T(1207), C/A(1209), T/C(1220)
		61–528, 815–1501	*B. anthracis* 1[b]	J. Jackman[f]	—
		61–528, 815–1501	*B. anthracis* 2[b]	J. Jackman[f]	—
Cereus A	Consensus[a]	1–1451	*B. cereus* NCTC 11143[c]	X55063	—
		49–1522	*B. cereus* WSBC 10037[g]	Z84576	A/G(178), G/T(1518)
		49–1522	*B. cereus* WSBC 10030[g]	Z84575	C/T(353), T/C(600), T/C(864), A/C(1146), G/T(1518)
		49–1489	*B. cereus* S-5[g]	AF390086	C/T(353), A/C(1146)
		28–1517	*B. cereus* AL1[g]	AY129651	A/T(1146), del(1459)
		28–1513	*B. cereus* (bovine serum isolate)[g]	AF206326	A/G(181), C/T(285), C/T(467), C/T(480), G/A(482), T/C(600), C/T(995), A/C(1146), T/C(1244), T/C(1345), delT(1459)
		105–1277	*B. cereus* ATCC 10702[g]	AF363440	—
		105–1277	*B. cereus* DL5[g]	AF363441	C/T(906), C/T(1035), G/A(1046), T/G(1141), T/A(1256), A/G(1263)
		105–1277	*B. cereus* DL115[g]	AF363442	A/C(462), ins A(471-472), A/C(623), T/C(746), C/A(771), T/C(872), C/T(906), C/T(1035), T/A(1256)
		1–1554	*B. cereus* ATCC 10987[d]	AE017264-66 AE017280	—
			B. cereus HFR1414[h]		?
		31–1462	*Bacillus* sp. strain JJ-1[g]	Y15466	—
		28–1534	*Bacillus* sp. strain BSID723[g]	AF027659	C/A(1232)
		18–1499	*Bacillus* sp. strain AH526[g]	AF290562	—
		52–1517	*Bacillus* sp. strain YSS/2001-3[g]	AF417847	A/T(1146), A/T(1155), T/G(1402), G/C(1426), ins G(1454-1455)
		228–1437	*Bacillus* sp. strain FO-011[g]	AF234842	ins T (276-277), ins C (280-281), C/T(293), C/A(303), A/T(323), C/T(350), G/T(416), C/T(498), C/A(520), A/T(568), G/A(1190), A/T(1205), ins T(1219-1220)
		11–1554	*B. thuringiensis* B8[b]	AF155955	—
Cereus B	**C/A(1015)**[q]	11–1554	*B. cereus* NCTC 9620[b]	AF155952	—
		11–1554	*B. cereus* T[b]	AF176322	—
		28–1513	*B. cereus* IAM 12605[i]	D16266	—
		1–1451	*B. cereus* NCDO 1771[c,i]	X55060	—
		17–1499	*B. cereus* ATCC 1778[i]	AF290546	—
		17–1499	*B. cereus* ATCC 4579[i]	AF290547	—
		49–1522	*B. cereus* ATCC 27877	Z84581	A/T(828), G/T(1518)
		28–1183	*B. cereus* (ocular isolate)	AF076031	C/T(498), del C(520), del (523), T/A(829), C/G(1167)
		17–1499	*B. cereus* ATCG 31293	AF290548	—
		1–1554	*B. cereus* BGSC 6A5[d]	AY224379-88	—
		8–1523	*B. cereus* SH 01[e]	AF522353	A/T(171)
		11–1249	*B. cereus* Tim-r01	AB050630	C/T(182)
		105–1277	*B. cereus* DL137	AF363444	A/T(170), G/C(407), A/C(623)
		105–1277	*B. cereus* DL122	AF363443	A/T(170), G/C(345), A/T(496), A/C(623)
		20–1534	*Bacillus* sp. strain P16	AY048782	G/A(28), T/C(30)

Continued on following page

TABLE 2—*Continued*

Subgroup name	Subgroup-specific signature(s) (position)[a]	Start and end of sequence	Organism[r]	GenBank accession no.	Position(s) of strain-specific variations
		11–1515	*Bacillus* sp. strain 82344	AF227848	—
		45–1513	*Bacillus* sp. strain F26	AF385082	T/C(49), G/C(530)
		30–1484	Glacial ice bacterium SB-12K-9-4	AF479367	—
		30–1481	Glacial ice bacterium G500K-2	AF479333	—
		30–1475	Glacial ice bacterium G50-TS3	AF479356	—
		47–1470	*Bacillaceae* bacterium PH27B	AF513473	ins GT(1379-1380)
		49–1517	Unident. HTA484 (Mariana Trench isol.)	AB002640	G/C(72), GC/CG(297,298), del (946), ins C(1111-1112), del (1279)
		49–1522	*B. thuringiensis* WS2614	Z84584	A/C(128), G/T(1518)
		49–1522	*B. thuringiensis* WS2617	Z84585	G/A(1153), G/T(1518)
		49–1522	*B. thuringiensis* WS2618	Z84586	A/G(725), G/T(1518)
		49–1522	*B. thuringiensis* WS2626	Z84588	G/T(1518)
Thuringiensis A	**C/A(1015)**, **C/T(192)**	49–1522	*B. thuringiensis* WS2623	Y18473	G/T(109), A/G(679), T/C(1228), A/G(1503)
		49–1522	*B. thuringiensis* WS2625	Z84587	C/T(565), G/T(1183), G/T(1518)
		18–1479	*B. thuringiensis* ATCC 33679	AF290549	C/Y(192)
			B. thuringiensis 4R1,[j] 4D1,[j] 4F1,[j] 4S2,[j] 4T1, 4W1,[j] and 4J4[j]		?
		22–1502	*Bacillus* sp. strain FPI/2002	AY124766	—
		8–1514	*Bacillus* sp. strain KPU-0013	AB067810	C/G(131), C/T(182), G/A(202), del (298), C/T(444), T/G(781), A/C(796), G/A(952), TC/CT(1036,1037), G/T(1313)
		55–1510	Unident. sp6 (bovine rumen isolate)	AB003391	C/T(63), del(108), C/T(182), C/G(227), GC/CG(297,298), del(565), del(1038)
		20–1530	*Bacillus* sp. strain CMB03	AF406633	G/A(28), T/C(30), ins C(730-731), A/T(1146), G/A(1254), T/A(1256), A/G(1263), G/A(1266), A/G(1269), G/A(1271), GG/TA(1282,1283), T/C(1287), T/C(1292), A/T(1299), C/T(1301), T/C(1317), T/C(1319), G/T(1322), C/A(1333), A/G(1336), A/G(1338), G/A(1451), T/A(1463), T/G(1477)
		18–1499	*B. cereus* ATCC 43881	AF290550	C/Y (182)
Thuringiensis B	**C/A(1015)**, **C/T(192)**, A/G(77), T/C(90), T/A(92)				
		11–1554	*B. thuringiensis* 4Q281[b]	AF155954	—
		28–1513	*B. thuringiensis* IAM 12077[k]	D16281	—
		1–1451	*B. thuringiensis* NCIMB 9134[c,k]	X55062	—
		18–1499	*B. thuringiensis* ATCC 10792[k]	AF290545	—
		37–1491	*B. thuringiensis* 82347	AF157112	C/Y(182), del C(303), del C(347), ins T(395-396), G/A(1029), C/T(1111), del G(1148), del G(1240), del C(1246), del G(1262), GG/AS(1321,1322), del G(1492)
		49–1522	*B. thuringiensis* (Pieris brassicae isolate)	AF160221	A/C(161), A/T(183), no C/T(192), G/T(1518)
		50–1513	*B. thuringiensis* Bactisubtil	AF172711	G/A(733), G/A(778), C/A(857)
		11–1052	*B. thuringiensis* HMB12389	AF501348	G/C(39)
			B. thuringiensis 9308,[l] 20,[l] Lb5,[l] 1230,[l] and L3[l]		?
			B. thuringiensis 4A1,[j] 4A7,[j] 4Q1,[j] 4Q2,[j] and 4M1[j]		?
			B. cereus Nagoya 126[m] and 127[m]		?

Continued on following page

TABLE 2—*Continued*

Subgroup name	Subgroup-specific signature(s) (position)[a]	Start and end of sequence	Organism[r]	GenBank accession no.	Position(s) of strain-specific variations
		11–1554	*B. medusa* ATCC 25621[b,n]	AF155958	No C/T (192)
		7–1440	*B. medusa* NCIMB10437[c,n]	X60628	del C(1038), A/G (1383)
		18–1499	*Bacillus* sp. strain AH540	AF290557	A/R(77), T/Y(90), T/W(92), T/A(1462)
		8–1497	*Bacillus* sp. strain Fa7	AY131217	C/A(19), del (28), no C/T(192)
		20–1499	*Bacillus* sp. strain SVM	AF503203	C/Y(192)
		8–1554	*Bacillus* sp. strain Kaza-37	AF441732	del (25), G/C(634), A/G(828), G/C(949), ins (955-956), del (963), del (982), G/A(983), ins T(990-991), ins T(1000-1001), ins AG(1048-1049), G/A(1049), G/A(1089)
		8–1554	*Bacillus* sp. strain Kaza-31	AF441728	no C/T(192), GGG/CCC(309-311), ins C(496-497), ins T(516-517), ins G(523-524), G/A(526), G/C(712), G/C(949), ins A(973-974), ins C(979-980), ins C(988-989), ins T(1009-1010), del G(1029), ins A(1055-1056), G/A(1098), delA(1269)
		28–1403	*Bacillus* sp. strain A23	AF397398	No T/A (92)
		26–1401	*Bacillus* sp. strain A24	AF397399	—
		18–1499	*Bacillus* sp. strain AH533	AF290556	C/Y(182)
		7–1551	*Bacillus* sp. strain Termite isolate bac	X81132	C/T(182), TT/GG(186,187), G/C(769), TA/CT(822,823), del A(875), G/C(897), TGG/CTA(1281–1283), del C(1301), ins GG(1428-1429), T/A(1461), del T(1463), CG/GT(1473,1474), del CT(1475,1476)
		28–1513	Bromate-reducing bacterium B6[e]	AF442522	G/K(61), G/S(66), G/R(93), G/S(100), G/R(141), C/T(182), G/C(255), T/K(264), G/S(297), G/A(307), G/R(334), C/A(1130), T/A(1462)
		30–1489	Glacial ice bacterium SB100-8-1	AF479369	C/T(182), T/A(1462)
		33–1485	Unident. bacterium V	AB004761	No T/A(92), G/C(667), T/A(1462)
		18–1499	B. cereus ATCC 53522	AF290551	CG/AM(1423,1424)
		18–1499	*B. cereus* AH527	AF290555	C/Y(182)
		29–1532	*B. cereus* ATCC 14893	AJ310098	CG/AY(43,44)
		28–1530	*B. cereus biovar toyoi* CNCM I-1012/NCIB 40112	AJ310100	—
		62–1511	*B. cereus* Biosubtil-Dalat	AJ277907	del T(76), G/A(388), G/A(402), del G(407), del G(425), G/T(436), G/A(769), G/C(772), G/A(787), ins C(788-789), C/T(1073)
		62–1511	*B. cereus* Bactisubtil	AJ277908	del A(70), del T(76), G/A(424), G/C(431), G/T(436), del G(680), G/A(769), G/C(772), C/T(1037), T/C(1039), C/T(1041), del AGCA(1045-1048), del A(1048), T/A(1052), AC/GA(1054,1055), del T(1072), C/T(1073), ins A(1103-1104), C/G(1113), ins A(1121-1122)
			B. thuringiensis BT3,[k,o] BT13,[o] BT15,[o] BT16,[o] BTT6,[o] and BTT8[o]		?
Mycoides A	**C/A(1015)**, **C/T(192)**, G/A(133), C/T(182), G/A(197), A/G(286), C/T(1029), G/A(1030), T/A(1464)	1–1451	*B. mycoides* DSM2048T[p]	X55061	—
		49–1551	*B. mycoides* MWS5303-1-4	Z84591	T/C(1454), G/T(1518)
		49–1523	*B. mycoides* DRC1	AF144645	C/G(63), G/A(1279), TAG/GTA(1319-1321), C/G(1398), A/C(1437), G/T(1441), G/T(1471), T/A(1477), G/A(1492)
		49–1522	*B. mycoides* MWS5303-2-51	Z84583	A/G(180), G/T(1518)

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TABLE 2—*Continued*

Subgroup name	Subgroup-specific signature(s)[a]	Start and end of sequence	Organism[r]	GenBank accession no.	Position(s) of strain-specific variations
		32–1544	*B. mycoides* ATCC 6462[p]	AB021192	—
		228–1415	*B. mycoides* FO-080	AF234860	G/T(289), G/C(297), del G(311), C/G(387), G/T(392), C/T(520), ins A(532-533), ins G(827-828), A/T(1112), C/T(1117), G/A(1134), G/C(1148), del G(1162), del G(1202)
		32–574	*B. mycoides* B10	BMY491827	ins G(111)
		16–340	*B. mycoides* SFLB6	BMY344516	A/T(50), A/G(69)
		14–1544	*B. weihenstephanensis* DSM11821	AB021199	—
		49–1522	*B. cereus* WSBC10201	Z84577	A/G(203), no A/G(286), A/G(1513), G/T(1518)
		49–1522	*B. cereus* WSBC10204	Z84578	A/G(128), G/T(1518)
		49–1554	*B. cereus* WSBC10206	Z84579	G/C(225), AG/GT(1517,1518)
		49–1522	*B. cereus* WSBC10210	Z84580	A/G(60), T/C(375), A/G(1298), G/T(1518)
		18–1499	*B. cereus* AH521	AF290554	—
		18–1499	*Bacillus* sp. strain AH628	AF290558	—
		18–1499	*Bacillus* sp. strain AH648	AF290559	—
		18–1499	*Bacillus* sp. strain AH665	AF290560	—
		18–1499	*Bacillus* sp. strain AH678	AF290561	—
		28–1517	*Bacillus* sp. strain Fa25	AY131220	—
Mycoides B	A/C(189), T/G(200), G/C(208), T/C(1036), A/G(1045)	11–1554	*B. mycoides* ATCC 6462m[b]	AF155956	—
		11–1554	*B. mycoides* ATCC 10206[b]	AF155957	—
		117–423	*B. mycoides* jshs5	AY039819	T/G(132), T/A(185), ins C(185-186), ins G(202-203), T/G(206), C/G(411)
		7–1520	*B. pseudomycoides* sp. nov.	AF013121	A/T(55), C/A(341), T/C(495), C/T(516), G/C(566), A/T(929), C/A(958), A/C(1017), T/C(1034), T/G(1040), G/C(1104), C/A(1110), A/C(1121), A/T(1128), C/G(1138), C/A(1232), C/A(1276), T/A(1281), T/A(1390), G/A(1441), G/A(1485), T/A(1508)
		34–1373	*B. cereus* Ki21	AJ288157	A/T(95), no T/G(200), del G(202), A/G(329), T/G(752), G/C(778), A/G(793), no T/C(1036), T/A(1350), T/A(1357)

[a] For more details, see Fig. 1.
[b] Sequenced in this work.
[c] Sequences need to be reexamined; see also Results.
[d] From sequences of whole genome; data represent average sequence of all available 16S rRNA genes.
[e] Sequence submitted in 3′ to 5′ form.
[f] J. Jackman, unpublished.
[g] Final discrimination from Anthracis subgroup will be done after sequencing of 23S rRNA gene (see Results).
[h] Not sequenced; identified through hybridization analysis (see Results).
[i] These four strains are identical and correspond to *B. cereus* DSM 31; see 23S rRNA sequence of this strain in Table 3.
[j] Not sequenced; assignment to subgroups Thuringiensis A or B was based on hybridization with subgroup-specific probes (S. Bavykin and J. Jackman, unpublished).
[k] These four strains are identical and correspond to *B. thuringiensis* DSM2046; see the 23S rRNA sequence of this strain in Table 3.
[l] Not sequenced; subgroup-specific signatures A/G(77), T/C(90), and T/A(92) were identified by hybridization with subgroup-specific probes (18).
[m] Not sequenced; subgroup-specific signatures A/G(77), T/C(90), and T/A(92) were identified by hybridization with subgroup-specific probes (55).
[n] According to *Bergey's Manual* (45), these two strains of *B. medusa* should be identical.
[o] Not sequenced; subgroup-specific signatures A/G(77), T/C(90), and T/A(92) were identified by hybridization with subgroup-specific probes (13).
[p] According to *Bergey's Manual* (45), these two strains of *B. mycoides* should be identical.
[q] Subgroup-specific mutations, which are highlighted in bold, were identical for two or more subgroups and are shown on separate lines to demonstrate connections between different subgroups.
[r] Selected abbreviations of collections and institutions appearing in titles of listed strains: ATCC, American Type Culture Collection, Rockville, Md; NCTC, National Collection of Type Cultures and Pathogenic Fungi, London, United Kingdom; DSM or DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; HER, Center of Reference bacteria and viruses, Laval University, Department of Microbiology, Quebec, Canada; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; NSDO or NSFB, National Collection of Food Bacteria, c/o NCIMB Ltd., Aberdeen, Scotland, United Kingdom; NCIMB or NCIB, National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland, United Kingdom; CNCM, National Collection of Microorganisms and Cell Cultures, Pasteur Institute, Paris, France; TIGR, The Institute for Genomic Research, Rockville, Md. W = A or T; Y = C or T; S = C or G; M = A or C. Del, deletion; ins, insertion.
[s] —, no strain-specific variations in this strain.

TABLE 3. Classification of bacteria in the *B. cereus* group according to 23S rRNA sequences

Subgroup name	Subgroup-specific signature(s) (position)[a]	Start and end of sequence	Organism	GenBank accession no.	Position(s) of strain-specific variation(s)
Anthracis	Consensus[a]	1–2922	*B. anthracis* Sterne[b]	AF267877	—[k]
		1–2922	*B. anthracis* Delta Ames-1[b]	AF267876	—
		1–2922	*B. anthracis* Ames ANR[b]	AF267734	—
		1–2922	*B. anthracis* Ames (TIGR)[c]	AE017024 to -26, AE017039	—
		15–2943	*B. anthracis* Sterne[d]	S43426	T/C(491), del CG(1413, 1414), T/C(2651)
Cereus A	**Y/C(594)**[j]	1–2923	*B. cereus* NCTC11143[d]	S43429	T/C(2651)
	G/A(1559)	1–2923	*B. cereus* ATCC 10987[c]	AE017264 to -66 AE017280	—
	Insertion G(1218-1219)	1–527	*B. cereus* WSBC10030[e]	Z84589	—
			B. cereus HER1414[f]		?
		1–2923	*B. thuringiensis* B8[b]	AF267880	—
Cereus B	**Y/C(594)**	1–2922	*B. cereus* NCTC9620[b]	AF267878	—
	G/A(1559)	1–2922	*B. cereus* T[b]	AF267879	G/R(1559)
	T/A(2153)	1–2787	*B. cereus* DSM31[g]	X94448	T/C(1275)
		24–2789	*B. cereus* LMG6923[g]	AJ310096	—
		1–2922	*B. cereus* ATCC 14579[c,g]	AF016998 to AF017000, AF017013	—
		1–2922	*B. cereus* BGSC 6A5[c]	AY224379 to AY224388	—
		18–2897	*B. cereus* Tim-r01	AB050631	ins G(1218–1219), G/T(1268), G/A(1557), ins T(1781–1782), T/A(1938)
		1–527	*B. thuringiensis* WS2617[e]	Z84594	—
		1–527	*B. thuringiensis* WS2614[e]	Z84593	—
Thuringiensis A	**T/A(2153)**		*B. thuringiensis* strs. 4R1,[h] 4D1,[h] 4F1,[h] 4T1,[h] 4W1,[h] 4S2,[h] and 4J4[h]		?
	Insertion G(1218–1219)				
Thuringiensis B	**Y/T(594)**	1–2922	*B. thuringiensis* 4Q281[b]	AF267881	G/R(1559)
	T/C(157)	24–2789	*B. thuringiensis* LMG7138[i]	AJ310738	G/R(546)
	G/A(921), A/G(1020), C/T(1037), G/A(1209), A/G(1251), T/C(1283) C/T(132), A/T(174), G/T(1250) T/A(2153)	1–2784	*B. thuringiensis* DSM2046[i]	X89895	C/T(57), T/G(413), ins AATA(479–480), del GG(541–542), G/A(646), C/G(670), G/A(1953), G/A(2055), ins AGT(2556–2557), del G(2573)
			B. thuringiensis 4A1,[h] 4A7,[h] 4Q1,[h] 4Q2,[h] and 4M1[h]		?
		1–2922	*B. medusa* ATCC 25621[b]	AF267885	—
		20–2790	*B. cereus* ATCC 14893	AJ310099	CA/TC(265,266), T/C(358), G/A(646), C/T(655), G/A(663), C/G(1816), G/C(1849)
		24–2799	*B. cereus* biovar toyoi CNCM 1-1012/NCIB 40112	AJ310101	CA/TC(265,266), G/A(646), C/T(655), G/A(663), C/G(1816), G/C(1849)
Mycoides A	**Y/T(594)**	1–527	*B. mycoides* DSM2048T	Z84592	—
	T/C(157)	1–527	*B. mycoides* MWS5303-1-4	Z84591	—
	G/A(921), A/G(1020), C/T(1037), G/A(1209), A/G(1251), T/C(1283)	23–2789	*B. mycoides* DSM2048	AJ310097	CA/AY(375, 376), T/C(1112), T/C(2651)
	CA/TC(265,266), GT/AC(364,365), C/G(1816), G/C(1849) C/T(132), A/T(174), G/T(1250) T/A(2153) C/T(375)	1–527	*B. cereus* WSBC10206	Z84590	—
Mycoides B	**Y/T(594)**	1–2922	*B. mycoides* ATCC 6462m[b]	AF267884	—
	T/C(157)	1–2922	*B. mycoides* ATCC 10206[b]	AF267883	—
	G/A(921), A/G(1020), C/T(1037), G/A(1209), A/G(1251), T/C(1283) CA/TC(265,266), GT/AC(364,365), C/G(1816), G/C(1849) GA/AG(346,347), TC/CT(358,359), C/A(482), C/T(672), A/T(1219), G/T(1268), A/G(2159)				

[a] For more details, see Fig. 2.
[b] 23S rDNA sequenced in this work.
[c] From sequences of whole genome; data represent average sequence of all available 23S rRNA genes.
[d] Need to be reexamined (see Results).
[e] Assigned to this subgroup in accordance with the 16S rRNA sequence (see Table 2).
[f] Not sequenced; identified through hybridization analysis (see Results).
[g] These three strains are identical and correspond to *B. cereus* IAM12605, *B. cereus* NCDO1771, *B. cereus* ATCC 11778, and *B. cereus* ATCC 14579; see 16S rRNA sequences of these strains in Table 2.
[h] Not sequenced; subgroup-specific signatures A/G(77), T/C(90), and T/A(92) were identified by hybridization with subgroup-specific probes (S. Bavykin and J. Jackman, unpublished).
[i] These two strains are identical and correspond to *B. thuringiensis* IAM12077, *B. thuringiensis* NCIMB9134, *B. thuringiensis* ATCC 10792, and *B. thuringiensis* BT3; see 16S rRNA sequences of these strains in Table 2. Y = C or T; R = G or A.
[j] See footnote *q* of Table 2.
[k] See footnote *s* of Table 2.

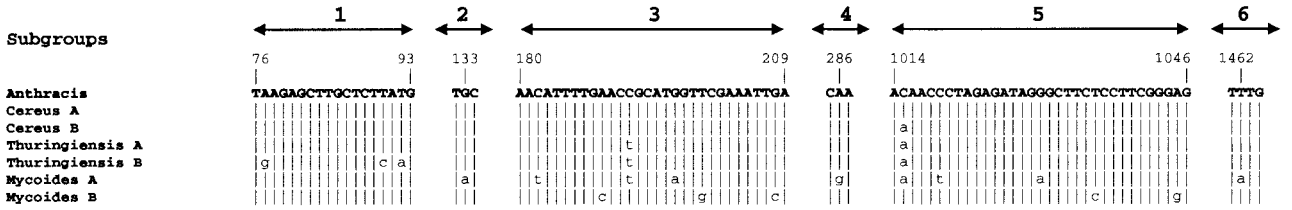


FIG. 1. Positions of subgroup-specific sequence differences in the 16S rRNA of *B. cereus* subgroups. The sequence of *B. anthracis* Sterne (GenBank accession no. AF 176321) has been used as the consensus sequence. Vertical lines indicate nucleotides identical to the consensus sequence. Arrows indicate regions containing subgroup-specific differences. For more details, see Table 2.

Analysis of the 23S rRNA sequences for the *B. cereus* group organisms revealed 12 regions within which the majority of the sequence variation occurred (Fig. 2 and Table 3). The differences within these regions were analogous to the subgroup-specific variants found in the 16S rRNA.

However, due to the limited number of 23S rRNA sequences in the database, it may be that not all of these differences are subgroup specific. Some of the regions which appear to contain subgroup-specific differences may actually contain strain-specific variations. For example, the Mycoides B subgroup signature contained five subgroup-specific differences in the 16S rRNA and nine subgroup-specific differences in 23S rRNA sequences that were not found in other subgroups (Fig. 2). However, available rRNA sequences for the Mycoides B subgroup currently include only five strains for which 16S rRNA sequences have been determined and two strains for 23S rRNA sequences (Tables 2 and 3). Among them, *B. mycoides* ATCC 6462m and *B. mycoides* ATCC 10206 had identical 16S and 23S rRNA sequences (Fig. 2; Tables 2 and 3) as well as 16S-23S rRNA spacers (GenBank accession numbers AF267905 and AF267906). However, they differed in colony morphology (see Materials and Methods). If additional members of the Mycoides B subgroup will be sequenced, we may find that some of the subgroup-specific differences are actually strain specific. Further work is needed to address this issue.

The most common subgroup-specific differences in 23S rRNA sequence occurred at positions 157 and 594 (Table 3; Fig. 2). The presence of these common variants among the subgroups supports a phylogenetic relationship among them.

Grouping of microorganisms in the *B. cereus* group according to 16S rRNA sequences. The *B. cereus* group can be divided into seven subgroups based on 16S rRNA sequence differences (Table 2). We have labeled each of these subgroups according to the name of the most common member of the subgroup: Anthracis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B.

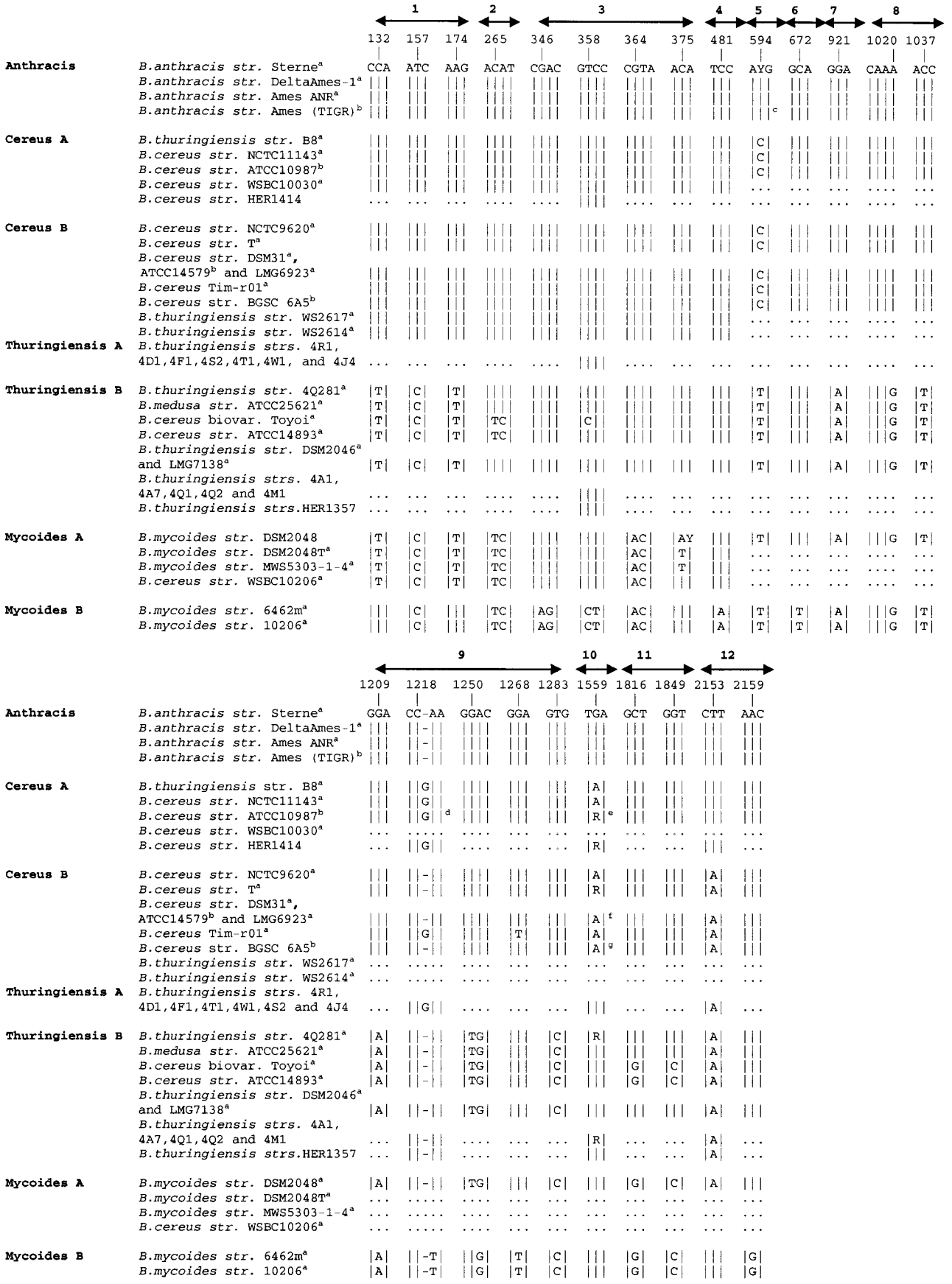
The following subgroups reflect the 16S rRNA sequence relationships (Table 2). Subgroup Anthracis includes eight strains of *B. anthracis*. Most of the published 16S rRNA sequences in this subgroup contain a polymorphic site at position 1146 or 1147.

Subgroup Cereus A includes 17 members which do not contain any subgroup-specific sequence differences from the *B. anthracis* consensus but which were not identified as *B. anthracis* by conventional taxonomic methods. Ten of these 17 isolates do contain strain-specific sequence differences. However, at least six of the other seven members, *B. cereus* NCTC 11143, *B. cereus* ATCC 10702, *B. thuringiensis* B8, *Bacillus* sp. strain JJ-1, and *Bacillus* sp. strain AH526, have sequences identical to subgroup Anthracis in the region of the 16S rRNA compared. Two isolates of the subgroup Cereus A, *B. cereus* WSBC10037 and *B. cereus* WSBC10030, have been previously characterized as mesophilic (33). *B. thuringiensis* B8 apparently represents a misclassification, because it does not contain any *cry* genes (J. Jackman, personal communication).

Subgroup Cereus B includes 23 strains of *B. cereus* and *B. thuringiensis* that differ from *B. anthracis* by a C-to-A change at position 1015. *B. cereus* NCTC 9620, *B. cereus* T, *B. cereus* IAM12605, *B. cereus* NCDO1771, *B. cereus* ATCC 11778, *B. cereus* ATCC 14579, *B. cereus* ATCC 31293, *B. cereus* BGSC 6A5, *Bacillus* sp. strain 82344, and glacial ice bacterium strains SB-12K-9-4, G500K-2, and G50-TS3 do not differ from one another in 16S rRNA sequence and, thus, they would be indistinguishable based on 16S rRNA hybridization. Strains *B. cereus* IAM12605, *B. cereus* NSDO1771, *B. cereus* ATCC 11778, and *B. cereus* ATCC 14579 represent the same strain and correspond to *B. cereus* DSM31 and *B. cereus* LMG6923, whose 23S rRNA sequences are considered below (Tables 2 and 3).

Subgroups Thuringiensis A and Thuringiensis B include, respectively, 15 and 42 strains of microorganisms which contain two and five subgroup-specific sequence differences, respectively, C/A (1015) and C/T (192) being shared among the two subgroups. These two subgroups include mainly *B. thuringiensis* strains. Six strains in the subgroup Thuringiensis B (*B. thuringiensis* 4Q281, *B. thuringiensis* IAM12077, *B. thuringiensis* NCIM9134, *B. thuringiensis* ATCC 10792, *Bacillus* sp. strain A24, and *B. cereus* biovar *toyoi* CNSMI-1012/NCIB40112) have identical 16S rRNA sequences. Strains *B. thuringiensis* IAM12077, *B. thuringiensis* NCIMB9134, *B. thuringiensis* ATCC 10792, and *B. thuringiensis* BT3 comprise the same strain and correspond to *B. thuringiensis* DSM2046, the 23S rRNA sequence of which is considered below (Tables 2 and 3). Two other members of this subgroup, *B. medusa* ATCC 25621 and *B. medusa* NCIMB10437, should be identical according to those in *Bergey's Manual* (45). However, according to our sequencing and hybridization studies (data not shown), strain *B. medusa* ATCC 25621 does not contain the subgroup-specific alteration C/T (192), whereas according to published sequences (7), *B. medusa* NCIMB10437 does contain this sequence variant.

In the last two subgroups, Mycoides A and Mycoides B, 18 *B. mycoides* strains group in subgroup Mycoides A and 5 fall under subgroup Mycoides B. Subgroups contain, respectively, nine and five subgroup-specific differences in their signatures. Strains *B. mycoides* DSM2048 and *B. mycoides* ATCC 6462


1 2 3 4 5 6 7 8
132 157 174 265 346 358 364 375 481 594 672 921 1020 1037
CCA ATC AAG ACAT CGAC GTCC CGTA ACA TCC AYG GCA GGA CAAA ACC
Anthracis
B.anthracis str. Sterne[a]
B.anthracis str. DeltaAmes-1[a]
B.anthracis str. Ames ANR[a]
B.anthracis str. Ames (TIGR)[b]
Cereus A
B.thuringiensis str. B8[a]
B.cereus str. NCTC11143[a]
B.cereus str. ATCC10987[b]
B.cereus str. WSBC10030[a]
B.cereus str. HER1414
Cereus B
B.cereus str. NCTC9620[a]
B.cereus str. T[a]
B.cereus str. DSM31[a], ATCC14579[b] and LMG6923[a]
B.cereus Tim-r01[a]
B.cereus str. BGSC 6A5[b]
B.thuringiensis str. WS2617[a]
B.thuringiensis str. WS2614[a]
Thuringiensis A
B.thuringiensis strs. 4R1, 4D1,4F1,4S2,4T1,4W1, and 4J4
Thuringiensis B
B.thuringiensis str. 4Q281[a]
B.medusa str. ATCC25621[a]
B.cereus biovar. Toyoi[a]
B.cereus str. ATCC14893[a]
B.thuringiensis str. DSM2046[a] and LMG7138[a]
B.thuringiensis strs. 4A1, 4A7,4Q1,4Q2 and 4M1
B.thuringiensis strs.HER1357
Mycoides A
B.mycoides str. DSM2048
B.mycoides str. DSM2048T[a]
B.mycoides str. MWS5303-1-4[a]
B.cereus str. WSBC10206[a]
Mycoides B
B.mycoides str. 6462m[a]
B.mycoides str. 10206[a]
9 10 11 12
1209 1218 1250 1268 1283 1559 1816 1849 2153 2159
GGA CC-AA GGAC GGA GTG TGA GCT GGT CTT AAC
Anthracis
B.anthracis str. Sterne[a]
B.anthracis str. DeltaAmes-1[a]
B.anthracis str. Ames ANR[a]
B.anthracis str. Ames (TIGR)[b]
Cereus A
B.thuringiensis str. B8[a]
B.cereus str. NCTC11143[a]
B.cereus str. ATCC10987[b]
B.cereus str. WSBC10030[a]
B.cereus str. HER1414
Cereus B
B.cereus str. NCTC9620[a]
B.cereus str. T[a]
B.cereus str. DSM31[a], ATCC14579[b] and LMG6923[a]
B.cereus Tim-r01[a]
B.cereus str. BGSC 6A5[b]
B.thuringiensis str. WS2617[a]
B.thuringiensis str. WS2614[a]
Thuringiensis A
B.thuringiensis strs. 4R1, 4D1,4F1,4T1,4W1,4S2 and 4J4
Thuringiensis B
B.thuringiensis str. 4Q281[a]
B.medusa str. ATCC25621[a]
B.cereus biovar. Toyoi[a]
B.cereus str. ATCC14893[a]
B.thuringiensis str. DSM2046[a] and LMG7138[a]
B.thuringiensis strs. 4A1, 4A7,4Q1,4Q2 and 4M1
B.thuringiensis strs.HER1357
Mycoides A
B.mycoides str. DSM2048[a]
B.mycoides str. DSM2048T[a]
B.mycoides str. MWS5303-1-4[a]
B.cereus str. WSBC10206[a]
Mycoides B
B.mycoides str. 6462m[a]
B.mycoides str. 10206[a]

according to *Bergey's Manual* (45) represent the same strain. Psychrotolerant isolates *B. weihenstephanensis* DSM11821 and *B. cereus* strains WSBC 10201, 10204, 10206, and 10210 were also included in subgroup Mycoides A. Nine isolates, *B. mycoides* strain DSM2048T, *B. mycoides* ATCC 6462, *B. weihenstephanensis* DSM11821, *B. cereus* AH521, and *Bacillus* sp. strains AH628, AH648, AH665, AH678, and Fa25 have identical 16S rRNA sequences. Subgroup Mycoides B contains *B. cereus* Ki21 and *B. pseudomycoides* sp. nov., which may have split off from the other three isolates in this subgroup rather early in their evolution, as they have a large number of strain-specific sequence differences (Table 2).

The same groups containing the same microorganisms were identified in a rooted phylogenetic tree that was generated with the minimum evolution method using 16S rRNA sequences of the *B. cereus* group with *Bacillus licheniformis* strain GA8 (GenBank accession no. AY162136), *Bacillus megaterium* strain C1 (GenBank accession no. AJ491841), and *B. megaterium* strain KL-181 (GenBank accession no. AY030336) 16S rRNA sequences as an outgroup. This grouping was also completely and independently confirmed by reconstructing an unrooted 16S rRNA tree with the maximum parsimony method (Fig. 3A).

Although the affiliations in the tree are consistent with those we defined earlier by signature analysis (Table 2 and Fig. 1), these groupings do not correspond exactly to the current taxonomy, which divides the *B. cereus* group into seven species: *B. anthracis, B. cereus, B. thuringiensis, B. medusa, B. mycoides, B. pseudomycoides* sp. nov., and *B. weihenstephanensis* (15, 16, 33, 38, 45, 51).

Grouping of microorganisms in the *B. cereus* group according to 23S rRNA sequences. For all microorganisms whose 23S rRNA genes were sequenced (Table 3), 16S rRNA sequences were also available. This finding gave us a unique opportunity to confirm the division of the *B. cereus* group into seven subgroups that was done according to 16S rRNA sequences (Fig. 1 and 3A; Table 2). Unfortunately, there were no 23S rRNA sequences available for any of the organisms from subgroup Thuringiensis A. Analyzing 23S rRNA signatures obtained for six subgroups did not reveal any serious contradictions with the groupings based on 16S rRNA sequence analysis (compare data in Table 2 and Fig. 3A with Fig. 2 and Table 3 data). However, in contrast to 16S rRNA sequences, 23S rRNA sequences of isolates in the Anthracis subgroup revealed a set of variations that provided the possibility for differentiation of the Anthracis subgroup from all the other six subgroups (Table 3; Fig. 2).

Subgroup Cereus A contains *B. cereus* ATCC 10987, *B. thuringiensis* B8, and *B. cereus* NCTC 11143 (Table 2). The 23S rRNA sequences of these two organisms include subgroup-specific alterations at positions 594 and 1559 and an insertion G(1218-1219) (Fig. 2; Table 3). The 23S rRNA sequence of *B. cereus* WSBC10030 was sequenced only partially (Fig. 2; Table 3). It does not contain any differences that are specific for any other subgroups, but also it does not cover subgroup-specific sites for the Cereus A subgroup.

Subgroup Cereus B contains seven strains. We observed that 23S rRNA sequences of this subgroup contain three subgroup-specific differences at positions 594, 1559, and 2153 (Fig. 2). Unfortunately, the 23S rRNA sequences available for *B. thuringiensis* WS2614 and *B. thuringiensis* WS2617 do not extend beyond position 527 from the 5′ end of the gene. Strains *B. cereus* DSM31, *B. cereus* ATCC 14579, and *B. cereus* LMG6923 represent the same strain that corresponds to *B. cereus* IAM12605, *B. cereus* NCDO1771, and *B. cereus* ATCC 11778 (Table 3), whose 16S rRNA sequences are also available (Table 2).

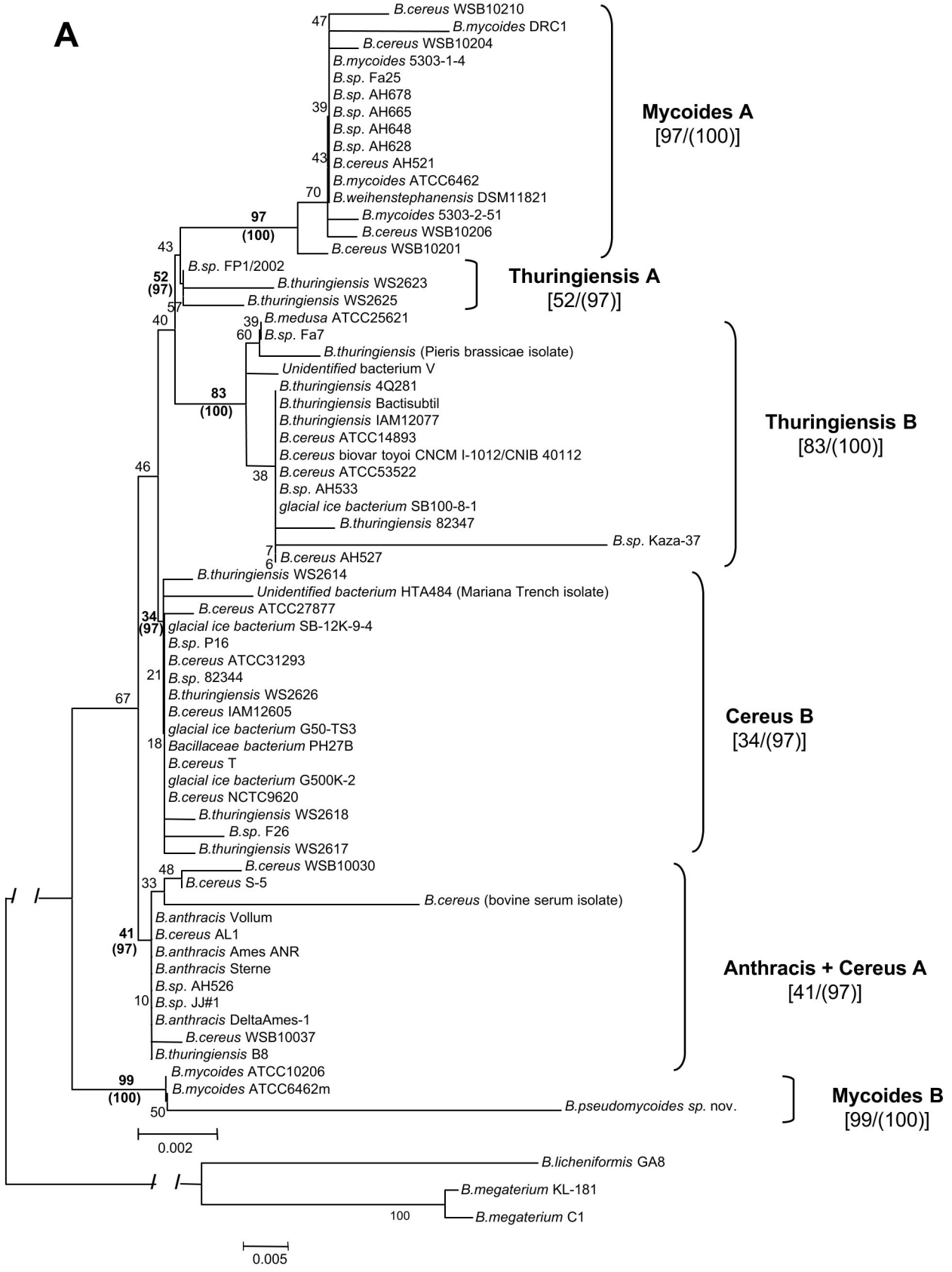
Subgroup Thuringiensis B consists of 10 microorganisms containing 12 subgroup-specific alterations. *B. thuringiensis* LMG7138 and *B. thuringiensis* DSM2046 (Table 3) represent one strain, whose synonyms are *B. thuringiensis* IAM 12077, *B. thuringiensis* NCIMB9134, *B. thuringiensis* ATCC 10792, and *B. thuringiensis* BT3, whose 16S rRNA sequences were considered above (Table 2).

B. mycoides DSM2048, *B. mycoides* DSM2048T, *B. mycoides* MWS5303-1-4, and *B. cereus* WSBC10206 form subgroup Mycoides A. Although only 527 nucleotides of sequence from the 5′ end of 23S rRNAs are available for the last three organisms, they revealed enough (8) variations to be discriminated from members of all other subgroups (Table 3; Fig. 2).

Subgroup Mycoides B includes only two microorganisms, *B. mycoides* ATCC 6462m and *B. mycoides* ATCC 10206. We found 23 specific differences in their 23S rRNA sequences (Table 3; Fig. 2).

The subgroups described above were consistent with the 23S rRNA phylogenetic trees obtained by two independent methods where the minimum evolution tree was rooted using *B. licheniformis* strain DSM 13 (GenBank accession no. X68433) and *Bacillus subtilis* strain W168 (GenBank accession no. K00637) as an outgroup (Fig. 3B). Phylogenetic analysis also revealed a division of *B. cereus* group bacteria into two clades which we called cluster I, which includes subgroups Anthracis, Cereus A, and Cereus B, and cluster II, which consists of subgroups Thuringiensis B, Mycoides A, and Mycoides B (Fig. 3B). Thuringiensis A could not be placed in a cluster due to the lack of 23S rRNA sequences for any microorganisms which were members of this subgroup (based on their 16S rRNA sequences). We also found that microorganisms *B. cereus* biovar *toyoi* and *B. cereus* 14893, which according to their 16S and 23S rRNA sequences unambiguously belonged to subgroup Thuringiensis B, also contained four alterations in their 23S rRNA (CA/TC [265, 266], C/G [1816], and G/C[1849]) that are

FIG. 2. Positions of subgroup-specific differences in the 23S rRNA sequences of *B. cereus* subgroups. The sequence of *B. anthracis* Sterne (GenBank accession no. AF176321) has been used as the consensus sequence. Arrows indicate regions containing subgroup-specific differences. Vertical lines indicate nucleotides identical to consensus sequences. Dots note nonsequenced regions. Superscript letters: a, 16S rRNA sequences are available (Table 2); b, whole-genome sequences are available (Table 3); c, 5 of 11 23S rRNA genes contain T(594); d, 4 of 12 23S RNA genes contain the insertion G(1218-129); e, 6 of 12 23S rRNA genes have A(1559); f, 7 of 11 23S rRNA genes in *B. cereus* ATCC 14579 reveal A(1559); g, 8 of 11 23S rRNA genes contain A(1559).


A
B.cereus WSB10210
B.mycoides DRC1
B.cereus WSB10204
B.mycoides 5303-1-4
B.sp. Fa25
B.sp. AH678
B.sp. AH665
B.sp. AH648
B.sp. AH628
B.cereus AH521
B.mycoides ATCC6462
B.weihenstephanensis DSM11821
B.mycoides 5303-2-51
B.cereus WSB10206
B.cereus WSB10201
Mycoides A
[97/(100)]
B.sp. FP1/2002
B.thuringiensis WS2623
B.thuringiensis WS2625
Thuringiensis A
[52/(97)]
B.medusa ATCC25621
B.sp. Fa7
B.thuringiensis (Pieris brassicae isolate)
Unidentified bacterium V
B.thuringiensis 4Q281
B.thuringiensis Bactisubtil
B.thuringiensis IAM12077
B.cereus ATCC14893
B.cereus biovar toyoi CNCM I-1012/CNIB 40112
B.cereus ATCC53522
B.sp. AH533
glacial ice bacterium SB100-8-1
B.thuringiensis 82347
B.sp. Kaza-37
B.cereus AH527
Thuringiensis B
[83/(100)]
B.thuringiensis WS2614
Unidentified bacterium HTA484 (Mariana Trench isolate)
B.cereus ATCC27877
glacial ice bacterium SB-12K-9-4
B.sp. P16
B.cereus ATCC31293
B.sp. 82344
B.thuringiensis WS2626
B.cereus IAM12605
glacial ice bacterium G50-TS3
Bacillaceae bacterium PH27B
B.cereus T
glacial ice bacterium G500K-2
B.cereus NCTC9620
B.thuringiensis WS2618
B.sp. F26
B.thuringiensis WS2617
Cereus B
[34/(97)]
B.cereus WSB10030
B.cereus S-5
B.cereus (bovine serum isolate)
B.anthracis Vollum
B.cereus AL1
B.anthracis Ames ANR
B.anthracis Sterne
B.sp. AH526
B.sp. JJ#1
B.anthracis DeltaAmes-1
B.cereus WSB10037
B.thuringiensis B8
Anthracis + Cereus A
[41/(97)]
B.mycoides ATCC10206
B.mycoides ATCC6462m
B.pseudomycoides sp. nov.
Mycoides B
[99/(100)]
0.002
B.licheniformis GA8
B.megaterium KL-181
B.megaterium C1
100
0.005

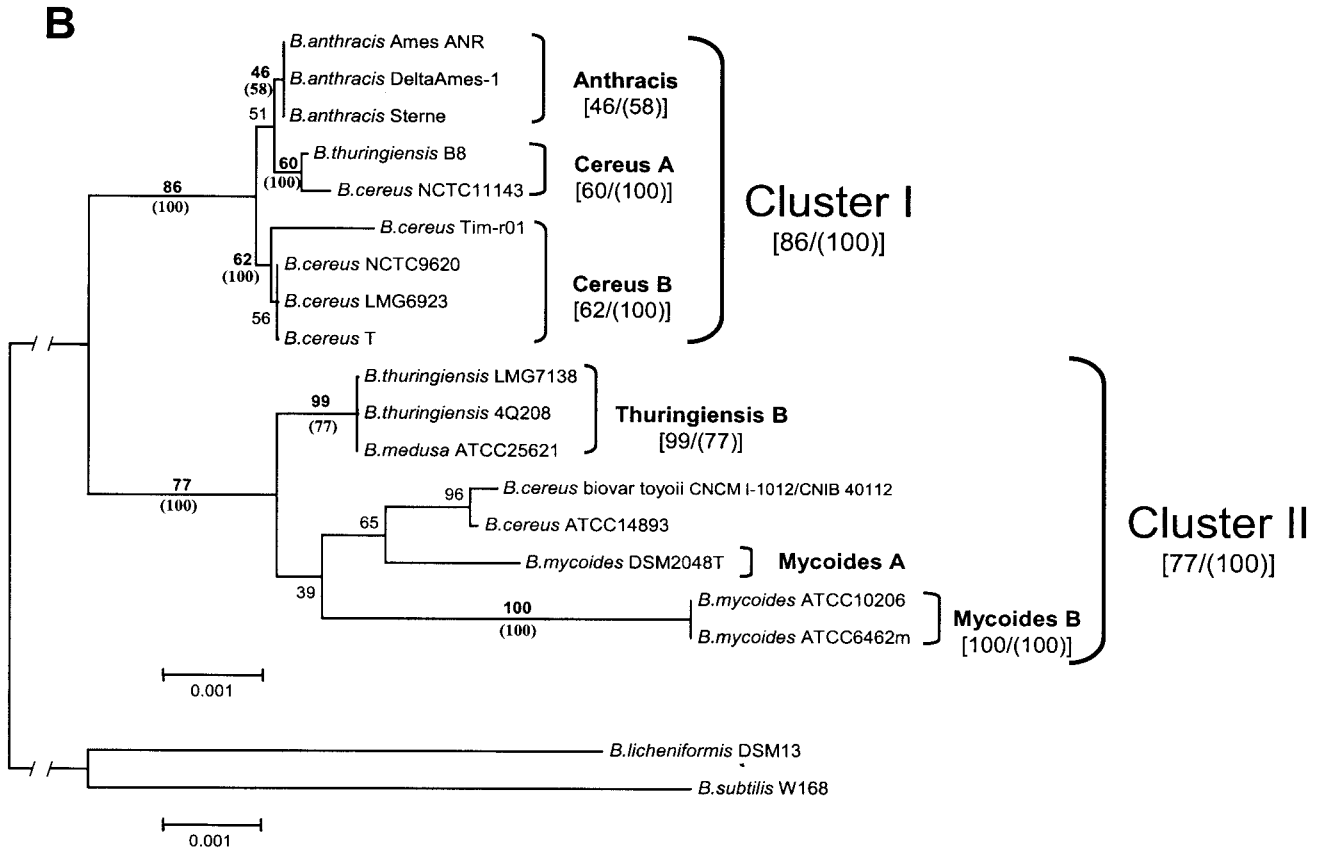


FIG. 3. Genetic relationship among *B. cereus* group strains. 16S rRNA (A) and 23S rRNA (B) phylogenetic trees obtained by minimum evolution method demonstrate the division of the *B. cereus* group into subgroups. Subgroup names are marked with bold on the right side of the brackets. Bootstrap volumes are reported on the branches. Numerals indicated in the quadrant parentheses denote the bootstrap volumes for each subgroup. During calculation of consensus parsimonious trees 7615 and 219 most-parsimonious trees were obtained for 16S rRNA and 23S rRNA, respectively. The percentages of most-parsimonious trees that support each subgroup in consensus parsimonious trees are presented in round parentheses. Bars indicate the scales of genetic distances.

typical for the Mycoides A and Mycoides B subgroups. The same connection between different subgroups was found for *B. cereus* Tim-r01 (subgroup Cereus B), which shares its G(1218-1219) insertion with subgroup Cereus A and variation G/T(1268) with subgroup Mycoides B (Fig. 2; Table 3).

Therefore, the division of the *B. cereus* group members into the specified subgroups was supported by both the 16S and 23S rRNA sequence analyses.

Subgroup identification through hybridization. Recently, two 16S rRNA probes containing A/G (77), T/C (90), and T/A (92) differences were described as a tool for discrimination of *B. thuringiensis* from *B. cereus* (18). In this study, we demonstrated that this signature is sufficient only for identification of bacteria included in the Thuringiensis B group (Fig. 1; Table 2). Using these probes, this signature was found in *B. thuringiensis* strains 9308, 20, Lb5, 1230, and L3 (18), in *B. thuringiensis* serotypes Galleriae and Israelensis (55) (Table 4), in *B. cereus* strains Nagoya 126 and 127 (55), and in *B. thuringiensis* strains BT3, BT13, BT15, BT16, BTT6, and BTT8 (13). We included all these microorganisms in the Thuringiensis B subgroup (Table 2).

We also included for consideration (Fig. 2; Tables 2 and 3) isolates *B. cereus* HER1414 and two sets of *B. thuringiensis* strains, *B. thuringiensis* strains 4R1, 4D1, 4F1, 4S2, 4T1, 4W1, and 4J4 and *B. thuringiensis* strains 4A1, 4A7, 4Q1, 4Q2, and 4M1, whose 16S and 23S rRNA sequences have not yet been sequenced. According to hybridization experiments of rRNA isolated from these bacteria with oligonucleotide probes specific to all described 16S rRNA subgroup-specific signatures (Fig. 1; Table 2), these strains were identified as belonging to subgroups Cereus A, Thuringiensis A, and Thuringiensis B, respectively. 23S rRNA signatures identified in these microorganisms after hybridization with probes specific to regions 3, 9, 10, and 12 are shown on Fig. 2 (S. Bavykin and J. Jackman, unpublished data).

Grouping of microorganisms in the *B. cereus* group according to *gyrB* gene sequences. Recently, a set of parallel 16S rRNA and partial (more then 60% of the whole gene length) *gyrB* sequences for *B. cereus* group isolates was placed in GenBank. We classified these microorganisms (Table 4) according to subgroup-specific signatures found for 16S rRNA sequences (Fig. 1 and Table 2). Additional information about the 23S rRNA obtained from whole-genome sequences of *B. cereus* ATCC 10987 made it possible to identify this organism as belonging to the Cereus A subgroup (Fig. 2; Table 3) and, therefore, to differentiate it from bacteria of the Anthracis

TABLE 4. Classification of *B. cereus* group bacteria with sequenced *gyrB* genes according to their 16S rRNA gene sequences

Subgroup name[a]	Organism[e]	GenBank accession no.	
		16S rRNA	*gyrB*
Anthracis	***B. anthracis* Ames**	AF155950	AE017024
	B. anthracis Pasteur 2	—[f]	AF090333
Cereus A	***B. cereus* ATCC 10987**[b]	NC003909	AE017264
Anthracis or Cereus A	*Bacillus* sp. strain H-01[c]	AY461742	AY461763
	Bacillus sp. strain H-03[c]	AY461744	AY461764
	Bacillus sp. strain H-05[c]	AY461746	AY461766
	Bacillus sp. strain H-06[c]	AY461747	AF136389
	Bacillus sp. strain H-07[c]	AY461748	AY461767
	Bacillus sp. strain H-08[c]	AY461749	AY461768
	Bacillus sp. strain H-09[c]	AY461750	AY461769
	Bacillus sp. strain H-12[c]	AY461753	AY461772
	Bacillus sp. strain H-17[c]	AY461758	AY461776
Cereus B	***B. cereus* ATCC 14579**	AF290547	AE016998
	B. thuringiensis serotype Aizawai	AY461760	AY461778
	Bacillus sp. strain H-04	AY461745	AY461765
	Bacillus sp. strain H-11	AY461752	AY461771
	Bacillus sp. strain H-13	AY461754	AY461773
	Bacillus sp. strain H-14	AY461755	AY461774
	Bacillus sp. strain H-15	AY461756	AY461775
Thuringiensis A	*Bacillus* sp. strain H-10	AY461751	AY461770
	Bacillus sp. strain H-18	AY461759	AY461777
Thuringiensis B	***B. thuringiensis* IAM12077**	D16281	AF090331
	B. thuringiensis serotype Galleriae	AY461761	AY461779
	B. thuringiensis serotype Israelensis	AY461762	AY461780
	Bacillus sp. strain ES-027	AY461789	AY461783
	Bacillus sp. strain H-16	AY461757	AF136387
Thuringiensis A or B	*Bacillus* sp. strain SAFN-003[d]	AY167823	AY461786
	Bacillus sp. strain 83-3C[d]	AF526900	AY461781
Mycoides A	***B. mycoides* ATCC 6462**	AB021192	AF090332
	Bacillus sp. strain SAFR-048	AY167860	AY461787
	Bacillus sp. strain FO-080	AY461791	AY461785

[a] Subgroups were identified in accordance with 16S rRNA sequences of the organisms.
[b] Belongs to subgroup Cereus A according to 16S rRNA and 23S rRNA sequences, which were extracted from whole-genome sequences of this organism (GenBank accession nos. AE017264, AE017265, AE01266, and AE017280).
[c] Because 23S rRNA genes of the organisms are not sequenced yet, the isolates were assigned to Anthracis or Cereus A subgroups.
[d] Because of incomplete 16S rRNA sequences, these two isolates were identified as belonging to Thuringiensis A or Thuringiensis B subgroup.
[e] Microorganisms whose names are marked in bold were also subgroup identified based on their 23S rRNA sequences.
[f] —, not sequenced.

subgroup. Because 23S rRNA genes for isolates *Bacillus* sp. strains H-01, H-03, H-05, H-06, H-07, H-08, H-09, H-12, and H-17 are not yet published, we identified these microorganisms as belonging to the Anthracis or Cereus A subgroups. The absence of 98 and 139 nucleotides in the beginning of the 16S rRNA sequences for isolates *Bacillus* sp. strain SAFN-003 and *Bacillus* sp. strain 83-3C, respectively, led us to identify these organisms as belonging to the Thuringiensis A or Thuringiensis B subgroups. Finally, among 30 microorganisms for which both 16S RNA and *gyrB* gene sequences were published, we identified members of six of the seven subgroups, identified using rRNA sequences (Tables 2 and 3 and Fig. 1 to 3). Unfortunately, there were no *gyrB* sequences available in GenBank for any members of the Mycoides B subgroup.

The same groups containing the same microorganisms were identified in rooted and unrooted phylogenetic trees that were generated with minimum evolution (compare Table 4 and Fig. 4), neighbor-joining, UPGMA, and maximum parsimony methods with the *gyrB* sequences of *B. cereus* group organisms and the *Bacillus pumilis* strain KL-052 *gyrB* sequence (GenBank accession no. AY167878) as an outgroup. All four trees revealed completely identical branching patterns (data not shown) with high bootstrapping values from all four methods for five of six subgroups (Fig. 4). The subgroups in all four trees were organized into two clusters, as was observed for the 23S rRNA tree (Fig. 3B). Cluster I contained subgroups Anthrax, Cereus A, and Cereus B, and Cluster II included subgroups Thuringiensis B and Mycoides A. Additionally, the Thuringiensis A subgroup was located in cluster I of the *gyrB* tree, but it was not completely separated from subgroup Cereus B. Unfortunately, as was mentioned above, there are no 23S rRNA sequences for Thuringiensis A subgroup organisms available.

Subgroups Anthracis and Cereus A were identified in the *gyrB* phylogenetic trees (Fig. 4) in accordance with the presence in these subgroups of *B. anthracis* AMES and *B. cereus* ATCC 10987, whose correspondence to subgroups Anthracis and Cereus A, respectively, were confirmed through independent grouping of 16S rRNA and 23S rRNA sequences (Tables 2 to 4). The presence of *B. anthracis* Pasteur 2 and *B. anthracis* Ames at the same subgroup (Fig. 4) also confirms this identification. Isolates *Bacillus* sp. strain SAFN-003 and *Bacillus* sp. strain 83-3C were found in the *gyrB* phylogenetic tree in subgroup Thuringiensis B (Fig. 4).

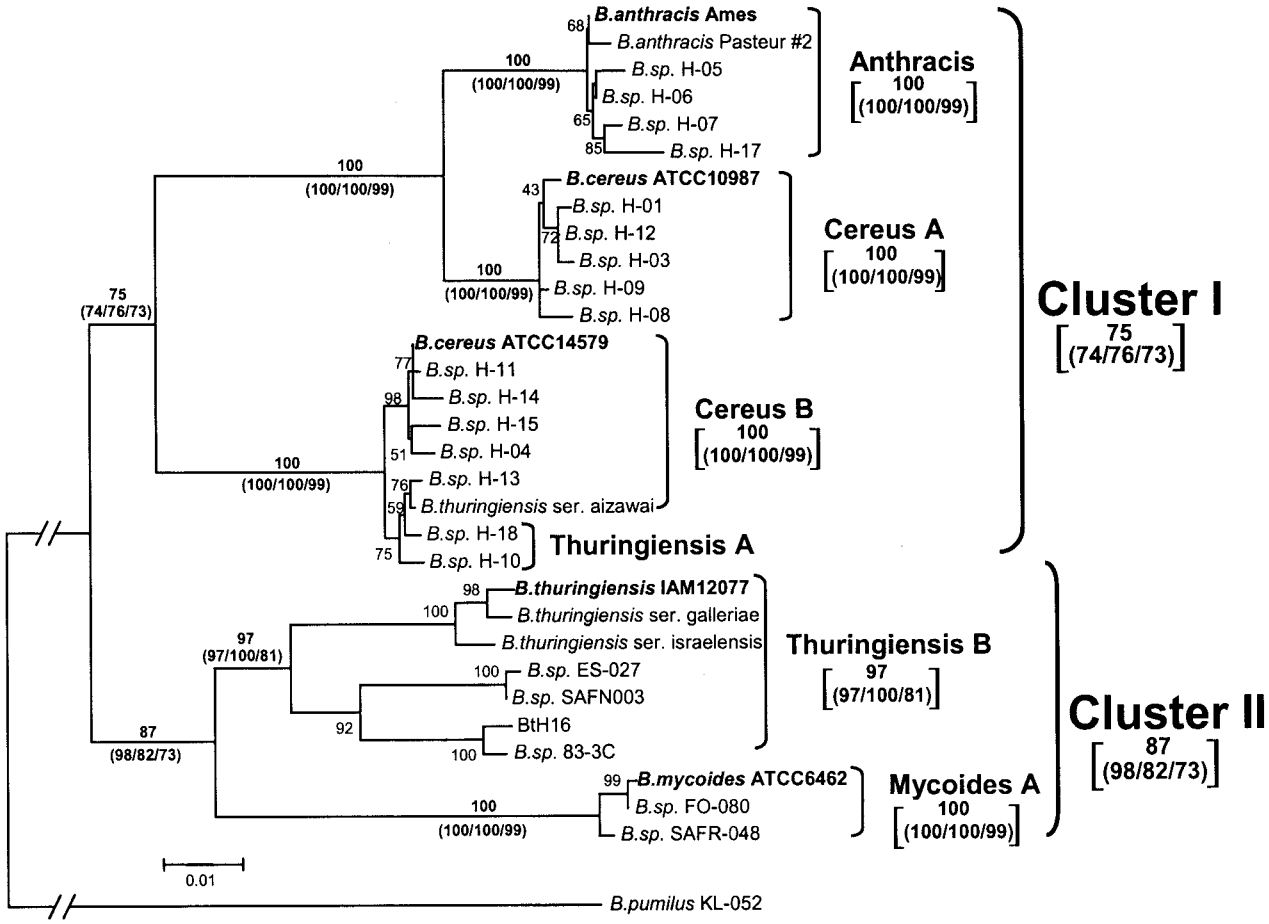


FIG. 4. Grouping of the *B. cereus* group strains based on *gyrB* gene sequences. Names of the subgroups were identified through 16S rRNA sequences of their members (Table 4) and are displayed with bold on the right sides of the brackets. Subgroup identities of the microorganisms, whose names appear in bold, were confirmed by their 23S rRNA sequences. The phylogenetic tree was constructed by the minimum evolution method. Bootstrap volumes are reported on the branches. Numerals indicated in the quadrant parentheses designate the bootstrap volumes for each subgroup. Phylogenetic trees obtained by neighbor-joining, UPGMA, and maximum parsimony methods gave identical branching patterns. Bootstrap numbers for these three methods are presented in round parentheses. Bar indicates scale of genetic distances.

***gyrB* as a phylogenetic marker for subgroup identification in the *B. cereus* group.** In accordance with our 16S rRNA analysis, we aligned *gyrB* sequences for 31 *B. cereus* group isolates. Analysis of the *gyrB* alignment resulted in identification of 93 unique subgroup-specific differences (Fig. 5). As shown in Table 5, we defined these differences for each subgroup as subgroup-specific signatures. Because the number of members of each subgroup was not statistically large enough, we defined subgroup-specific signatures according to the rule that all members of the subgroup should contain all of the selected subgroup-specific differences. In total, we identified 15 unique subgroup-specific *gyrB* differences for the Anthracis subgroup (Table 5) which were absent from the other five subgroups (Fig. 5). For subgroups Cereus A, Thuringiensis B, and Mycoides A, we identified 12, 13, and 32 unique subgroup-specific differences, respectively (Table 5). For subgroups Cereus B and Thuringiensis A, we did not find significant differences in the *gyrB* sequences. For this reason, we defined one set of signatures for these two subgroups (Table 5).

We also found that isolates *B. sp*. SAFN-003 and *B. sp*. 83-3C, which were found in the Thuringiensis B subgroup in the *gyrB* phylogenetic tree, included all 13 subgroup-specific differences (Fig. 5) that were present in subgroup Thuringiensis B (Table 5). This confirmed that these two isolates belonged in the Thuringiensis B subgroup.

DISCUSSION

Identification of subgroups in the *B. cereus* group. Based on analysis of the 16S rRNA sequence alignments, we divided the *B. cereus* group into the seven above-mentioned subgroups, each containing microorganisms with similar 16S rRNA gene sequences. The strains within each subgroup include all or most of the alterations specific for that subgroup (Fig. 1 and Table 2).

Some of the 16S rRNA subgroup-specific differences, indicated in Table 2, have already been used by other researchers for identification of certain *Bacillus* sp. (18, 33, 41), but a systematic analysis of all *B. cereus* group microorganisms in accordance with their 16S and 23S rRNA sequences had not been done before.

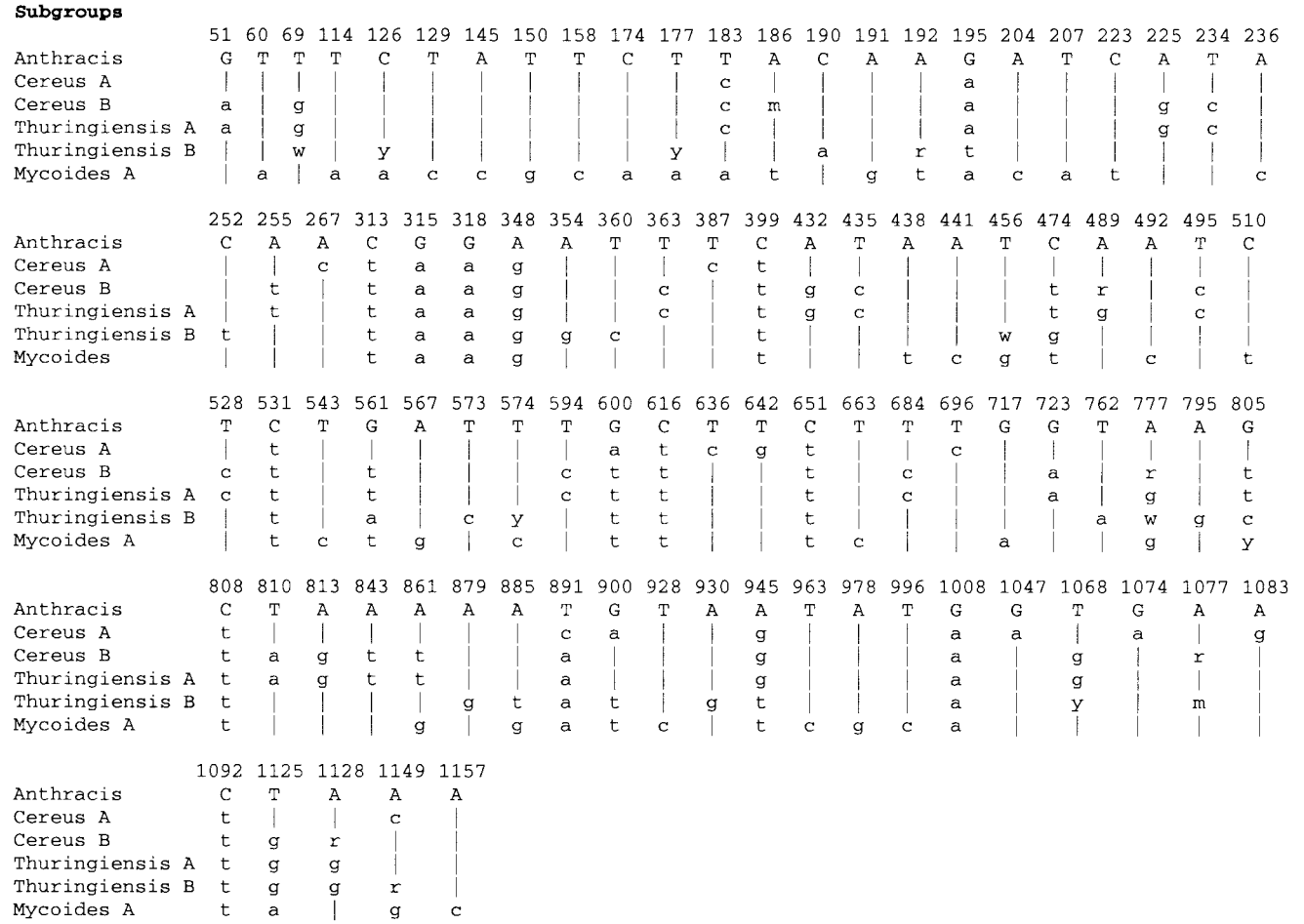


FIG. 5. Positions of subgroup-specific differences in the *gyrB* gene in the *B. cereus* group. Only differences that are unique for one of the subgroups are shown. (Members of each subgroup are displayed in Fig. 4.) Differences indicated for each subgroup were present in *gyrB* sequences of all members of that subgroup. The sequence complementary to the *B. anthracis* Ames *gyrB* gene (GenBank accession no. AE017024) has been used as the consensus sequence. Vertical lines indicate nucleotides identical to the consensus sequence. Multiple nucleotide substitutions (w, y, m, and r) indicate that more than one subgroup member revealed alternative differences. w, a or t; y, c or t; m, a or c; r, a or g.

We have also sequenced the 23S rRNA gene for a selected set of members of the *B. cereus* group. During 23S rRNA sequence alignment analysis of these and GenBank sequences, we did not find any conflicts between subgroupings based on 16S rRNA and subgroupings based on 23S rRNA (Tables 2 and 3 and Fig. 1 and 2). We also found that isolates from subgroup Cereus A, which have the same 16S rRNA sequence as *B. anthracis* Sterne (Fig. 1 and Table 2), have three sub-

TABLE 5. Subgroup-specific signatures in *gyrB* in *B. cereus* group microorganisms

Subgroup name	Subgroup-specific signatures
Anthracis	Consensus[a]: G(195), C(313), G(315), G(318), A(348), C(399), C(531), G(600), C(616), C(651), C(808), T(891), A(945), G(1008), C(1092)
Cereus A	A/C(267), T/C(387), G/A(600), T/C(636), T/G(642), T/C(696), T/C(891), G/A(900), G/A(1047), G/A(1074), A/G(1083), A/C(1149)
Cereus B and Thuringiensis A	G/A(51), T/G(69), A/G(225), T/C(234), A/T(255), T/C(363), A/G(432), T/C(435), T/C(495), T/C(528), T/C(594), T/C(684), G/A(723), T/A(810), A/G(813), A/T(843), A/T(861), T/G(1068)
Cereus B	A/M(186), A/R(489), A/R(777), A/R(1077), A/R(1028)
Thuringiensis A	A/G(489), A/G(777), A/G(1128)
Thuringiensis B	C/A(190), G/T(195), C/T(252), A/G(354), T/C(360), C/G(474), G/A(561), T/C(573), T/A(762), A/G(795), A/G(879), A/T(885), A/G(930)
Mycoides A	T/A(60), T/A(114), T/C(129), A/C(145), T/G(150), T/C(158), C/A(174), T/A(177), T/A(183), A/T(186), A/G(191), A/T(192), A/C(204), T/A(207), C/T(223), A/C(236), A/T(438), A/C(441), T/G(456), A/C(492), C/T(510), T/C(543), A/G(567), T/C(663), G/A(717), A/G(861), A/G(885), T/C(928), T/C(963), A/G(978), T/C(996), A/C(1157)

[a] For more details, see Fig. 5.

group-specific changes in the 23S rRNA sequence in comparison with *B. anthracis* Sterne (Fig. 2 and Table 3). Our study demonstrated that members of subgroups Anthracis and Cereus A may not be differentiated according to their 16S rRNA subgroup-specific signatures (Fig. 1; Table 2). However, we have demonstrated that *B. cereus* ATCC 10987, *B. cereus* NCTC 11143, *B. thuringiensis* B8, and *B. cereus* HER1414, which belong to the Cereus A subgroup, may be differentiated from members of subgroup Anthracis by using the 23S rRNA subgroup-specific changes Y/C(594), G/A(1559), and insertion G(1218-1219), in combination with 16S rRNA-targeted probes (Fig. 1 and 2; Tables 2 and 3). Strain-specific variations also may be used for these purposes (Tables 2 and 3). At the same time, because we do not have 23S rRNA sequences for most members of Cereus A subgroup we are not excluding the possibility that some nonpathogenic strains of *B. anthracis*, which have transcriptionally inactive toxin genes or which have lost their virulence plasmids (52), may be found besides the isolates that were placed in subgroup Cereus A according to their 16S rRNA sequences (Table 2).

Phylogenetic analysis of 16S rRNA sequences with two independent methods, minimum evolution and maximum parsimony, confirmed the presence of subgroups Mycoides A and B, Thuringiensis A and B, Cereus B, and joint subgroup Anthracis-Cereus A in the *B. cereus* group (Fig. 3A). Both methods generated subgroups with identical microbial content, which corresponded to the results of 16S and 23S rRNA alignment analyses (Tables 2 and 3 and Fig. 1 and 2). Low bootstrapping volumes for Anthracis-Cereus A (41%) and Cereus B (34%) subgroups obtained with the minimum evolution method reflect an absence of subgroup-specific differences between subgroups Anthracis and Cereus A and a strongly reproducible but small (one base only) difference between joint subgroup Anthracis-Cereus A and subgroup Cereus B in their 16S rRNA sequences (Table 2; Fig. 1). However, grouping of 16S rRNA sequences obtained with the maximum parsimony method strongly confirmed the data from the minimum evolution analysis (Fig. 3A).

In the rooted minimum evolution phylogenetic tree generated for 23S rRNA sequences of the *B. cereus* group, we found more significant differentiation of the Anthracis, Cereus A, Cereus B, Thuringiensis B, Mycoides A, and Mycoides B subgroups (Fig. 3B) according to their bootstrapping volumes because of the presence in these subgroups of a considerable amount of subgroup-specific changes in the 23S rRNA signatures (Table 3 and Fig. 2). The unrooted 23S rRNA phylogenetic tree obtained with the maximum parsimony method strongly confirmed this grouping (Fig. 3A and B). A rooted 23S rRNA tree also indicated that the *B. cereus* group divided into two clades, cluster I and cluster II, which included subgroups Anthracis, Cereus A, and Cereus B and Thuringiensis B, Mycoides A, and Mycoides B, respectively (Fig. 3B). The existence of microorganisms that sometimes shared their subgroup-specific alterations in 16S rRNA signatures between subgroups Thuringiensis B and Mycoides A (*B. thuringiensis* 82347, *Bacillus* sp. strain AH540, *Bacillus* sp. strain AH533, *Bacillus* sp. strain Termite isolate bac, bromate-reducing bacterium B6, glacial ice bacterium SB100-8-1, unidentified bacterium V, and *B. cereus* AH527) (Table 2), bacteria that shared some of their changes in 23S rRNA signatures between the Thuringiensis B and Mycoides A and B subgroups (*B. cereus* biovar *toyoi* and *B. cereus* 14893), and isolate *B. cereus* Tim-r01 (subgroup Cereus B), which shares a G(1218-1219) insertion in its 23S rRNA sequence with Cereus A subgroup (Table 3 and Fig. 2) confirm the division of the *B. cereus* group into these two clusters.

Comparative analysis of rRNA and *gyrB* sequences demonstrated excellent correlation for the grouping of bacteria from the *B. cereus* group. The single-copy *gyrB* gene is rather well conserved; however, for the *B. cereus* group, it displayed more nucleotide variations than the rRNA genes. The relatively high degree of variation in *gyrB* sequences probably explains the fact that analysis of *gyrB* sequences by four different methods (minimum evolution, neighbor-joining, UPGMA, and maximum parsimony [Fig. 4]) produced identical branching patterns (data not shown) and was supported with higher bootstrapping values than those obtained for rRNA analysis (Fig. 3). The rooted phylogenetic *gyrB* trees (Fig. 4) also showed the same organization of subgroups into two clusters that was found with the 23S rRNA phylogenetic analysis (Fig. 3B). At the same time, questions remain about the nature of isolates *Bacillus* sp. strains H-05, H-06, H-07, and H-17. Further investigation should be performed to clarify whether these microorganisms might represent strains of *B. anthracis* that have lost their virulence.

In summary, the existence of seven subgroups within the *B. cereus* group was confirmed by six independent methods: 16S rRNA, 23S rRNA, and *gyrB* sequence alignment analysis (Fig. 1, 2, and 5; Tables 2, 3, and 5) and phylogenetic analysis of all three sets of these sequences (Fig. 3 and 4).

In total we analyzed 183 16S and 74 23S rRNA sequences for 155 strains of *B. cereus* group bacteria, including 50 *Bacillus* isolates of unknown species and nine isolates of unknown genus (Tables 2 and 3). Identification of some unidentified microorganisms as members of the *B. cereus* group brought some interesting information. Particularly, we found that among *B. cereus* group bacteria there exist bromate-reducing (*Bacillus* sp. strain B6) and sulfur-degrading (*Bacillus* sp. strain KPU-0013) microorganisms. Some of the *B. cereus* group isolates were found in Cold Desert, India (*Bacillus* sp. strain Kaza-31 and *Bacillus* sp. strain Kaza-37), as intestinal symbionts (*Bacillus* sp. strain JJ-1, unidentified bacterium V, and unidentified bacterium sp6 [bovine rumen isolate]), on the surface of strawberry plants (*Bacillus* sp. strain Fa7), and even in the deepest sea mud of the Mariana Trench (unidentified bacterium HTA484). We also recognized that subgroup Cereus B was organized not later then 500,000 years ago, because glacial ice bacterium G500K-2 that belongs to this subgroup was isolated from more-than-500,000-year-old glacial ice from Gulia, China (Table 2; Fig. 3A).

Taxonomy of the *B. cereus* group. Recent studies demonstrated that the species *B. cereus, B. thuringiensis*, and *B. mycoides* represent genetically heterogeneous groups of microorganisms (12–14, 21–23, 28, 40, 50, 55) which, according to their rRNA sequences, comprise a group of close relatives (5–7) (Tables 2 and 3; Fig. 1 and 2). These findings resulted in several suggestions to consider *B. cereus* and *B. thuringiensis* (8, 12, 58), or these two bacteria together with *B. anthracis* (14, 21), as one species. Results of our analysis of 16S and 23S rRNA sequences also show disagreement with the current tax-

onomic classification of species within the *B. cereus* group. Traditionally, classification of microorganisms in the *B. cereus* group has been based on morphological, physiological, and immunological data. However, recent data suggest that use these criteria for current *B. cereus* group species identification may have some obstacles. For example, *B. thuringiensis* has been traditionally distinguished from *B. cereus* by the production of a parasporal crystal of a protein that is toxic for *Lepidoptera*, *Diptera*, and *Coleoptera* larvae. The capacity to form crystals is plasmid encoded (3), however, and some plasmids may be lost by laboratory culturing (4, 45). Moreover, authentic cultures of *B. cereus* can acquire the ability to produce crystals as a result of growing in mixed culture with *B. thuringiensis* (19). Based on our findings, we suggested that plasmid exchange apparently exists inside and between subgroups Cereus B, Thuringiensis A, and Thuringiensis B only, because simultaneously *B. cereus* and *B. thuringiensis* strains were found only in these three subgroups (Tables 2 and 3 and Fig. 3). Thus, the discrimination of *B. cereus* from *B. thuringiensis* is a difficult task by any method, and the fact that they have grouped together in our analysis (Fig. 1 to 3; Tables 2 and 3) and in other recent studies (12–14, 21–23, 28, 40, 50, 55) is not surprising. At the same time, we do not exclude that after resequencing their 16S rRNAs some *B. thuringiensis* strains may be moved from subgroup Cereus B to subgroup Thuringiensis A, which differ from each other by only one subgroup-specific signature, C/T (192) (Table 2).

For *B. mycoides*, sporadic loss of the ability to form rhizoid colonies has been observed in several strains (45), indicating an instability of morphology in this species. In this case, *B. mycoides* becomes morphologically similar to *B. cereus*. Bacterial isolates can also undergo physiological changes after the loss or acquisition of plasmids coding for toxins, sporulation, or antibiotic resistance (48). Therefore, flexible colony morphology may be a reason why strains of *B. mycoides* and *B. cereus* are present together in subgroups Mycoides A and Mycoides B (Tables 2 and 3 and Fig. 2 and 3).

Therefore, the current division of the *B. cereus* group into seven species (*B. anthracis, B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis*, and *B. medusa*) seems to be insolvent from genomic as well as phenotypic points of view.

Demonstrating that 16S rRNA and 23S rRNA sequences independently confirm separation of the *B. cereus* group into seven distinct subgroups, we tried to find additional confirmation for this division among other genomic sequences and morpho-physiological evidence. The Anthracis subgroup can be taken as an example. This subgroup is represented by one species only, *B. anthracis*, which is distinguishable from members of other subgroups based on its plasmid content and ability to induce anthrax (36, 42, 49, 51, 52).

We found some unique properties of subgroup Thuringiensis B in the literature. Two recent studies (13, 55) exploited differences in the *gyrB* gene as well as a 16S rRNA signature of A/G (77), T/C (90), and T/A (92) (unique for the Thuringiensis B subgroup in our classification) for discrimination of *B. cereus* and *B. thuringiensis*. Both studies demonstrated a good correlation between these two parameters but not with the current nomenclature, which is based on phenotypic characterization. We also analyzed *gyrB* sequences for a set of 30 *B. cereus* group isolates for which both 16S rRNA and *gyrB* gene sequences were deposited recently in GenBank (Table 4). This provided the unique possibility to identify a number of strong phylogenetic markers represented by *gyrB* subgroup-specific signatures (Table 5) for most of the described subgroups (Tables 2 and 3; Fig. 3 and 4). Each signature contained between 12 and 32 unique subgroup-specific differences and strongly differentiated its own subgroup from all others, with the exception of subgroups Cereus B and Thuringiensis A (Table 5). Because *gyrB* sequences were available for only two members of subgroup Thuringiensis A (Table 4; Fig. 4) and because the published *gyrB* sequences represent only about 65% of the whole length of the *gyrB* gene, we are unable to draw a final conclusion about the relationship between subgroups Cereus B and Thuringiensis A. However, if more-thorough sequencing of 23S rRNA, *gyrB*, and other genes does not reveal more differences between subgroups Cereus B and Thuringiensis A, this would suggest that subgroup Thuringiensis A should not be considered as a separate subgroup but should instead be characterized as a variation of subgroup Cereus B. At the same time, the 15 subgroup-specific differences found in the *gyrB* gene for subgroup Anthracis and the 12 differences observed for subgroup Cereus A provide us numerous possibilities for differentiation of members of these two subgroups (Table 5). Subgroups Thuringiensis B and Mycoides A and the joint Cereus B-Thuringiensis A subgroup also included a number of differences (13, 32, and 18, respectively) which could be useful for discrimination of these subgroups (Table 5).

Another distinguishing characteristic that relates to *B. mycoides* is psychrotolerance. Recent DNA relatedness studies have indicated that the *B. mycoides* species actually consists of two genetically distinct groups (37, 38). The fact that our study placed *B. mycoides* strains into two subgroups, Mycoides A and Mycoides B, supports this finding. The type strain of *B. mycoides* group 1 (*B. mycoides* sensu stricto), *B. mycoides* ATCC 6462 (37, 38), was included in our Mycoides A subgroup (Table 2; Fig. 3A), and the type strain of *B. mycoides* group 2 (*B. pseudomycoides*), *B. mycoides* NRRL B-617 ^{T} (*B. pseudomycoides* sp. nov.), is a member of our Mycoides B subgroup (Table 2; Fig. 3A). According to our classification scheme (Table 2), four representatives of psychrotolerant strains of *B. cereus* (WSBC10201, WSBC10204, WSBC10206, and WSBC10210), which were recently named as the new species *B. weihenstephanensis* (33), fall under subgroup Mycoides A. This finding suggests that species *B. weihenstephanensis* may be one of the *B. mycoides* strains that belongs to the subgroup Mycoides A. This suggestion was confirmed by the high degree of similarity of genomic DNA sequences (85 to 88%) between *B. cereus* strains WSBC10201, WSBC10204, and WSBC10206 and *B. mycoides* DSM2048 (33), which is also located in subgroup Mycoides A. In addition, based on the ability to grow at low temperature, *B. mycoides* is the most closely related species to *B. weihenstephanensis* in the *B. cereus* group (33). At the same time, the mesophilic strains *B. cereus* WSBC10030, *B. cereus* WSBC10037, and *B. cereus* HER1414 (33) were included in subgroup Cereus A, and mesophilic *B. cereus* ATCC 27877 (33) and *B. cereus* ATCC14579 (17) were found in subgroup Cereus B (Table 2; Fig. 3A). However, mesophilic strains (17, 41) are also widely represented in other subgroups. Thus, *B. thuringiensis* WS2614, *B. thuringiensis* WS2617, *B.*

thuringiensis WS2618, and *B. thuringiensis* WS2626 (41) were included in subgroup Cereus B, *B. thuringiensis* WS2625 (41) is a member of subgroup Thuringiensis A, and *B. thuringiensis* ATCC 10792 (17) is situated in subgroup Thuringiensis B. Interestingly, representative of Mycoides B subgroup, *B. pseudomycoides* sp. nov. has a minimum growth temperature of 15°C (38) and is therefore a mesophilic strain. Summarizing the above results, we can stress that currently we did not find any mesophilic representatives in the Mycoides A subgroup, nor did we find any psychrotolerant strains outside of this subgroup.

Experience with thousands of strains from several hundred species led taxonomists to conclude that 70% or higher DNA-DNA relatedness with 5% or less divergence within related sequences, together with 97% or higher 16S rRNA sequence similarity, is the best means of defining a species (10). Our findings indicated that 16S rRNA of *B. cereus* from subgroup Cereus B may differ from that of *B. thuringiensis* from subgroup Thuringiensis A in one base only (Table 2). Even *B. cereus* from the Mycoides A subgroup may have only nine substitutions (equivalent to 0.6% of 16S rRNA) in comparison with *B. thuringiensis* from Thuringiensis A subgroup (Table 2), in spite of a great difference between representatives of these two subgroups in phenetic (46) relationship that includes differences both in genotype and in phenotype. However, we found *B. cereus* strains in six of the seven subgroups and *B. thuringiensis* in three of the subgroups, whereas members of the different subgroups are considerably different both in phenotype and in rRNA sequences. For example, *B. cereus* strains are present in Thuringiensis A and B as well as in Mycoides A and B subgroups. At the same time, DNA-DNA relatedness between *B. thuringiensis* and *B. cereus* may range from 54.3 to 96.4% (33), with divergence inside of these two species of up to 45% (33, 37). In these terms, unification of all strains of *B. cereus* with all strains of *B. thuringiensis*, even without *B. anthracis*, as one species is rather questionable. Apparently, this is a case when only polyphasic (consensus) taxonomy (10), which integrates all available data, may provide the best opportunity to find the right solution.

The results of this work demonstrated the potential for the use of 16S rRNA, 23S rRNA, and *gyrB* gene sequences for identification of the members of the *B. cereus* group and, especially, differentiation of *B. anthracis* from other relatives, something which has for a long time been considered impossible by using rRNA sequences (5–7). Further work is needed to determine how the differences in rRNA genes, which have been revealed in our study, relate to differences in phenotypic traits and to determine what kind of revisions are necessary in the taxonomy of the *B. cereus* group.

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