

# Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of *Bacillus anthracis*

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In a bioterrorism event, a tool is needed to rapidly differentiate *Bacillus anthracis* from other closely related spore-forming *Bacillus* species. During the recent outbreak of bioterrorism-associated anthrax, we sequenced the 16S rRNA genes from these species to evaluate the potential of 16S rRNA gene sequencing as a diagnostic tool. We found eight distinct 16S types among all 107 16S rRNA gene sequences that differed from each other at 1 to 8 positions (0.06% to 0.5%). All 86 *B. anthracis* had an identical 16S gene sequence, designated type 6; 16S type 10 was seen in all *B. thuringiensis* strains; six other 16S types were found among the 10 *B. cereus* strains. This report describes the first demonstration of an exclusive association of a distinct 16S sequence with *B. anthracis*. Consequently, we were able to rapidly identify suspected isolates and to detect the *B. anthracis* 16S rRNA gene directly from culture-negative clinical specimens from seven patients with laboratory-confirmed anthrax.

The gram-positive, rod-shaped, and spore-forming bacterium *Bacillus anthracis* is the cause of the acute and often lethal disease anthrax. Phenotypic characteristics commonly used to differentiate *B. anthracis* from closely related *B. cereus* and *B. thuringiensis*, such as susceptibility to  $\beta$ -lactam antibiotics, lack of motility, lack of hemolysis on sheep blood agar (SBA) plate, and susceptibility to  $\gamma$ -phage lysis, may vary among different *Bacillus* species strains, hampering their identification and differentiation. Phenotypically and genotypically *B. thuringiensis* can be differentiated from *B. cereus* by the presence of the CRY crystal protein and plasmid-encoded *cry* genes (1), but if this plasmid were lost, *B. thuringiensis* could no longer be distinguished from *B. cereus* (1). The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. The 16S rRNA gene sequences of *B. anthracis*, *B. cereus*, and *B. thuringiensis* have high levels of sequence similarity (>99%) that support their close relationships shown by DNA hybridization (2–7). A limited number of 16S rRNA sequences of *B. anthracis* (7 sequences), *B. cereus* (34 sequences), and *B. thuringiensis* (16 sequences) have been available at GenBank. Although those sequences are of different lengths and qualities, in complementary regions they differ from each other by no more than a few nucleotides. Therefore, this minimal level of diversity seen in the 16S rRNA of *B. anthracis*, *B. cereus*, and *B. thuringiensis* was thought to be an obstacle for using 16S rRNA gene sequencing to identify and differentiate these three species. The bioterrorism events of October 2001

prompted us to evaluate several new molecular approaches to rapidly identify *B. anthracis*. We determined the entire 16S rRNA sequences in a large number of representative strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* to evaluate the potential of 16S rRNA sequencing not only to rapidly identify *B. anthracis* in culture, but also to detect *B. anthracis* directly in clinical specimens.

## Materials and Methods

### Bacterial Strains

A total of 107 strains were included in this study. Of 86 *B. anthracis* isolates analyzed (Table 1), 18 were selected to represent a wide range of temporal (1937–1997), geographic (16 countries), and source diversity (soil, animals, or humans). Fourteen reference and standard strains, such as the Vollum, Ames, Pasteur, New Hampshire, V770, and Sterne strains, were also included. The remaining 54 strains were isolated from October to December 2001 during the bioterrorism-associated anthrax outbreak in the United States. Ten *B. cereus* and 11 *B. thuringiensis* strains were also analyzed by 16S rRNA sequencing. All strains were identified by standard microbiologic procedures and according to the Laboratory Response Network diagnostic criteria (9,10).

### Clinical Specimens

We analyzed 198 clinical specimens (76 blood, 30 tissue, 16 pleural fluid, 37 serum, 6 cerebrospinal fluid, and 33 other specimens). Sixty-nine specimens were obtained from patients with laboratory-confirmed anthrax (55 specimens from 11 inhalational cases and 14 from 7 cutaneous cases). DNA was extracted from fluid (200  $\mu$ L) or small tissue specimens (<5 mm<sup>3</sup>) according to manufacturer's instructions with a Qiagen DNA Mini Kit (Qiagen, Valencia, CA). All 198 DNA samples

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BIOTERRORISM-RELATED ANTHRAX

Table 1. Descriptions and GenBank accession numbers of 107 *Bacillus* species strains analyzed in this study

Species	No.	Identification	GenBank 16S rRNA gene accession number	Geographic and/or temporal origin <sup>a</sup>	16S rRNA type	MLVA genotype <sup>b</sup>		
<i>B. anthracis</i>	Diversity collection	2000031650	AY138379	Human, Turkey, 1991	6	23		
		2000031651	AY138372	Bovine, France, 1997	6	80		
		2000031652	AY138374	Human, US, 1952	6	68		
		2000031653	AY138373	Wool, Pakistan, 1976	6	69		
		2000031655	AY138376	Cow, China	6	57		
		2000031656	AY138375	Ames	6	62		
		2000031657	AY138382	Bovine	6	10		
		2000031659	AY138377	Human, Turkey, 1984	6	28		
		2000031660	AY138378	Bovine, US, 1937	6	25		
		2000031661	AY138369	Human, South Korea, 1994	6	34		
		2000031662	AY139368	Zebra, Namibia	6	35		
		2000031663	AY138381	Bovine, Poland	6	15		
		2000031664	AY138383	Porcine, German, 1971	6	38		
		2000031665	AY138366	Bovine, Argentina	6	45		
		2000031666	AY138371	UK	6	77		
		2000031667	AY138380	Sheep, Italy, 1994	6	20		
		2000031670	AY138367	Human, Turkey, 1985	6	41		
		2000031671	AY138370	Bovine, Zambia	6	30		
		Standard and reference strains	14	Ames	AY138358	Ames	6	62
				2002007651	AY138355	Sterne, Chile	6	ND
2002007650	AY138356			Sterne, Chile	6	ND		
2002007649	AY138357			Pasteur, Chile	6	ND		
2000031887B	AY138347			Vaccine	6	ND		
2000031666	AY138352			Vollum	6	77		
2000031368	AY138350			Vollum	6	77		
2000031244	AY138354			Vollum	6	*		
2000031078	AY138351			Vollum M36	6	ND		
2000031076	AY138353			Vollum	6	*		
2000031259	AY138346			Pasteur	6	**		
2000031075	AY138345			Sterne	6	*		
2000031887	AY138348			V770-NP1-R	6	*		
2000031136	AY138349	New Hampshire	6	73				
Outbreak strains	54		AY138291 to AY138344	US Oct/Dec 2001	6	62		
<i>B. cereus</i>	10	2000031486	AY138272	Human, US, 1994	12	NA		
		2000031491	AY138276	Human, US, 1997	7	NA		
		2000031498	AY138274	Human, US, 1979	9	NA		
		2000031503	AY138277	Human, US, 1999	7	NA		
		2000031513	AY138279	Human, US, 1986	13	NA		
		G3317	AY138278	Human, Israel, 1989	7	NA		
		G8639	AY138271	Milk, Bolivia, 1993	3	NA		
		G9667	AY138273	Human, US, 1995	12	NA		
		H1439	AY138270	Human, US, 2000	2	NA		
		ATCC 14579	AY138275	1887	9	NA		
<i>B. thuringiensis</i>	11	2000031482	AY138290	Human, US, 1989	10	NA		
		2000031485	AY138289	Spray, US, 1993	10	NA		
		2000031494	AY138288	Human, US, 1985	10	NA		
		2000031496	AY138287	Human, US, 1981	10	NA		

Table 1 continued. Descriptions and GenBank accession numbers of 107 *Bacillus* species strains analyzed in this study

Species	No.	Identification	GenBank 16S rRNA gene accession number	Geographic or temporal origin <sup>a</sup>	16S rRNA type	MLVA genotype
		2000031508	AY138286	Human, US, 1985	10	NA
		2000031509	AY138285	Human, US, 1985	10	NA
		2002007400	AY138283	Powder, US, 2001	10	NA
		2002017401	AY138284	Powder, US, 2001	10	NA
		2000032755	AY138282	Environment, US, 2000	10	NA
		2000032757	AY138280	Environment, US, 2000	10	NA
		2000032756	AY138281	Human, US, 1981	10	NA

<sup>a</sup> Date and source of isolation are provided when available; \*, lacking pXO2; \*\*, lacking pXO1.

<sup>b</sup>ND, MLVA (8).

NA, not applicable.

were analyzed for 16S rRNA gene amplification and products sequenced.

### Polymerase Chain Reaction (PCR)

A 1,686-bp fragment of DNA, including the 1,554-bp 16S rRNA gene, was amplified from all 107 *Bacillus* species strains by using primers 67F and 1671R (Table 2). For clinical samples, we used the initial DNA amplicon as a template in a nested PCR with a second set of internal primers, 23F and 136R (Table 1). Both sets of primers were designed from the *B. anthracis* genome sequence (<http://www.tigr.org>). The full-length size of *B. anthracis* 16S rRNA gene (1,554 bp) was determined from an alignment of the 16S rRNA genes from *Escherichia coli*, *Neisseria gonorrhoeae* (GenBank accession nos. J01859 and X07714, respectively), and the 16S rRNA gene regions of the *B. anthracis* genome sequence (<http://www.tigr.org>). Whole cell suspensions or DNA extracts were used for PCR of isolates or clinical samples, respectively. For whole cell suspensions, a single colony from an SBA plate was resuspended in 200  $\mu$ L of 10 mM Tris, pH 8.0. The suspension was put in a Millipore 0.22- $\mu$ m filter unit (Millipore, Bedford, MA), heated at 95°C for 20 min, centrifuged at 8,000 rpm for 2 min, and then used for PCR. Each final PCR reaction (100  $\mu$ L) contained 5 U of Expand DNA polymerase (Roche, Mannheim, Germany); 2  $\mu$ L of whole cell suspension or DNA; 10 mM Tris-HCl (pH 8.0); 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; 200  $\mu$ M (each) dATP, dCTP, dGTP, and dTTP; and 0.4  $\mu$ M of each primer. Reactions were first incubated for 5 min at 95°C. Then 35 cycles were performed as follows: 15 s at 94°C, 15 s at the annealing temperature of 52°C, and 1 min 30 s at 72°C. Reactions were then incubated at 72°C for another 5 min. The annealing temperature for the nested PCR was 50°C. PCR products were purified with Qiaquick PCR purification kit (Qiagen).

### 16S rRNA Sequence Determination

The amplified products of approximately 1,686 bp (1,656 bp for nested PCR) were sequenced by using a modification of 16 primers as described (Table 2) (11). Sequencing was performed by using a Big Dye terminator cycle sequencing kit (Applied BioSystems, Foster City, CA). Sequencing products

were purified by using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ) and were resolved on an Applied BioSystems model 3100 automated DNA sequencing system (Applied BioSystems). The length of sequences obtained differed for each primer but were sufficient to provide 5- to 8-fold sequence coverage. An inner fragment of 1,554 bp was

Table 2. Primers used for amplification and sequencing of the 16S rRNA gene of *Bacillus anthracis*, *B. thuringiensis*, and *B. cereus*<sup>a</sup>

Generic primers used for 16S rRNA amplification	
8F	5' AGT TGA TCC TGG CTC AG 3'
1492R	5' ACC TTG TTA CGA CTT 3'
Primers for amplification of the 16S rRNA gene	
67F	5' TGA AAA CTG AAC GAA ACA AAC 3'
1671R	5' CTC TCA AAA CTG AAC AAA ACG AAA 3'
Inner primers used for nested PCR on clinical samples	
23F	5' ACA AAC AAC GTG AAA CGT CAA 3'
136R	5' AAA CGA AAC ACG GAA ACT T 3'
Primers used for sequencing of the 16S rRNA gene	
104F	5' GGA CGG GTG AGT AAC ACG TG 3'
104R	5' CAC GTG TTA CTC ACC CGT CC 3'
1230F	5' TAC ACA CGT GCT ACA ATG 3'
1390F	5' GGG CCT TGT ACA CAC CG 3'
1390R	5' CGG TGT GTA CAA GGC CC 3'
8F	5' AGT TGA TCC TGG CTC AG 3'
357F	5' TAC GGG AGG CAG CAG 3'
357R	5' CTG CTG CCT CCC GTA 3'
530F	5' CAG CAG CCG CGG TAA TAC 3'
530R	5' GTA TTA CCG CGG CTG CTG 3'
790F	5' ATT AGA TAC CCT GGT AG 3'
790R	5' CTA CCA GGG TAT CTA AT 3'
981F	5' CCC GCA ACG AGC GCA ACC C 3'
981R	5' GGG TTG CGC TCG TTG CGG G 3'

<sup>a</sup>Primers 67F and 1671R or primers 23F and 136R were also used for 16S sequence on isolates or clinical samples, respectively.

obtained and analyzed by using the GCG (Wisconsin) Package, v. 10.1, (Genetics Computer Group, Madison, WI). A number was assigned for each allele of 16S rRNA gene sequence in order of elucidation; a single base change or a mixed base (more than one nucleotide determined at a single position) was considered a new 16S type. When a novel 16S type, mixed base pairs, or any discrepancies in the alignment were obtained, the 16S rRNA gene amplification and sequencing of the entire gene or parts containing the problematic region were repeated.

#### GenBank 16S rRNA Gene Sequences and Accession Numbers

Sixty 16S rRNA gene sequences of *B. anthracis*, *B. cereus*, and *B. thuringiensis* were available in GenBank. Thirty-nine of these sequences were incomplete, contained a large number of undetermined nucleotides, or were not associated with a specific strain identification, and therefore were not used in this study. The remaining 21 sequences were identified as eight *B. anthracis* (AF155950 [Ames]), (AF155951 [Delta Ames]), (AF176321 [Sterne]), (AF290552 [Sterne]), (AF290553 [Vollum]), (AF155950 [Ames]), (AF155951 [Delta Ames]), and (AF176321 [Sterne]); eight *B. cereus* (AF155952, AF155958, AF176322, AF290546, AF290547, AF290548, AF290550, and AF290555); three *B. thuringiensis* (AF155954, AF155955, and AF290549); and two *B. mycooides* (AF155956 and AF155957). A total of 114 16S rRNA gene sequences were determined in this study (107 from isolates [GenBank accession nos. in Table 1] and 7 from clinical specimens [GenBank accession nos. AY138359 to AY138365]).

## Results

### 16S rRNA Gene Sequence Diversity

The 1,554-bp nucleotide sequences of the entire 16S rRNA gene from all 107 *Bacillus* species strains were aligned and compared. Differences were found at eight single nucleotide positions (positions 1, 2, 3, 4, 6, 9, 12, and 13), and no gaps were present. When 21 *Bacillus* 16S rRNA sequences from GenBank were added to the alignment, five additional positions with differences (positions 5, 7, 8, 10, and 11) were located (Table 3). The 13 positions of differences were distributed throughout the gene (Table 3). In six of these positions (positions 1, 2, 3, 4, 6, and 12), more than one nucleotide was detected (mixed nucleotides) (Table 3). These results indicated that the strain contained multiple rRNA operons with slightly different 16S rRNA gene sequences.

We found eight different 16S types among the 107 16S rRNA genes from our collection of isolates (Table 3). All 86 *B. anthracis* had an identical sequence, 16S type 6, containing a single mixed base, a W(A/T) at position 12, not found in the other two species. 16S type 10 was seen in all 11 *B. thuringiensis* strains, and a single mixed base pair was identified in all strains at position 6. Six other 16S types were found among the 10 *B. cereus* strains. Three additional 16S types were found

among the 18 GenBank sequences that we analyzed. 16S types 1, 4, and 5 correlated to *B. mycooides*, *B. thuringiensis*, and *B. cereus*, respectively (Table 3). Five *B. anthracis* sequences from GenBank were identical to the 16S type 6 found in all our 86 *B. anthracis* isolates, and three were identical to the 16S type 7 found in *B. cereus*.

### 16S rRNA Sequencing Directly in Clinical Specimens

We detected 16S rRNA genes in 7 (3.5%) of 198 clinical samples: all were 16S type 6 characteristic for *B. anthracis*. None of the seven specimens were culture positive (Table 4), although all specimens had been collected from patients with laboratory-confirmed anthrax.

## Discussion

The goal of this study was to evaluate the potential of 16S rRNA sequencing to rapidly identify *B. anthracis* in cultures. We found that 16S rRNA genes of *B. anthracis* were highly conserved; only one 16S type (16S type 6) was identified in all 86 strains tested. However, not all *B. anthracis* 16S rRNA genes sequences in GenBank are type 6. Three of the eight *B. anthracis* 16S rRNA sequences are reported as type 7, a type that, in our study, we found exclusively among the *B. cereus* strains. The only difference between type 7 and type 6 is a mixed base pair at position 1146. The strain designations of two of these three 16S type 7 *B. anthracis* strains in GenBank are Ames and Sterne. We did not acquire these particular strains from the submitting laboratory, but the one Ames and two Sterne strains (obtained from different sources) in our collection were consistently type 6. A third Sterne strain 16S rRNA sequence in GenBank is also type 6.

One possible explanation for these different 16S rRNA sequencing results may be the use of different sequencing approaches, such as using cloned DNA versus genomic DNA as template. In sequencing clones, one allele may be missed if only a few clones are sequenced, not representing the total diversity. In this case, the position with the mixed base would not be detected. If both types 6 and 7 exist in *B. anthracis*, the difference may be due to recombination, mutation, or loss of an allele. The type 7 *B. anthracis* sequences in GenBank are unpublished; therefore, we do not know if the genes were cloned and, if so, how many clones were sequenced.

The complete *B. anthracis* genome was posted at <http://www.tigr.org/tigr-scripts/ufmg/ReleaseDate.pl> on May 7, 2002. The genome has 11 rRNA operons. There are 10 positions in the 16S rRNA gene where the nucleotides are not identical among the 11 rRNA operons, but the DNA sequencing software scores only one of them as a mixed base 100% of the time. This position is 1146, where five 16S rRNA genes contain Ts and six have As in a 54%:46% ratio. In this case, the base-calling software (GCG; Genetics Computer Group) always assigns a W at that position. At position 1137, there are seven Gs and four As, a 64%:36% ratio, but the position is scored as a G, the predominant base. In eight positions, a 9%:91% ratio is present. For example, at position 1047 are one

Table 3. 16S rRNA gene types identified among 125 *Bacillus* spp. strains analyzed in this study (n=107) and available at GenBank (n=18)

16S type	<i>Bacillus</i> species	No. of strains	Positions <sup>a</sup>												
			1 (77)	2 (90)	3 (92)	4 (182)	5 (189)	6 (192)	7 (200)	8 (208)	9 (1,015)	10 (1,036)	11 (1,045)	12 (1,146)	13 (1,462)
16S types identified in 107 strains in this study															
2	<i>cereus</i>	1	R <sup>b</sup>	Y	W	C <sup>c</sup>	A	T	T	G	A	T	A	A	A
3	<i>cereus</i>	1	G	C	A	Y	A	T	T	G	A	T	A	A	A
6	<i>anthracis</i>	86	A	T	T	C	A	C	T	G	C	T	A	W	T
7	<i>cereus</i>	3	A	T	T	C	A	C	T	G	C	T	A	A	T
9	<i>cereus</i>	2	A	T	T	C	A	C	T	G	A	T	A	A	T
10	<i>thuringiensis</i>	11	A	T	T	C	A	Y	T	G	A	T	A	A	T
12	<i>cereus</i>	2	A	T	T	Y	A	T	T	G	A	T	A	A	T
13	<i>cereus</i>	1	A	T	T	C	A	C	T	G	C	T	A	T	T
16S types identified in strains available at GenBank <sup>d</sup>															
1	<i>mycoides</i>	2	A	T	T	C	C	C	G	C	C	C	G	A	- <sup>e</sup>
4	<i>thuringiensis</i>	3	G	C	A	C	A	T	T	G	A	T	A	A	-
5	<i>cereus</i>	8	G	C	A	C	A	C	T	G	A	T	A	A	-
7	<i>anthracis</i>	3	A	T	T	C	A	C	T	G	C	T	A	A	T

<sup>a</sup>Numbers refer to the number of positions where mismatches are found. Numbers in parentheses refer to positions in the 16S rRNA gene.

<sup>b</sup>R refers to a purine (A or G) at that position; Y refers to a pyrimidine (C or T) at that position; and W refers to an A or T at that position.

<sup>c</sup>A, C, G, and T refer to the four deoxynucleotides that DNA comprises.

<sup>d</sup>Five additional positions of differences (positions 5, 7, 8, 10, and 11) were found when GenBank sequences were used.

<sup>e</sup>The last position (position 13) on 16S types 1, 4, and 5 is missing because those GenBank sequences are shorter.

T and 10 Cs. In these cases, the nucleotide is called as the predominant base by the base-calling software.

The quality of DNA sequences generated in laboratories has been greatly improved by the introduction of automated sequencing systems and DNA alignment software, but other factors, such as the purity of the DNA template and number of overlapping nucleotide fragments in the alignment, contribute to the reliability of the final sequence. Mixed base pairs are clearly the result of sequence differences between different rRNA operons and not due to any sequencing artifacts. In this study, the length of the fragment sequences varied for each primer, but they were of sufficient length to provide 5- to 8-fold sequence coverage in both directions. This 5–8 sequence overlap simplifies identifying and clarifying positions with double signals, increasing the confidence in our final consensus sequence. The occurrence of mixed base pairs in rRNA sequences is well known and accepted (15–19). The Ribosomal Database Project Web site shows that operon heterogeneity has been documented in several different bacterial species ([http://rrndb.cme.msu.edu/rrndb/rrn\\_table.pdf](http://rrndb.cme.msu.edu/rrndb/rrn_table.pdf)). In addition, we did not observe mixed base pairs in single-copy genes such as *pagA* and a variety of others. A previous study of a small set of *Bacillus* strains isolated from soil demonstrated the diversity of 16S rRNA genes of both *B. cereus* and *B. thuringiensis* (15). Our results confirm the diversity among *B. cereus* strains, although we did not find diversity among *B. thuringiensis* strains. The lack of diversity in our collection of *B. thuringiensis* strains may be associated with natural selection with

human host; 6 of 11 of our *B. thuringiensis* strains were isolated from humans.

#### Direct Amplification of 16S rRNA from Clinical Samples

Even though *B. anthracis* is present at high levels (up to 10<sup>8</sup>/mL) in the blood of patients with anthrax and will readily grow on standard bacteriologic media, as for other bacteria, specimens collected after the administration of antimicrobial therapy may fail to grow *B. anthracis*. Laboratory confirmation for the two patients with inhalational anthrax whose specimens were analyzed (patient #10i [12], and patient #11i [13]) was achieved by isolation and identification of *B. anthracis* from clinical samples at the medical facility where the patients were treated. Generally, for all patients, isolates themselves were forwarded to the appropriate public health laboratory and then to the Centers for Disease Control and Prevention for confirmatory identification and molecular subtyping, but the initial clinical specimens were not sent along with the isolates. With few exceptions, clinical specimens available for analysis from these two patients and from other patients with inhalational anthrax were collected after initiation of antimicrobial therapy, resulting in few culture-positive results. For 3 of the 11 inhalational patients, laboratory confirmation was based on two of three available supportive tests, including PCR targeting two plasmid and one chromosomal target (14), immunohistochemistry or a reactive anti-protective antigen titer (immunoglobulin G ELISA) (12,20). Laboratory confirmation for the two cutaneous cases with skin biopsies analyzed in this

Table 4. Results of laboratory testing on seven clinical samples in which 16S type 6 was identified

ID <sup>a</sup>	Patient		Clinical specimens		
	Diagnosis	Laboratory confirmation <sup>b</sup>	Type	Culture	<i>Bacillus anthracis</i> -specific PCR <sup>b</sup>
2i	Inhalational anthrax	IHC + PCR of pleural fluid; serology	Tissue	Neg	Neg
10i	Inhalational anthrax	<i>B. anthracis</i> isolated from blood and pleural fluid	Pleural fluid	Neg	Pos
			Pleural fluid	Neg	Pos
			Blood	Neg	ND
			Lymph node	Neg	Pos
11i	Inhalational anthrax	<i>B. anthracis</i> isolated from blood	Lymph node	Neg	Pos
7c	Cutaneous anthrax	IHC + PCR on skin biopsy	Skin from forehead	Neg	Pos

<sup>a</sup>Patient identification numbers are described in references 12 and 13; I, inhalational case; C, cutaneous case; PCR, polymerase chain reaction.

<sup>b</sup>The immunohistochemical (IHC), serologic, and PCR results are described in reference 14.

study was indeed achieved by these supportive laboratory tests: one case was confirmed by immunohistochemistry and a reactive anti-protective antigen titer (IgG ELISA). For the other case all three supportive laboratory tests were positive.

Previously, strains having <3% difference between their 16S rRNA genes were considered the same species (21). However, differences between 16S rRNA genes for some *Bacillus* species, such as *B. anthracis*, *B. cereus*, and *B. thuringiensis*, are <1% (1). Such small differences (e.g., one base between sequences or partial matches at a single nucleotide position in the 16S rRNA gene) have not been used for species differentiation. Our study clearly demonstrates that such small differences might be important for species identification. DNA-DNA hybridization and 16S rRNA sequencing studies have shown that these three *Bacillus* species are closely related and probably represent a single species (3,6,7). If the three were classified as a single species, 16S rRNA sequencing appears to have the potential to differentiate strains at the subspecies level.

Although pXO1 and pXO2 plasmids must be detected to confirm the virulence of *B. anthracis*, 16S rRNA sequencing has a powerful capacity to rapidly identify *B. anthracis* and other species. Although further studies are needed to fully evaluate 16S sequencing as a diagnostic assay, its value as a tool for rapid initial screening in outbreak investigations has been demonstrated.

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# EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

## Instructions for Authors

### Types of Articles

**Perspectives.** Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Synopses.** Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Research Studies.** Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

**Policy and Historical Reviews.** Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

**Dispatches.** Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

**Commentary.** Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

**Another Dimension.** Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

**Letters.** This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

**Book Reviews.** Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

**News and Notes.** We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.) In this section, we also include summaries (500–1,000 words) of emerging infectious disease conferences. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.