APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2000, p. 2627–2630 Vol. 66, No. 6
0099-2240/00/$04.00+0

Bacillus anthracis, *Bacillus cereus*, and *Bacillus thuringiensis*—One Species on the Basis of Genetic Evidence

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Received 28 December 1999/Accepted 19 March 2000

***Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* are members of the *Bacillus cereus* group of bacteria, demonstrating widely different phenotypes and pathological effects. *B. anthracis* causes the acute fatal disease anthrax and is a potential biological weapon due to its high toxicity. *B. thuringiensis* produces intracellular protein crystals toxic to a wide number of insect larvae and is the most commonly used biological pesticide worldwide. *B. cereus* is a probably ubiquitous soil bacterium and an opportunistic pathogen that is a common cause of food poisoning. In contrast to the differences in phenotypes, we show by multilocus enzyme electrophoresis and by sequence analysis of nine chromosomal genes that *B. anthracis* should be considered a lineage of *B. cereus*. This determination is not only a formal matter of taxonomy but may also have consequences with respect to virulence and the potential of horizontal gene transfer within the *B. cereus* group.**

The spore-forming bacterium *Bacillus anthracis* is the cause of the acute and often lethal disease anthrax. It is therefore of concern as a possible agent in biological warfare. Virulent forms of *B. anthracis* harbor two plasmids, pXO1 of 181 kb and pXO2 of 93.5 kb (22), which recently have been completely sequenced (14). A sequencing project aimed at determining the total genome of a plasmid-cured strain of *B. anthracis* is also under way. The closest relatives of *B. anthracis* are the two species *B. thuringiensis* and *B. cereus. B. thuringiensis* is a very useful source of insecticidal toxins, often in the form of spore-containing preparations of crystal protein toxins that are spread from airplanes over fields. *B. cereus* is a ubiquitous soil bacterium and an opportunistic human pathogen, causing contamination problems in the dairy industry and paper mills. The only established difference between *B. cereus* and *B. thuringiensis* strains is the presence of genes coding for the insecticidal toxins, usually present on plasmids. If these plasmids are lost, *B. thuringiensis* can no longer be distinguished from *B. cereus* (22).

Multilocus enzyme electrophoresis (MEE) comparing the allozyme patterns of 10 to 20 housekeeping genes has for decades been used extensively in phylogenetic investigations of bacterial populations (20). We have previously employed MEE analysis to establish the relationships between 36 strains of *B. cereus* and *B. thuringiensis*, mostly from reference strain collections, and shown that the strains appear to belong to the same species (4). Analysis of *B. cereus* and *B. thuringiensis* strains isolated from soil demonstrated a very high diversity in multilocus genotypes, indicating that *B. cereus* and *B. thuringiensis* exhibit a low degree of clonality and that exchange of genetic material occurs frequently in their natural environment (9).

We present here evidence for a close similarity of the genomes of *B. anthracis* strains to those of *B. thuringiensis* and *B. cereus* strains, demonstrating that they should be considered as belonging to one and the same species. What distinguishes them functionally are mostly genes carried on plasmids. In view of their natural competence, horizontal spreading of plasmids may take place and has in fact been demonstrated for *B. thuringiensis* and *B. cereus* (6, 7, 19, 23). What may seem to be a minor problem of taxonomy may therefore have serious implications for virulence and pathogenicity.

Protein extracts of the isolates were electrophoresed on starch-gel, and selective enzyme staining was performed as described by Selander and coworkers (20). The 13 enzymes were assayed as previously described (9).

Oligonucleotide primers were selected on the basis of previously determined gene sequences from *B. cereus* ATCC 10987 (15) using Primer3 (S. Rozen and H. J. Skaletsky [http://www.genome.wi.mit.edu/genome_software/other/primer3.html]) and synthesized at the DNA Synthesis Laboratory, Biotechnology Centre of Oslo, Oslo, Norway. PCR was run for 40 cycles in a 50-μl volume using 0.8 mM each deoxynucleoside triphosphate, 0.4 μM each primer, 50 ng of genomic DNA, and 1 U of Dynazyme (Finnzymes Oy, Espoo, Finland). The appropriate annealing temperature was determined for each primer set.

PCR products were purified using a QIAquick purification kit (Qiagen, Hilden, Germany), after Seakem GTG (FMC) agarose gel electrophoresis (1× Tris-acetate-EDTA or 1× Tris-borate-EDTA running buffer), when necessary. Sequencing reactions were performed on an ALF sequencer (Pharmacia, Uppsala, Sweden) using fluorescein isothiocyanate-end-labeled oligonucleotide primers corresponding to the primers used in PCR, employing a Thermo Sequenase Cycle Sequencing kit (Vistra Systems, Amersham, Buckinghamshire, United Kingdom). DNA sequences were analyzed and assembled using GeneSkipper software (European Molecular Biology Laboratory, Heidelberg, Germany).

Preliminary sequence data of *B. anthracis* were obtained from The Institute for Genomic Research website (http://www.tigr.org).

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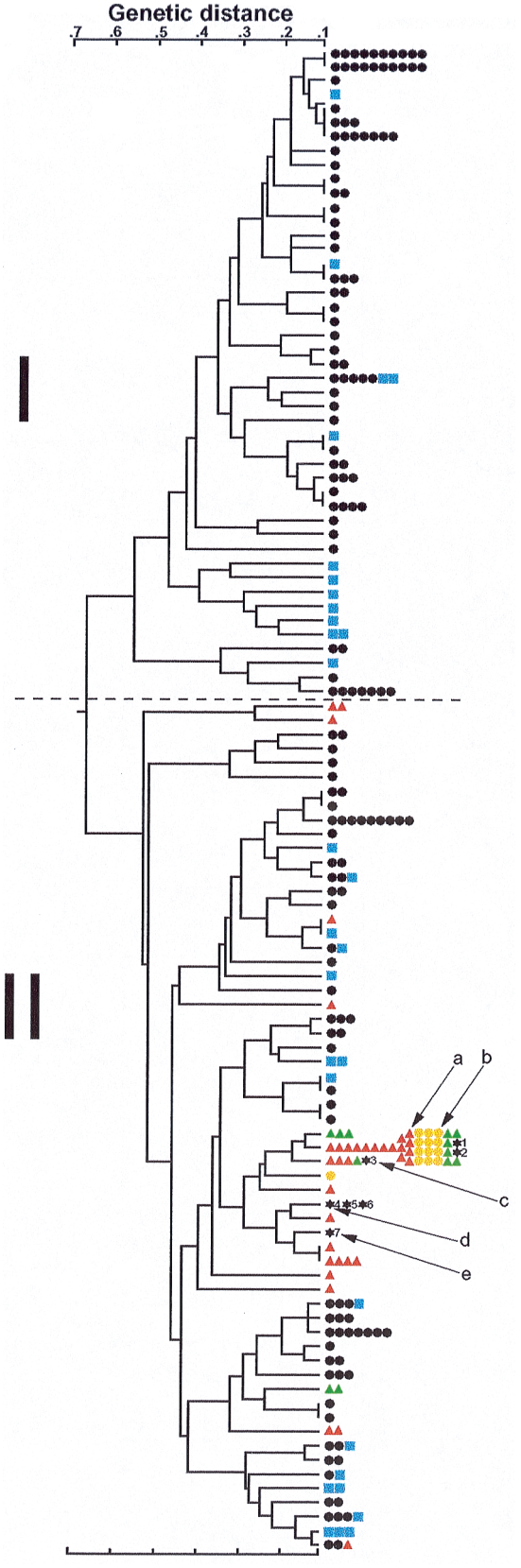

FIG. 1. MEE analysis. Genetic relationships between 239 strains of *B. cereus*, *B. thuringiensis*, and *B. anthracis*. The dendrogram was generated by the average-linkage method of clustering (unweighted-pair group matrix analysis) (19), from a matrix of genetic-distance coefficients based on 13 enzyme loci, using the Molecular Evolutionary Genetics Analysis package (12). The dendrogram generates two main clusters, I and II, with a genetic distance of 0.65. Isolates were placed on the same branch when the genetic distance was less than 0.1. Sources of the isolated strains are indicated with the following symbols: red triangles, patients (*B. cereus*); black circles, soil samples (*B. cereus* and *B. thuringiensis*); blue boxes, dairies (*B. cereus* and *B. thuringiensis*); yellow circles, *B. anthracis*; green triangles, Ba813-positive *B. cereus* strains isolated from *B. anthracis* outbreak areas; star 1, *B. cereus* ATCC 4342; star 2, *B. thuringiensis* subsp. *thuringiensis* (HD2); star 3, *B. cereus* ATCC 10987; star 4, *B. thuringiensis* subsp. *kurstaki* (HD1); star 5, *B. thuringiensis* subsp. *subtoxicus* (HD109); star 6, *B. thuringiensis* subsp. *entomocidus* (HD9); star 7, *B. cereus* ATCC 14579. Arrows indicate strains analyzed for the results shown in Fig. 2. Arrow a, *B. cereus* periodontitis strain; arrow b, *B. anthracis* 7700; arrow c, *B. cereus* ATCC 10987; arrow d, *B. thuringiensis* subsp. *kurstaki* (HD1); arrow e, *B. cereus* type strain ATCC 14579.

In the present study we have analyzed 13 *B. anthracis* strains using MEE by comparing the allozyme patterns of 13 enzyme loci to those of 227 *B. cereus* and *B. thuringiensis* strains. The multilocus genotypes of all but one of the *B. anthracis* strains were identical and were, except in one locus for which no enzymatic activity was detected, indistinguishable from the genotype of the clone of *B. cereus* most frequently isolated from patients (Fig. 1). The remaining *B. anthracis* strain (Davis TE 702) differed from the other strains by presenting distinct alleles at two enzyme loci and clustered at a genetic distance of 0.23. *B. thuringiensis* subsp. *thuringiensis* (HD2) from the Bacillus Genetic Stock Center, previously shown to be closely related to *B. cereus* strains (4), was also closely related to the *B. anthracis* cluster (Fig. 1). Ten *B. cereus*-like strains isolated from sites of anthrax outbreaks were positive for the chromosomal marker Ba813 (17) but lacked the two plasmids which are necessary for full virulence of *B. anthracis* (17). These strains exhibited multilocus genotypes located within or near the *B. anthracis* cluster (Fig. 1). Previous studies using other techniques to analyze the relationships between *B. anthracis* strains have all stated that *B. anthracis* is very homogenous and perhaps the most monomorphic species so far identified, with the relationship to *B. cereus* and *B. thuringiensis* being more remote (2, 3, 8, 12). Our results confirm the genetic homogeneity of *B. anthracis* but demonstrate that its apparent relatedness to *B. cereus* and *B. thuringiensis* is highly dependent on the choice of strains studied.

We have further analyzed DNA sequences from nine genes to investigate the genetic relationship between a more narrow selection of members of the *B. cereus* group. A collection of gene loci were amplified by PCR and analyzed by direct DNA sequencing. Dendrograms were subsequently constructed using cluster analysis, based on pairwise similarities of strains. The nine genes were selected from 86 previously sequenced genes from the *B. cereus* ATCC 10987 genome (15), and the genes were scattered on the chromosome (Fig. 2a). Four additional strains were selected for the analysis: *B. anthracis* 7700 (5), the *B. cereus* type strain ATCC 14579, *B. thuringiensis* subsp. *kurstaki*, which is widely used for the preparation of biopesticides, and a *B. cereus* strain isolated from a patient with periodontitis (10). Pairwise similarities between the PCR-amplified nucleotide sequences were used to construct distance matrices for phylogenetic analysis, based on percentages of divergence between the sequences. By separate examination of each gene locus, the DNA sequences were highly conserved among the five strains, exhibiting between 92.2 and 99.6% pairwise identity (Fig. 2b). The protein sequences were similarly conserved, with only 25 differences among a total of 1,128 amino acid positions in the nine deduced sequences and with

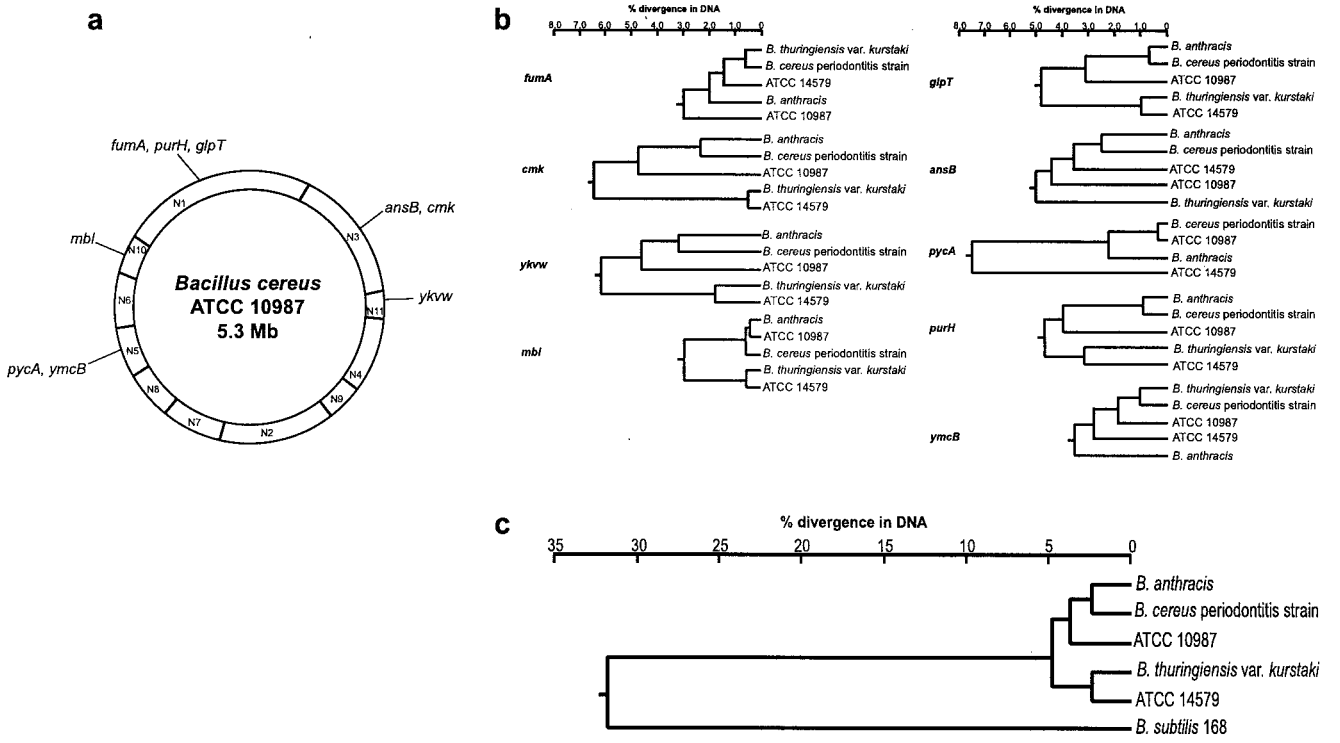

FIG. 2. Sequence analysis of genes. (a) Locations of genes used for the sequence analysis on a physical map (*Not*I restriction fragments) of the *B. cereus* ATCC 10987 chromosome (14). (b) Single-gene dendrograms based on DNA sequences from nine genes (sizes of sequences in base pairs are in parentheses): *fumA* (354), *cmk* (271), *ykvW* (415), *mbl* (568), *glpT* (309), *ansB* (414), *pycA* (437), *purH* (336), and *ymcB* (299) from *B. cereus* ATCC 10987, *B. anthracis* 7700, *B. cereus* periodontitis strain, *B. thuringiensis* subsp. *kurstaki* (HD1), and *B. cereus* type strain ATCC 14579. (c) Dendrogram based on DNA sequences from seven genes, *cmk*, *ymcB*, *ykvW*, *mbl*, *glpT*, *ansB*, and *purH*, including homologous gene sequences from *B. subtilis* 168 forming an outgroup in the analysis. Neither the *fumA* nor *pycA* gene was included in this dendrogram, since no *fumA* sequence homolog exists in *B. subtilis* 168 and *pycA* was not amplified from *B. thuringiensis* subsp. *kurstaki*. All dendrograms were constructed with the Molecular Evolutionary Genetics Analysis package (12) and show proportional divergence between strains by the unweighted-pair group matrix analysis (19).

14 of the substitutions being conservative (Table 1). The analysis further showed that evolutionary relationships estimated on the basis of the DNA sequence data correlated well with the results from the MEE analysis, with *B. anthracis* 7700 grouping together with the periodontal *B. cereus* isolate, and that the *B. cereus* type strain ATCC 14579 was most similar to *B. thuringiensis* subsp. *kurstaki* (Fig. 2b). *B. subtilis* 168, which exhibits an isoenzyme pattern too divergent from that of the *B. cereus* group to be included in the analysis of the MEE data (Fig. 1) (4), also formed the outgroup in the sequence analysis (Fig. 2c). Similarly, the 86 putative genes previously identified from *B. cereus* ATCC 10987 (15) were used to search the nonannotated DNA sequence set representing a triple coverage of the *B. anthracis* genome, available at The Institute for Genome Research website (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi).

Putative orthologs were detected for 69 of the genes, while 17 genes were either not present in the *B. anthracis* strain or missed due to physical or sequence gaps in the preliminary data set. The sequence identities between the *B. cereus* ATCC 10987 and *B. anthracis* orthologs were high, averaging 96.5% at the amino acid level. DNA sequences were equally similar.

The results presented in this study clearly reveal that *B. anthracis* appears to be genetically indistinguishable from members of the *B. cereus-B. thuringiensis* group. The results are in agreement with earlier results from DNA-DNA hybridization analysis showing high identity among *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains (11, 18). Furthermore, our results are in agreement with the view of *B. cereus* as the more ancestral species, with many of the strains belonging to the variants *B. anthracis* and *B. thuringiensis* encoding their most characteristic phenotypic properties from extrachromosomal DNA. Other characteristics that have been used to differentiate *B. anthracis* from *B. cereus* and that may be chromosomally encoded, such as sensitivity to β-lactam antibiotics and lack of motility and hemolytic activity, may be caused by differences in a single gene(s). For instance, 3 to 5% of *B. anthracis* strains are penicillin resistant (16), which dismisses this as a characteristic feature of the bacterium. Interestingly, PlcR, a transcriptional regulator of putative extracellular virulence factors in *B. cereus* and *B. thuringiensis*, is mutated and nonfunctional in *B. anthracis* strains (1). These mutations may thus be at least partly responsible for some of the features often associated with *B. anthracis*, like the lack of lecithinase and hemolytic activity.

We have demonstrated that *B. anthracis* is genetically very closely related to some *B. cereus* and *B. thuringiensis* strains usually regarded as rather harmless and even beneficial. Horizontal transfer of plasmids may dramatically alter their phenotypes. It is, however, possible that for receiving and retaining the virulence plasmids of *B. anthracis*, additional genetic features of the chromosome are needed. Such factors remain to be elucidated.

TABLE 1. Amino acid differences in nine genes from five strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis*[a]

Gene	Length (aa)	Position (aa)	aa difference in:					Conserved substitution
			B. cereus type strain ATCC 14579	*B. thuringiensis* subsp. *kurstaki* (HD1)	*B. cereus* ATCC 10987	*B. cereus* periodontitis strain	*B. anthracis* 7700	
ansB	137	3	E	E	**Q**	E	E	−
		132	I	V	I	I	I	+
cmk	89	22	N	N	K	K	K	−
		49	D	D	E	E	E	+
		76	K	K	E	E	E	−
		77	K	N	K	K	K	−
glpT	102	101	A	A	A	**V**	A	+
fumA	117	32	I	I	I	I	**V**	+
		44	E	**D**	E	E	E	+
mbl	189	14	S	S	T	T	T	+
purH	112	47	T	T	A	A	A	−
		54	M	I	I	M	M	+
		61	V	V	**I**	V	V	+
pycA	145	1	V		V	**I**	V	+
		57	**A**		S	S	S	−
		82	**E**		K	K	K	−
		85	**K**		E	E	E	−
		108	**E**		D	D	D	+
		124	**I**		M	M	M	+
ykvW	138	9	I	I	I	I	**V**	+
		27	G	G	D	D	E	−
		49	**D**	E	E	E	E	+
ymcB	99	38	E	E	E	E	**D**	+
		66	S	A	A	A	S	−
		72	P	P	P	P	**S**	−

[a] Boldface letters indicate that the amino acid (aa) substitution was observed in only one strain.

The work was supported by grants to A.-B.K. from The Norwegian Research Council and an EMBO short-term fellowship to E.H.

Preliminary sequence data of *B. anthracis* was obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *B. anthracis* was accomplished with support from the Office of Naval Research. We thank J. Vaissaire (AFSSA, Maisons-Alfort, France) for providing Ba813-positive *B. cereus* strains.

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