Comparative Analysis of Bacillus anthracis, Bacillus cereus, and Related Species on the Basis of Reverse Transcriptase Sequencing of 16S rRNA

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The primary structures of the 16S rRNAs of Bacillus anthracis, Bacillus cereus, Bacillus mycoides, and Bacillus thuringiensis were determined by using the reverse transcription-dideoxy sequencing method. All of the strains exhibited very high levels of sequence similarity (>99%) that were consistent with the close relationships shown by previous DNA hybridization studies. The sequences of B. anthracis Sterne and B. cereus emetic strain NCTC 11143 were found to be identical for a continuous stretch of 1,446 bases and differed from the sequence of B. cereus NCDO 1771T (T = type strain) by only a single nucleotide. The 16S rRNA sequences of B. mycoides and B. thuringiensis differed from each other and from the sequences of B. anthracis and B. cereus by four to nine nucleotides.

The species Bacillus anthracis, Bacillus cereus, Bacillus mycoides, and Bacillus thuringiensis were originally described on the basis of their habitats, their pathogenicity for mammals or insects, and their morphological and physiological characteristics. However, the taxonomic interrelationships of these species are equivocal. All four species share many phenotypic properties, and several workers have questioned their status as separate species (6, 11, 12). DNA-DNA hybridization studies on strains of B. anthracis, B. cereus, and B. thuringiensis have also shown that these organisms share relatively high levels of chromosomal base sequence similarity (7, 10, 13). However, inconsistencies in reported levels of DNA relatedness make it difficult to draw firm conclusions regarding species differentiation within this group of organisms.

Small-subunit rRNA is now recognized as a powerful molecular chronometer (16). Degrees of sequence conservation, ranging from highly variable to highly conserved regions, enable systematists to measure small as well as great genealogical distances. In this study we determined partial primary 16S RNA sequences of B. anthracis, B. cereus, B. mycoides, and B. thuringiensis in order to investigate the genealogical interrelationships of these organisms.

MATERIALS AND METHODS

Cultures and cultivation. Details concerning the test strains which we examined are shown in Table 1. Strains were grown in shake flasks containing nutrient broth no. 2 (Oxoid) to late exponential phase at 30°C.

Extraction and sequence determination of 16S rRNA. Total cellular rRNA was extracted from ca. 2 g of wet cells by mechanical disruption, using glass beads and a Braun homogenizer, and was purified as described by Embley et al. (5). Nucleotide sequences were determined by the Sanger dideoxynucleotide method (9) directly from cellular rRNA, using avian myeloblastosis virus reverse transcriptase (8). The sequences of the oligonucleotide primers were the same as those described by Embley et al. (5). In addition, the following primer was used: 5'TCACCAACTAGCTAATG, which is complementary to positions 258 to 242 (Escherichia coli nomenclature). This primer was included to enable determination of the sequence between positions 100 and 150, which could not always be fully established by using the primer at position 357 described by Lane et al. (8).

Nucleotide sequence accession numbers. The 16S rRNA sequences have been deposited in the EMBL Data Library (accession numbers X55059 to X55063).

RESULTS AND DISCUSSION

The 16S rRNA sequences of B. anthracis Sterne, B. cereus NCDO 1771T (T = type strain), B. cereus NCTC 11143 (emetic strain), B. mycoides DSM 2048T, and B. thuringiensis NCIMB 9134T were determined.

TABLE 1. Similarity values and numbers of nucleotide differences for a 1,446-nucleotide region of 16S rRNAs of B. anthracis and related species

<table>
<thead>
<tr>
<th>Organism</th>
<th>B. anthracis Sterne</th>
<th>B. cereus NCDO 1771T</th>
<th>B. cereus NCTC 11143</th>
<th>B. mycoides DSM 2048T</th>
<th>B. thuringiensis NCIMB 9134T</th>
<th>B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. anthracis Sterne</td>
<td>99.9</td>
<td>100</td>
<td>99.5</td>
<td>99.7</td>
<td>94.1</td>
<td></td>
</tr>
<tr>
<td>B. cereus NCDO 1771T</td>
<td>1</td>
<td>99.9</td>
<td>99.6</td>
<td>99.7</td>
<td>94.0</td>
<td></td>
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<tr>
<td>B. cereus emetic strain NCTC 11143</td>
<td>0</td>
<td>1</td>
<td>99.5</td>
<td>99.7</td>
<td>94.0</td>
<td></td>
</tr>
<tr>
<td>B. mycoides DSM 2048T</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>99.4</td>
<td>94.0</td>
<td></td>
</tr>
<tr>
<td>B. thuringiensis NCIMB 9134T</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>94.1</td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>85</td>
<td>87</td>
<td>87</td>
<td>88</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

* The values on the upper right are percentages of similarity, and the values on the lower left are numbers of nucleotide differences. The actual numbers of base differences between strains may be marginally higher because approximately 2% of the nucleotides were not determined.
COMPARATIVE ANALYSIS OF BACILLUS SPECIES

FIG. 1. Primary structures of 16S rRNAs of *B. anthracis* Sterne (B. an), *B. cereus* NCDO 1771T (B. ce), *B. cereus* NCTC 11143 (B. c. em), *B. mycoides* DSM 2048T (B. my), and *B. thuringiensis* NCIMB 9134T (B. th) as determined by reverse transcription. The first and last nucleotides in the sequences are analogs to the nucleotides at positions 1 and 1,440 of the *E. coli* 16S rRNA sequence (2). N, undetermined nucleotide.

The strains of *B. mycoides* and *B. thuringiensis* NCIMB 9134T were investigated by performing reverse transcription experiments. Each of the sequences shown in Fig. 1 comprises a continuous stretch of 1,446 bases (ranging from position 1 to position 1,440 on the *E. coli* numbering system), which corresponds to approximately 94% of the total 16S rRNA primary sequence. A comparison of the sequence alignments revealed very high levels of sequence similarity among all of the strains examined (Table 1). Only 11 base substitution points on the sequences were identified, and these occurred in variable domains V1, V2, and V6. Of the variable positions, four involved base pairs (77, 92, 100, 920 [E. coli numbering]). Table 2 shows the locations of the base differences in the strains. The sequences of *B. anthracis* Sterne and *B. cereus* emetic strain NCTC 11143 were found to be identical and differed from the *B. cereus* NCDO 1771T sequence in only a single nucleotide (position 1,005 [E. coli numbering]). In contrast, the type strains of *B. mycoides* and *B. thuringiensis* differed from each other and from *B. anthracis* and *B. cereus* strains by a slightly greater number of nucleotides (ca. four to nine bases) (Tables 1 and 2).

Previous chromosomal DNA-DNA hybridization investigations indicated that *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. thuringiensis* are closely related and probably repre-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nucleotides at the following positions*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77 - 92</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne</td>
<td>A</td>
</tr>
<tr>
<td><em>B. cereus</em> NCDO 1771T</td>
<td>A</td>
</tr>
<tr>
<td><em>B. cereus</em> NCTC 11143</td>
<td>A</td>
</tr>
<tr>
<td><em>B. mycoides</em> DSM 2048T</td>
<td>A</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> NCIMB 9134T</td>
<td>G</td>
</tr>
</tbody>
</table>

* Based on *E. coli* numbering (2).
sent a single species (10, 13). Our 16S rRNA sequence data also clearly demonstrate that these “species” form a gene-
alogically tight group of microorganisms, with the number of base differences comparable to the numbers found at the intraspecific level for many other gram-positive species (for example, zero to six differences in Lactococcus lactis [3] and Streptococcus parasanguis [14] and zero to nine differences in Mycobacterium intracellulare [1]). It must be emphasized however that it is not possible to draw firm conclusions regarding species differentiation on the basis of rRNA sequence relatedness data alone (because of differing rates of sequence divergence). Several other distinct genospecies are known which have similarly small numbers of nucleotide differences (e.g., members of the Enterococcus avium group of species [15], Enterococcus casseliflavus and Enterococcus gallinarum [15], and Aerococcus viridans genospecies 1 and 2 [4]). Clearly a comprehensive reexamination of the levels of DNA-DNA relatedness among members of the B. cereus complex will be required to resolve the specific interrelationships of these organisms.

ACKNOWLEDGMENTS

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REFERENCES


