Bacillus anthracis Virulent Plasmid pX02 Genes Found in Large Plasmids of Two Other *Bacillus* Species

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In order to cause the disease anthrax, *Bacillus anthracis* requires two plasmids, pX01 and pX02, which carry toxin and capsule genes, respectively, that are used as genetic targets in the laboratory detection of the bacterium. Clinical, forensic, and environmental samples that test positive by PCR protocols established by the Centers for Disease Control and Prevention for *B. anthracis* are considered to be potentially *B. anthracis* until confirmed by culture and a secondary battery of tests. We report the presence of 10 genes (*acpA*, *capA*, *capB*, *capC*, *capR*, *capD*, IS*1627*, ORF 48, ORF 61, and *repA*) and the sequence for the capsule promoter normally found on pX02 in *Bacillus circulans* and a *Bacillus* species closely related to *Bacillus luciferensis*. Tests revealed these sequences to be present on a large plasmid in each isolate. The 11 sequences consistently matched to *B. anthracis* plasmid pX02, GenBank accession numbers AF188935.1, AE011191.1, and AE017335.3. The percent nucleotide identities for *capD* and the capsule promoter were 99.9% and 99.7%, respectively, and for the remaining nine genes, the nucleotide identity was 100% for both isolates. The presence of these genes, which are usually associated with the pX02 plasmid, in two soil *Bacillus* species unrelated to *B. anthracis* alerts us to the necessity of identifying additional sequences that will signal the presence of *B. anthracis* in clinical, forensic, and environmental samples.

Long considered a biowarfare agent, *Bacillus anthracis* was used in 2001 in an act of bioterrorism. After bacterial endospores were placed into envelopes and delivered by the United States postal system to unsuspecting victims, 22 people were infected of whom 11 developed inhalational anthrax and 5 died (14, 15). As a result, first responders delivered hundreds of thousands of environmental specimens to laboratories across the nation that are part of the National Laboratory Response Network (LRN), a coordinated system of sentinel, reference, and national laboratories established by the Centers for Disease Control and Prevention (CDC) (6, 7). Of the three Florida Department of Health (FDOH) laboratories with biosafety level 3 facilities designated to receive these specimens, the FDOH Tampa Laboratory received and analyzed 1,046 environmental samples from first responders across west-central Florida over the last 3 months of 2001. Among these specimens, 19 loose powders or swab samples of powders that were brought in by local law enforcement officers and considered plausible threats initially tested positive for potentially carrying a *B. anthracis* isolate.

Because of the initial positive results, the 19 powders and swabs were cultured and all isolates were tested further using the LRN tests, consisting of nonspecific phenotypic physical/biochemical tests and specific molecular methods for *B. anthracis* such as gamma phage susceptibility testing, cell wall and capsule detection by direct fluorescence antibody tests (DFA), and amplification of targeted DNA sequences by PCR (31). After more tests demonstrated that none of these were *B. anthracis*, the 19 isolates were given to the Center for Biological Defense for further examination. By in-house real-time PCR assays, 14 of the 19 isolates and respective powders tested positive only for the Ba813 chromosomal element, which is considered to be unique to *B. anthracis* (19, 22, 23). These were not studied further. Yet, five environmental isolates of the *Bacillus* genus tested positive for Ba813 and pX02 by PCR and/or showed a positive reaction for the capsule by DFA. The isolates were later identified as three *B. cereus* isolates, one *B. circulans* isolate, and one *Bacillus* isolate not determined to species level.

The DNA targets used for the LRN PCR assay for *B. anthracis* are sequences that are not specified to LRN personnel and others. Yet it is known that one DNA target sequence is located on the chromosome and the other two targets on the two virulence plasmids, pX01 and pX02 (31). The "toxin" plasmid pX01 contains the *lef*, *pag*, and *cya* genes encoding lethal factor, protective antigen, and edema factor, respectively, and is the larger (184 kb) and purportedly more numerous (24 to 243 copies) of the two plasmids (8). The second plasmid, pX02, is smaller (97 kb) and lower in copy number (1 to 32) and contains a total of 85 open reading frames (ORFs) including the genes that comprise the capsule operon (8). The pX02 plasmid has been reported in the highly related species of *B. cereus* and *B. thuringiensis*, but it has never been reported in *Bacillus* species outside of the *B. cereus* group (23). Although some of the pX02 plasmid genes have also been re-

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TABLE 1. Results of initial tests of two non-*B. anthracis* *Bacillus* species and two control *B. anthracis* strains[a]

Strain	Species	Result[b] by:						γ phage susceptibility	Hemolysis	Motility
		PCR				DFA				
		pX01	pX02	Chromosome	Ba813	Capsule	Cell wall			
63	*B. anthracis*	−	+	+	+	+	+	+	NH[c]	−
118	*B. circulans*	−	+	+	+	+[d]	−	−	NH	−[e]
119	*Bacillus* sp.[f]	−	+	+	+	−	−	−	NH	−
131	*B. anthracis*	−	+	+	+	+	+	+	NH	−

[a] PCR assays, DFA, and gamma phage testing were performed by FDOH following LRN protocols (31), while Ba813 testing was performed by following a method previously published (17). All of the LRN tests were repeated by the Center for Biological Defense immediately after receiving these isolates from FDOH personnel.
[b] −, negative result; +, positive result.
[c] NH, nonhemolytic on media with 5% sheep blood cells.
[d] CBD 118 was positive for capsule DFA at initial testing; subsequent testing after cryostocking resulted in negative DFA tests. CBD protocols allow minimum subculturing (one to four subcultures) per cryostock vial.
[e] CBD 118 appeared to be nonmotile at first examination, but later testing revealed that the cells were motile.
[f] CBD 119 was identified as closely resembling *B. luciferensis*.

ported in the *B. cereus* and *B. thuringiensis* chromosomes, none of the capsule genes have been reported in other *Bacillus* species (3, 5, 10, 21).

Because our initial tests suggested that the two isolates carried one capsule gene, we hypothesized that the full capsule operon of *B. anthracis* was present on either the chromosome or plasmid. We attempted to identify the capsule operon genes and other ORFs in the two isolates and to determine whether the genes were located on a plasmid or the chromosome.

(These data were presented as abstract Q-195 at the 105th General Meeting of the American Society for Microbiology, Atlanta, Ga., 5 to 9 June 2005.)

MATERIALS AND METHODS

Bacteria culture and phenotypic characterization. Two unidentified *Bacillus* isolates (CBD 118 and CBD 119) were isolated from two different environmental samples that were received by the FDOH, Bureau of Laboratories, on two different days from local law enforcement personnel. The sample harboring CBD 118 was a "dirty swab" laden with a brown-gray powder, and the other sample containing CBD 119 was a white powder within a small container. These isolates and powders were then given to the Center for Biological Defense because, although the isolates proved not to be *B. anthracis*, they had given positive results for the pX02 plasmid and chromosomal element by PCR and/or the DFA used in the LRN protocols for ruling out *B. anthracis* (31) (Table 1). *Bacillus* control strains used were two *B. anthracis* Pasteur strains, CBD 63 (derived from CDC BC 3132) and CBD 131, *B. cereus* CBD 55 (derived from ATCC 14579), *B. cereus* CBD 58 (derived from CDC BC 3133), *B. circulans* CBD 389 (derived from ATCC 4513; American Type Culture Collection, Manassas, VA), *B. luciferensis* CBD 980 (derived from JCM 12212; Japanese Collection of Microorganisms, Saitama, Japan), *B. megaterium* CBD 395 (derived from ATCC 14581), and *B. thuringiensis* CBD 61 (derived from ATCC 35646).

Isolates were grown on Trypticase soy agar (TSA) supplemented with 5% sheep red blood cells (Remel, Lenexa, KS) overnight and were evaluated for Gram's stain reaction, cellular and colony morphology, and hemolysis pattern (11, 17, 18, 26, 31). Motility was determined by examination of overnight growth in Trypticase soy broth (TSB) (Becton Dickinson and Company, Sparks, MD [BD]) using phase-contrast microscopy of log-phase cells as described previously (20). Sporulation was induced on nutrient agar (BD) supplemented with 5 mg/liter $MnSO_4$ and incubated for 48 to 72 h. Endospore shape and location, swelling of the vegetative cell, and the presence of parasporal bodies were observed in wet mounts by phase-contrast microscopy. Lecithinase production, mannitol fermentation, and tolerance of polymyxin B were tested on mannitol-egg yolk-polymyxin B agar, as previously described (20). Acid production from 49 carbohydrates or carbohydrate derivatives after 48 h of incubation at 30°C was tested using the API 50 CH panel with API CHB/E medium in combination with 11 biochemical tests from the API 20E kit, including tests for β-galactosidase activity, citrate utilization, and urea and gelatin hydrolysis, the Voges-Proskauer test for acetoin production, and nitrate reduction testing, by following the man-

ufacturer's instructions (bioMerieux, Inc., Hazelwood, MO). Profiles were analyzed and identifications obtained using the APILAB Plus identification program, V.3.3.3/3.0 (bioMerieux).

To determine whether there was capsule production by the two *Bacillus* isolates, they were grown on Biolog universal growth agar (BUG) and TSA with and without 0.8% sodium bicarbonate in 5% CO_2 at 35°C for 15 h by following the LRN protocol (31). In addition, capsule formation was examined after growth in an approximately 20% CO_2 atmosphere as previously described (12). Isolated colonies were suspended in 60 μl sterile water and adjusted to a 2 McFarland standard optical density. One drop of India ink (Becton Dickinson) was added to the bacterial suspension and mixed. Three microliters of this bacterium-ink mixture was placed on a microscope slide and observed at 1,000× with phase contrast. Control strains were *B. anthracis* Pasteur CBD 63 (positive) and *B. cereus* CBD 58 (negative). All DFA tests were repeated by FDOH personnel following the LRN protocols and using the same DFA reagents and control strains.

16S rRNA gene sequencing. DNA was extracted from cells grown overnight in TSB using the MasterPure gram-positive DNA purification kit (Epicenter, Madison, WI). PCR for the 16S rRNA gene amplicon used primers TU102 (5′-AGA GTT TGA TCA/C TGG-3′) and TU103 (5′-TAC CTT GTT ACG ACT-3′), comparable to primers 27F and 1492R, respectively, used by Herrick et al. (13). The PCR parameters were as follows: initial heating at 94°C for 4 min followed by 30 cycles of 1 min at 94°C, 2 min at 37°C, and 2 min at 72°C and a final extension at 72°C for 6 min. The amplicon was purified with Montage PCR centrifugal filter devices (Millipore Corporation, Billerica, MA). Sequencing was performed by the Oklahoma Medical Research Foundation DNA sequencing facility using the ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA) with primers TU102 (27F), TU108 (926F), D907R, and 704F (13, 16). Sequencher (Windows version 4.2; Gene Codes Corp., Ann Arbor, MI) was used to assemble the fragments and to examine the sequences for the presence of conserved regions. The sequencing data were analyzed by comparison of the consensus sequences with the GenBank database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST/) using BLASTN 2.2.12 software (1, 4), and the percentages of similarity to closely related sequences were determined using 1,495 bp (01/06). GenBank bl2seq (29) was used to make the pairwise alignments (01/2006).

Retesting original sample powders. Total DNA was extracted from the original environmental specimens and used as template in a PCR assay for Ba813, an intergenic chromosomal marker for *B. anthracis*, as previously described (19, 22). In addition, the PCR assays for the pX01 and pX02 plasmids and chromosomal marker were repeated following LRN procedures (31).

DNA extraction. Total DNA was extracted from overnight bacterial growth in TSB using the MasterPure gram-positive DNA purification kit (Epicenter) by following the manufacturer's directions. Plasmid DNA was obtained using the QIAGEN Inc. (Valencia, CA) plasmid Midi kit by following the manufacturer's "very low copy number plasmid" protocol. Concentrated DNA was stored at −30°C, while diluted DNA used for PCR and sequencing was stored at 4°C until needed.

Plasmid restriction digestion. Restriction digestions were performed with 0.3 μl bovine serum albumin (10 mg/ml), 3 μl 10× buffer, 20 to 24 units of restriction

enzyme, and approximately 1 μg plasmid DNA in 30-μl volumes (9). Enzymes used were EcoNI (New England BioLabs, Beverly, MA), KpnI, MluI, NotI, PvuI, and XhoI (Promega, Madison, WI). We chose these enzymes after performing a virtual digestion on both the LaserGene MapDraw program (DNAStar, Madison, WI) and a free website at http://restrictionmapper.org (last update 18 January 2003 and last accessed 5 April 2006). Digestion mixtures were incubated for 4 h at optimal temperature for complete digestion and stored at −20°C until needed. *B. anthracis* Pasteur CBD 63 and *B. anthracis* CBD 131 were used for comparison. The expected number of DNA bands produced by each digestion was determined by the restrictionmapper and MapDraw programs and were as follows: EcoNI, 10 bands (108, 229, 1,328, 3,344, 4,445, 8,487, 10,658, 16,355, 18,608, and 32,658 bp); KpnI, 4 bands (3,184, 7,712, 23,206, and 62,129 bp); MluI, 3 bands (2,136, 36,871, and 57,224 bp); NotI, 1 band (96,231 bp); PvuI, 4 bands (11,833, 26,226, 26,889, and 31,282 bp); XhoI, 2 bands (14,426 and 81,805 bp). KpnI, MluI, NotI, and XhoI were not expected to cut within any of the targeted sequences. PvuI was expected to cut into the *capD* gene at location 53115. EcoNI was expected to cut into three targeted genes, *repA* (location 32669), *capD* (location 53577), and *capA* (location 54905). Digestions were repeated to confirm all findings.

PCR. Besides the capsule operon genes, we chose ORFs on pX02 that, to our knowledge, have not been previously identified in *B. cereus* or *B. thuringiensis* in either the chromosome or plasmids (21). Together, these sequences comprise only 10.3% of the total plasmid. Primers were designed using LaserGene (DNAStar) and the *B. anthracis* pX02 sequence obtained from GenBank (accession number NC_002146) (Table 2 and Fig. 1). PCRs on 10-μl mixtures (1.5 mM MgCl₂, 1× buffer, 2 μM deoxynucleoside triphosphate, 1.3 μM primers, 0.025 U *Taq* DNA polymerase; Takara, Madison, WI) were carried out using 10 to 100 ng plasmid-rich template in a T1 thermocycler (Biometra, Horsham, PA) with the following parameters: initial heating for 2 min at 94°C, followed by 40 cycles of 20 seconds at 94°C, 20 seconds at 58 to 70°C, and 3 min at 72°C, with a final extension at 72°C for 7 min. When the desired amplicon was >1 kb in length, the melting and annealing times were increased to 1 min each. Positive (*B. anthracis* Pasteur CBD 63 and *B. anthracis* CBD 131) and negative (*B. cereus* CBD 58 and distilled water [dH₂O]) controls were used for all assays.

Electrophoresis. The plasmid preparations and genomic DNA were electrophoresed on a 0.7% DNA grade agarose gel (Fisher Scientific International, Inc., Hampton, NH) (0.5× Tris-borate-EDTA [TBE]) for 90 to 120 min at 60 V constant voltage. Digested DNA was electrophoresed for 4 h at 100 V constant voltage. DNA bands were visualized with UV light after staining with 0.5 μg/ml ethidium bromide for 15 min and destained twice for 15 min in dH₂O. Photographs of the stained gels were made using the GelDoc (Bio-Rad, Hercules, CA). PCR products were separated on a 1% agarose gel (0.5× TBE with 0.05 μg/ml ethidium bromide) for 60 min at 100 V constant voltage. Staining and destaining were not necessary for the 1% gels.

DNA-DNA hybridization. DNA dot blots containing 10 to 100 μg purified total or plasmid DNA and Southern blots from gels were made using an Immobilon-nYplus nylon membrane (Millipore, Bedford, MA) or Roche nylon membrane (Roche Diagnostics, Indianapolis, IN) and prepared by following standard protocols (25). The DNA was bound to the membrane by UV irradiation using the Spectrolinker XL1000 (Spectronics Corporation, Westbury, NY). Oligonucleotide probes specific for internal portions of the targeted genes were designed with LaserGene (DNAStar) (Table 2) and labeled with digoxigenin using the DIG oligonucleotide tailing kit (Roche) in accordance with the manufacturer's instructions. Oligonucleotide probes specific for Ba813 and LRN chromosomal sequence were used to ensure that there was no chromosomal contamination of Southern blots (19). The different probes detected as little as 2 ng of positive DNA. Positive (*B. anthracis* Pasteur CBD 63 and CBD 131) and negative controls (*B. cereus* CBD 58 and dH₂O) were used with each assay.

Sequencing. Different combinations of forward and reverse primers were tested to determine which pairs produced large amplicons that could be used as a template for the sequencing PCRs (Tables 2 and 3 and Fig. 1). The amplicons from the PCR assays were purified using the Wizard PCR prep kit (Promega, Madison, WI) in accordance with the manufacturer's protocol before use as a template. Additional oligonucleotide primers were designed (Table 2 and Fig. 1) and were used in 25-pmol amounts in dye incorporation PCR cycle sequencing using the CEQ DTCS Quick Start kit (Beckman-Coulter, Fullerton, CA). Sequencing conditions were as follows: 30 cycles of 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 min. The amplicon was held at 4°C until it was precipitated, dried, and resuspended in sample loading solution (Beckman) overlaid with mineral oil and loaded into the CEQ 8000 sequencer (Beckman-Coulter) by following the company protocol. All reactions were performed multiple times, and sequences were obtained in both directions. Contiguous sequences were assembled with the SeqMan program of LaserGene (DNAStar)

and compared with four *B. anthracis* plasmid GenBank sequences for *B. anthracis* Pasteur (accession numbers NC_002146.1), *B. anthracis* Ames (accession number AE017335.3), *B. anthracis* A2012 (accession number AE011191.1), and *B. anthracis* (accession number AF188935.1) using the NCBI BLASTN software (1, 4) (http://www.ncbi.nlm.nih.gov/BLAST/) (01/06). Sequences from CBD 118 and 119 have been submitted to GenBank.

Nucleotide sequence accession numbers. Nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers DQ374636 and DQ374637 (16S rRNA gene sequences of CBD 118 and CBD 119, respectively). GenBank accession numbers for CBD 118 (*B. circulans*) gene sequences obtained in this study are as follows: DQ517343 (*acpA*), DQ517347 (for *capA*, *capB*, and *capC* together), DQ517352 (*capD*), DQ517350 (*capR*), DQ517353 (*repA*), DQ517341 (ORF 48), DQ517345 (ORF 61), DQ517355 (IS*1627*), and DQ517357 (capsule promoter sequence). GenBank accession numbers for CBD 119 (*Bacillus* species) are as follows: DQ517344 (*acpA*), DQ517348 (for *capA*, *capB*, and *capC* together), DQ517351 (*capD*), DQ517349 (*capR*), DQ517354 (*repA*), DQ517342 (ORF 48), DQ517346 (ORF 61), DQ 517356 (IS*1627*), and DQ517358 (capsule promoter sequence).

RESULTS

Both CBD 118 and CBD 119 were nonhemolytic, penicillin susceptible, and nonmotile on the first examination. Using our laboratory protocol for determining motility, CBD 118 was clearly motile while CBD 119 was confirmed to be nonmotile. Both isolates produced ellipsoidal, subterminal endospores without evidence of parasporal crystal formation. Endospore-driven swelling of CBD 118 was subtle to occasionally pronounced, while overt swelling was seen for CBD 119. Both CBD 118 and 119 failed to grow on the mannitol-egg yolk-polymyxin B media, whereas *B. cereus* CBD 55, *B. anthracis* Pasteur CBD 63, and *B. thuringiensis* CBD 61 thrived, fermented mannitol, and were lecithinase⁺. CBD 118 was twice identified by the API system as *Bacillus circulans* biotype 1 ("very good identification"; % confidence in identification [%ID] = 99.4%). Two tests of CBD 119 in the API panels resulted in an "identification not valid," with *Bacillus amyloliquefaciens* (%ID = 77.6%), *B. subtilis* (%ID = 9.4%), *B. lentus* (%ID = 5.8%), *B. cereus* (%ID = 3.3%), and *Brevibacillus laterosporus* (%ID = 3.1%) proposed as significant taxa, and a "doubtful profile" with the significant taxon of *B. laterosporus* (%ID = 99.4%).

When 1,495 bp of 16S rRNA gene sequence was compared to those in the GenBank database, CBD 118 shared 1,454/1,458 bp (99.7%) with unpublished *Bacillus benzoevorans* (accession number AY043085), 1,477/1,495 bp (98.7%) with *Bacillus circulans* N3 (accession number AB215100), and 1,470/1,489 bp (98.7%) with *B. circulans* ATCC 4513 (accession number AY724690). When compared with *B. anthracis* Ames (accession number AE017024), CBD 118 had less than the requisite 97% sequence similarity (1,399/1,490 bp [93.9%]) for relatedness at the species level (27, 28). Based upon the API system results and 16S rRNA gene sequence similarities, CBD 118 was identified as a *Bacillus circulans*.

CBD 119 shared 1,478/1,488 bp (99.3%) with *Bacillus luciferensis* LMG 18422 (accession number AJ419629), the only named *Bacillus* sp. with which the isolate had greater than 97% sequence similarity. CBD 119 shared 1,414/1,487 bp (95.1%) with *B. anthracis* Ames (accession number AE017024) and 1,196/1,311 bp (91.2%) with *B. laterosporus* BL-2 (accession number DQ371289). No *Brevibacillus* sp. in the GenBank database shared ≥97% 16S sequence similarity with CBD 119. Therefore, CBD 119 was identified by 16S rRNA gene se-

TABLE 2. Oligonucleotides used as primers and probes for PCR assays for plasmid sequences

Gene or ORF	Primer[b]	Sequence (5′→3′)	Location	Length (bp)	Source or reference
acpA (partial)	acpA2F	AGC-CGA-AAA-ATT-GAT-TTG-TTA-AAT-ATC-CTA-ATT	68924–68956	1,653	This study
	acpA5R	CAC-TCC-TGG-TAA-TAT-CAT-CGG-AAA-TCG-GTT-AT	70546–70577		This study
	acpA3F[a]	GAA-TTG-TAG-GGG-ATG-AAT-TTC-AAA-TTA-TAC-TAA-TG	68324–68358		This study
	acpA3R[a]	TTC-CCC-CTC-TTT-GTA-AAG-TAC-TAA-TTC-TTC-TTG	69731–69763		This study
acpA (partial)	acpA1F	GTT-ATG-TTT-GAT-ATC-CCC-TGA-TGT-TAC-CAA-TT	68536–68567	1,657	This study
	acpA4R	CTC-CTA-CAA-ATT-TTA-ATT-GAT-CGC-CAT-AAC-G	70163–70193		This study
	acpA3F[a]	GAA-TTG-TAG-GGG-ATG-AAT-TTC-AAA-TTA-TAC-TAA-TG	68324–68358		This study
	acpA3R[a]	TTC-CCC-CTC-TTT-GTA-AAG-TAC-TAA-TTC-TTC-TTG	69731–69763		This study
capA	capA1F	CAA-CAT-TTG-CAA-TCA-TGA-ATA-TTT-ATT-ACT-TAT	55630–55662	1,291	This study
	capA2R	AGT-TGT-TGT-CTC-CAC-TGA-TAC-TTG-ATT-TTC	54381–54410		This study
	capA2F[a]	CGG-ATT-ATG-GTG-CTA-AGG-GAA-CTA-AAG-A	55135–55162		This study
	capCR[a]	CGC-AGC-TAT-TAA-TAT-AAC-TGC-GAT-AAG	55494–55520		This study
capB	capB1F	CTT-AAT-AAG-CAA-GAA-ATC-GAA-AAG-CAG-GAA-GG	57294–57325	2,204	This study
	capA1R	AGG-CCT-TTA-TTG-TAT-CTT-TAG-TTC-CCT-TAG	55121–55150		This study
	capB1R[a]	CAA-GCG-ATG-CAT-TAT-CTG-GGA-AGA-CCA	56631–56657		This study
	capCF[a]	GGA-AGA-ACA-AAT-TGG-CAA-AAA-GC	56106–56128		This study
capC	capB2F	GGT-CTT-CCC-AGA-TAA-TGC-ATC-GCT-TG	56631–56656	1,535	This study
	capA1R	AGG-CCT-TTA-TTG-TAT-CTT-TAG-TTC-CCT-TAG	55121–55150		This study
	capA1F[a]	CAA-CAT-TTG-CAA-TCA-TGA-ATA-TTT-ATT-ACT-TAT	57294–57325		This study
capC	capCF	GGA-AGA-ACA-AAT-TGG-CAA-AAA-GC	56106–56128	635	This study
	capCR	CGC-AGC-TAT-TAA-TAT-AAC-TGC-GAT-AAG	55494–55520		This study
	capB2R[a]	TTC-TTT-CTG-TAA-AAA-TAA-GGC-TCA-GTG-TAA-CTC-CT	56005–56039		This study
capC	RcapCF	ACT-CGT-TTT-TAA-TCA-GCC-CG	55942–55961	264	23
	RcapCR	GGT-AAC-CCT-TGT-CTT-TGA-AT	55698–55717		23
	capA1F[a]	CAA-CAT-TTG-CAA-TCA-TGA-ATA-TTT-ATT-ACT-TAT	57294–57325		This study
capD	dep1F	CGA-ACG-TTT-AAA-GGT-CCC-CAT-TGT	52697–52720	1,600	This study
	dep4R	TTG-CGG-TTG-AGG-AAG-GTA-TGA-AA	54190–54212		This study
	dep2F[a]	ATC-GTT-TCC-CCA-TCT-TTC-TTT-AAT-ACA-GTG	53114–23143		This study
	dep3R[a]	CAT-ATT-GGA-GTA-CCC-GGA-TTT-GTG-G	53892–54006		This study
capR	capR1F	GGC-GGG-TTT-TCC-TTT-GTT-CC	35917–35936	2,408	This study
	repA1R	TGG-CAA-ACT-ATC-AGC-AAA-CAT-TTC-TCG-TA	33528–33556		This study
	capR1R[a]	ATG-GCT-AGC-GAT-TAT-GTT-CTT-GTT-GC	35507–35532		This study
	capR2F[a]	ATC-GCT-AGC-CAT-TGC-AGA-ACT-TTT-T	35494–35518		This study
IS*1627*	IS1627F	TAA-ACG-GGG-CAC-CTA-TTA-AAA-CAC-CTA-AA	39718–39746	479	This study
	IS1627R	TTG-AAG-CGT-CAG-TCC-CAA-ATG-AAA-AG	40171–40196		This study
	ORF43-1[a]	GGA-TAC-TTT-TGT-TAC-CTT-TTG-TGA-ATA-GAA	39814–39843		This study
	ORF43-2[a]	GAT-GAA-GAC-AGT-AAA-ACA-AGT-AAT-GAG-AGG	40016–40045		This study
Promoter	procapF	AAA-TAA-TTG-CAA-TCT-TTG-GAT-CAG-AAT-CTA-A	58104–58134	443	This study
	procapR	AAG-AAA-AAC-GCA-ATT-TAC-AGA-ACT-CCT-CT	57684–57722		This study
	procap-1[a]	CTA-AGA-ATA-GAT-TTC-AAT-TTA-AAG-GGG-GTG	57894–57923		This study
ORF 48	ORF48-F	GGA-GGA-CAA-AGC-ATG-ACT-AAA-GTT-GAT-AAG-GTA-A	44465–44498	669	This study
	ORF48-R	AAT-GTC-TCC-CCC-TCA-TAA-ACA-GAA-GAT-ACT-TTT-T	45130–45163		This study
	ORF48-1[a]	GCC-TAC-GAC-AAA-AGT-GGT-GAG-ATT-ATG-TGG	44645–44674		This study
ORF 61	ORF61-F	CTT-TCT-CTG-TCT-TTA-CCG-ACC-TTT-TAT-ACT-CA	62793–62824	459	This study
	ORF61-R	AGA-AAT-CAA-ATG-CCT-TCT-TTG-TCG-AT	63221–63426		This study
	ORF61-3[a]	CTT-AAC-CAA-TGC-TTG-GTA-TTG-CTG-TAA-ATC	62910–62939		This study
repA	repA1F	GAA-GAT-TTA-AGG-GCT-AAG-TTT-GGG-ACT-CG	33913–33941	1,350	This study
	repA4R	GCC-CTT-CCA-ATA-TGC-CGT-ATA-GTG-TTT-C	32591–32618		This study
	repA2R[a]	TGA-AAA-CAC-GCT-AAA-GAA-AGG-GTG-AAA-AT	33211–33239		This study
	repA4F[a]	GCC-CTT-CCA-ATA-TGC-CGT-ATA-GTG-TTT-C	33006–33026		This study

[a] Oligonucleotide probes were internal to, and specific for, the target sequence as determined by N-blast with the NCBI library. Probes were used alone or with other oligonucleotide in a "cocktail" for optimal results. Oligonucleotides were end labeled with digoxigenin by following the manufacturer's directions. Probes were also used for DNA-DNA hybridization of dot blots made from either genomic or plasmid DNA.
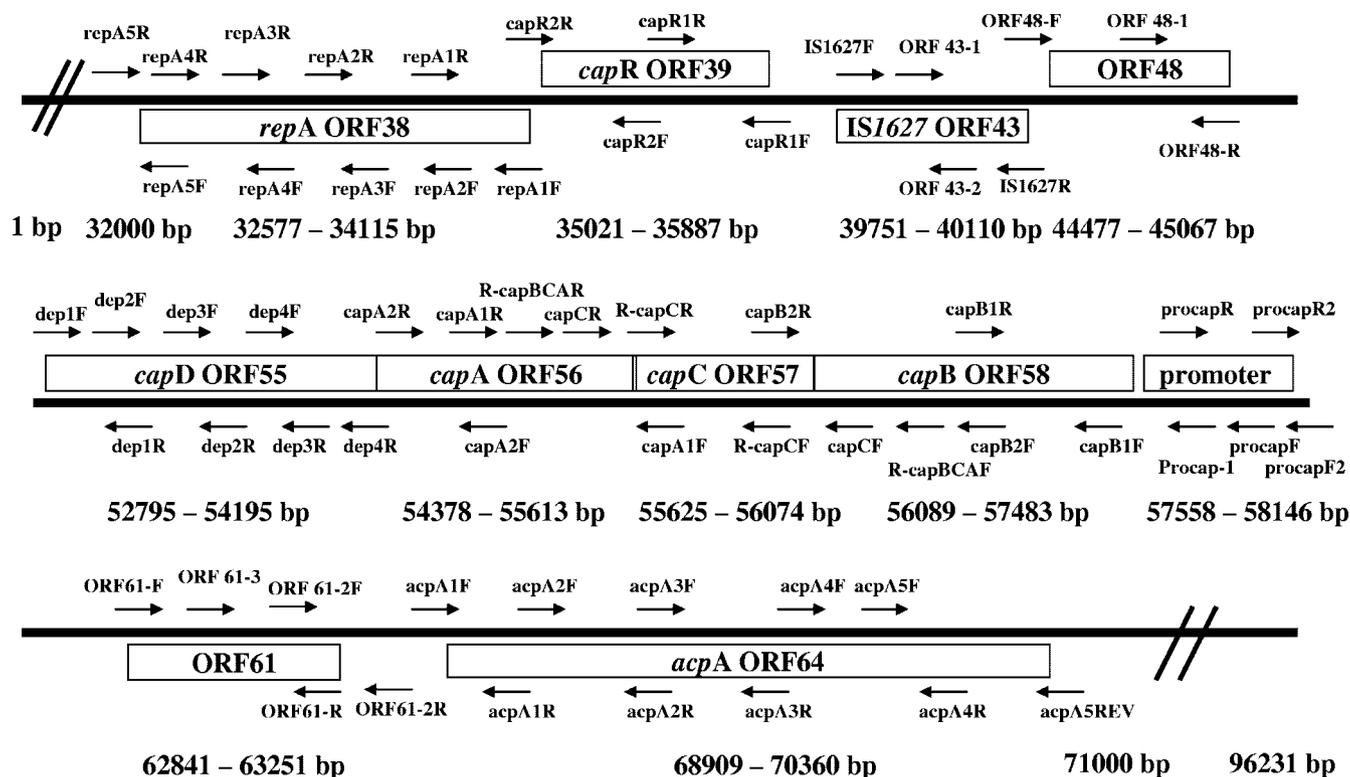[b] The position and orientation of each primer can be noted in Fig. 1.

FIG. 1. Map of oligonucleotide primers and probes for selected genes and ORFs on pX02. The genes were selected because they have not been previously described in the genomes or plasmids of *Bacillus* species other than *B. anthracis*, *B. cereus*, and *B. thuringiensis*.

quence similarity as a *Bacillus* sp. closely related to *B. lucife-rensis*.

CBD 118, although positive by the capsule DFA test, did not display a visible capsule in India ink mounts by phase contrast after growing overnight on TSA and/or BUG supplemented with 0.8% sodium bicarbonate with 5% or approximately 20% CO_2 at 35°C. After growing in either concentration of CO_2 on media containing sodium bicarbonate, colonies of the positive control CBD 63 (*B. anthracis* Pasteur) appeared to generate a distinct wet coating. When observed microscopically, the cells incubated in 5% CO_2 produced obvious thin-to-moderate capsules on approximately 50% of cells per microscope field (1,000×). When incubated at the higher CO_2 concentration, most of the bacterial cells seen per field possessed thicker capsules. In contrast, the negative controls CBD 58 (*B. cereus*) and CBD 389 (*B. circulans*) failed to produce discernible capsules under identical conditions. CBD 119 and CBD 980 (*B. luciferensis*) failed to thrive on media supplemented with 0.8% sodium bicarbonate. Therefore, they were tested after growing on BUG or TSA plates without added CO_2 or in 5% CO_2. Although CBD 119 was negative for the *B. anthracis* capsule by DFA, the strain produced thin but omnipresent capsules with or without CO_2. CBD 980 failed to produce capsules under all conditions tested.

After repeated testing of initial assay mixtures, CBD 118 and 119, as well as the original specimens from which they came, were consistently positive by PCR for Ba813, pX02, and the LRN chromosomal marker for *B. anthracis*, while both isolates were negative for gamma phage susceptibility

and the *B. anthracis* cell wall by DFA (Table 1). Only CBD 118 was positive for the capsule by the initial DFA testing. The test on CBD 118 was performed more than once since it was an unexpected result. When the cryostock vials from the original culture and the official CBD collection were later subcultured (one or two passages) and examined, the DFA results for both CBD 118 and 119 were negative. The same DFA reagents were used throughout the tests although lot numbers did change.

Using previously published primers (23), we established that the two *Bacillus* isolates also gave positive PCR results for *capC* (Table 4). Labeled oligonucleotide probes designed from the sequences of 11 ORFs that are located on pX02 hybridized with the dot blots of total DNA and Southern blots of uncut plasmid DNA (Table 4). Probes specific for chromosomal DNA targets hybridized with the total DNA blots only. PCR assays for the 11 ORFs yielded positive results for both isolates (Table 4), while PCR for the chromosomal marker was consistently negative when using the plasmid DNA as a template. Hybridization with labeled probes specific for internal sequences of the different ORFs confirmed that the PCR amplicons were the desired targets.

Both isolates carried a large plasmid that migrated the same distance as the *B. anthracis* Pasteur (CBD 63) plasmid when electrophoresed on a 0.7% agarose gel. DNA bands larger than 23 kb were not easily resolved unless the DNA was electrophoresed for longer than 4 h. The uncut plasmid was usually still supercoiled, migrated faster on the gels, and appeared to be closer to the 23-kb position. Occasionally, the plasmid

TABLE 3. Additional oligonucleotides used for sequencing[a] the capsule operon and related genes[b] in the two *Bacillus* spp.

Oligonucleotide	Sequence (5′→3′)	Location	Reference
acpA1R	TCA-ATT-TTT-CGG-CTA-ATA-TCT-TTT-TCC-ATA-ATT	68905–68937	This study
acpA2R	ATA-ATT-TGA-AAT-TCA-TCC-CCT-ACA-ATT-CTT-AAT-G	69318–69351	This study
acpA4F	TTT-ACA-AAG-AGG-GGG-AAA-GCA-CAA-CTT-A	69747–69774	This study
acpA5F	TTG-CTT-ATT-ACC-GGT-GAA-GGA-AAA-AGT-TGG	70103–70132	This study
capA2F	CGG-ATT-ATG-GTG-CTA-AGG-GAA-CTA-AAG-A	55135–55162	This study
RcapBCA-F	GAA-ATA-GTT-ATT-GCG-ATT-GG	56315–56334	23
RcapBCA-Fcomp	CCA-ATC-GCA-ATA-ACT-ATT-TC	56315–56334	23
RcapBCA-Rcomp	CGT-ACA-GAA-GCA-GTA-GCA-CC	55462–55481	23
capB1F-comp	CCT-TCC-TGC-TTT-TCG-ATT-TCT-TGC-TTA-TTA-AG	57294–57325	This study
capR2R	TTT-TTG-GAG-GGA-TAC-ATA-TGG-AAC-ACA	35004–35030	This study
dep1R	GGC-CCC-CAC-TGT-ATT-AAA-GAA-AGA-T	53125–53149	This study
dep2R	ATG-ATG-TTT-ATA-CCG-CTC-CAC-CAC-CTT	53580–53606	This study
dep3F	ACG-TAA-TTT-GGA-TCC-CCT-AGG-TTT-TTC-T	53423–53450	This study
dep4F	TAA-GGT-TCT-CGC-TAA-ATC-TGT-CTG-GA	53752–53777	This study
dep4R-comp	TTT-CAT-ACC-TTC-CTC-AAC-CGC-AA	54190–54212	This study
procapRcomp	AGA-GGA-GTT-CTG-TAA-ATT-GCG-TTT-TTC-TT	57684–57722	This study
repA1Fcomp	CGA-GTC-CCA-AAC-TTA-GCC-CTT-AAA-TCT-TC	33913–33941	This study
repA2F	AGG-CGG-TGT-ACG-TAT-CAA-ACA-ACA-AGA-ACT	33596–33625	This study
repA2Fcomp	AGT-TCT-TGT-TGT-TTG-ATA-CGT-ACA-CCG-CTT	33211–33239	This study
repA3F	GCC-TTC-CGT-GCG-CCT-TCT-GTA	33242–33265	This study
repA3R	GTT-AAG-CGC-TTC-GCA-CAG-TTC-TGA-T	32856–32880	This study
repA5F	ACG-TTC-CCC-GCA-GCA-CAT	32840–32857	This study
repA5R	TTT-ACC-CAA-TTT-TAG-CAC-CAC-CAA	32366–32389	This study
orf61-2F	TCA-GGT-ACA-TAA-TAA-AAG-CAG-AAA-GT	63089–63114	This study
orf61-2R	GAG-AGG-AGG-GTT-GTA-AAT-GAA-GTT	63826–63849	This study

[a] In accordance with the manufacturer's directions, oligonucleotides were used to generate sequences that were assembled using the SeqMan program of DNAStar. In order to prevent potential PCR errors, sequences were determined multiple times in both directions.
[b] The position and orientation of each primer can be noted in Fig. 1.

would get torn or nicked and would migrate much more slowly and appear to be larger than the supercoiled DNA. When the CBD 63 plasmid was digested with EcoNI and electrophoresed on the gel, the two smallest DNA bands that were expected (108 and 229 bp) never appeared in any of our attempts (Fig. 2). The bands may have migrated off the gels before the ladder or larger bands were better resolved or the control strain may have lost restriction sites. If loss of restriction sites occurred, the small sections of DNA would still be attached to a larger band and would probably not affect the migration of the larger DNA band by any appreciable distance. The band at about 1.3 kb was seen as a very faint band on the gel and never was as intense as the larger bands. The band expected at about 10.6 kb was never seen in any EcoNI digestions, suggesting the loss of another restriction site. The other six bands (3.328, 4.445, 8.487, 16.355, 18.608, and 32.658 kb) expected in the EcoNI digestion were present (Fig. 2, lane 2). The digestion of CBD 63 with KpnI produced three, not four, DNA bands: a light one at about 11 kb and two large ones that migrated closely to-

gether (23.206 and 62.129 kb). It is possible that this control strain lost the restriction site to produce a 10.8-kb band from the missing 3.184- and 7.712-kb bands. The digestion with MluI produced two large intense bands that migrated closely together. These bands were hard to resolve unless electrophoresed for a much longer time. The small 2.136-kb band appears to be missing. The PvuI digestion yielded one intense band in which the 26.226-kb and 26.889-kb bands migrated together and were closely followed by another large intense band (31.282 kb). The expected 11.833-kb band was not seen on any repeated digestions with PvuI, suggesting a loss of restriction site. The XhoI digestion yielded two bands that migrated close to the 23-kb position. A large 81.8-kb band was expected and should have been visible and clearly distinct from the 23-kb position. It is possible that the control strain has gained a restriction site to produce the two bands. It appears from our gels that the sequence of pX02 in the *B. anthracis* Pasteur strain in our collection may have some differences from those sequences in the databases upon which the digestions are

TABLE 4. Results of PCR, Southern, and dot blot hybridization for 10 ORFs and the capsule promoter of *B. anthracis* pX02

Strain	Organism	Result for indicated ORF or capsule promoter:										
		ORF 38 (*repA*)	ORF 39 (*capR*)	ORF 43 (IS*1627*)	ORF 48	ORF 55 (*capD*)	ORF 56 (*capA*)	ORF 58 (*capB*)	ORF 57 (*capC*)	Capsule promoter	ORF 61	ORF 64 (*acpA*)
63	*B. anthracis*	+	+	+	+	+	+	+	+	+	+	+
118	*B. circulans*	+	+	+	+	+	+	+	+	+	+	+
119	*Bacillus* sp.	+	+	+	+	+	+	±[a]	+	+	+	+
131	*B. anthracis*	+	+	+	+	+	+	+	+	+	+	+

[a] The dot blot hybridization was negative for *capB*, but PCR and subsequent hybridization of Southern blots were positive. Sequencing attempts revealed the full sequence for *capB* in CBD 119.
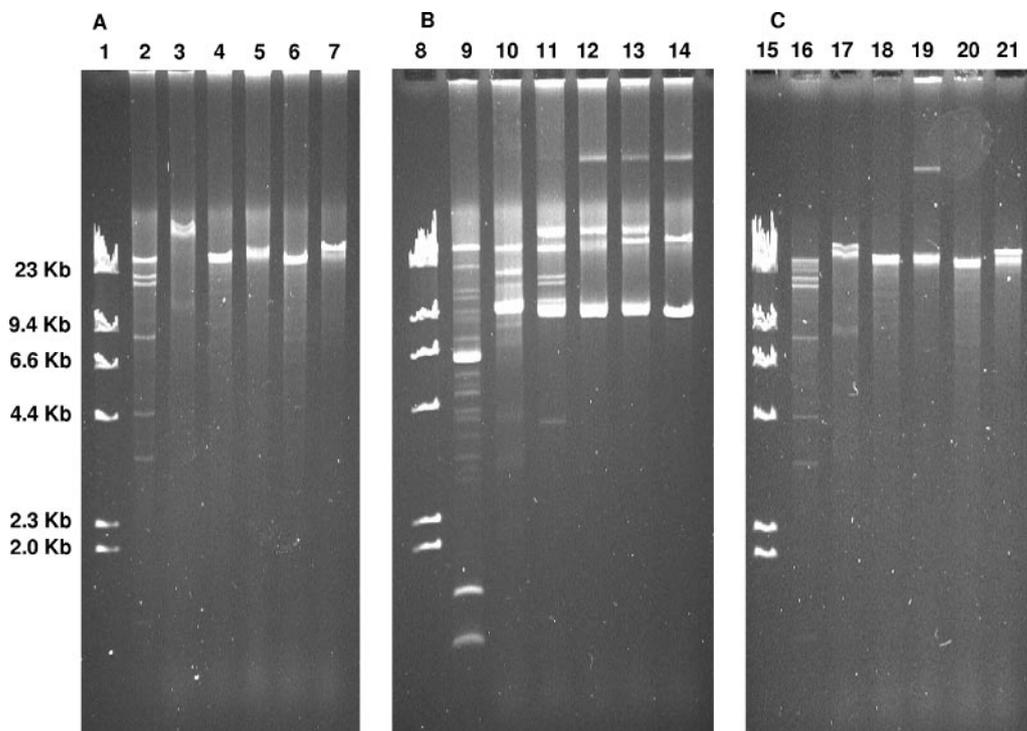
FIG. 2. Restriction enzyme-digested plasmid DNA on 0.7% agarose gels after electrophoresis. (A) *B. anthracis* CBD 63; (B) CBD 118; (C) CBD 119. Lanes 1, 8, and 15, HindIII-digested Lambda DNA marker; lanes 2, 9, and 16, plasmid DNA digested with EcoNI; lanes 3, 10, and 17, plasmid DNA digested with KpnI; lanes 4, 11, and 18, plasmid DNA digested with MluI; lanes 5, 12, and 19, plasmid DNA digested with NotI; lanes 6, 13, and 20, plasmid DNA digested with PvuI; lanes 7, 14, and 21, plasmid DNA digested with XhoI.

based, even though the sequences of the genes examined to date are identical.

CBD 118 presented a very different restriction pattern than that of CBD 63 (Fig. 2). All of the restriction enzyme digestions produced more DNA bands in CBD 118 than in CBD 63. For EcoNI, there were 19 bands seen: 1 large intense band >23 kb, 3 bands between 23 and 9.4 kb, 1 band close to 9.4 kb, 2 bands between 9.4 and 6.6 kb, 1 intense band about 6.6 kb, 3 bands between 6.6 and 4.4 kb, 1 band about 4.4 kb, 5 bands between 4.4 and 2.3 kb, and 2 intense bands <2.0 kb (Fig. 2). The KpnI digestion produced nine bands (one band >23 kb, two bands between 23 and 9.4 kb, one band about 9.4 kb, two bands between 9.4 and 6.6 kb, and three light bands <4.4 kb), while the MluI digestion produced seven bands (two bands >23 kb, two bands midway between 23 and 9.4 kb, two close bands about 9.4 kb, and one band <4.4 kb. The NotI digestion produced four bands (one band ≫23 kb, two bands close to 23 kb, and one band about 9.4 kb), while the PvuI digestion also produced four bands (one band ≫23 kb, two bands >23 kb, and one band about 9.4 kb). Lastly, the XhoI digestion yielded three bands (one band ≫23 kb, one band close to but more than 23 kb, and one band about 9.4 kb). The number of DNA bands and very different intensities seen in the DNA bands suggest that there may be more than one large plasmid present or that the plasmid is much larger than pX02 (Fig. 2).

The restriction pattern of the CBD 119 plasmid was similar but not identical to that obtained from *B. anthracis* CBD 63 (Fig. 2). Two extra bands were seen in the EcoNI digestion, one at the 23-kb position and one slightly larger. There appears

to be one extra band (about 8 kb) in the KpnI digestion, yielding a total of four bands (about 8 kb, 10 kb, 23 kb, and perhaps 62 kb). The MluI and PvuI digestions appeared to produce the same number of DNA bands of similar sizes and intensities for CBD 119 as for CBD 63 (Fig. 2). The digestion with XhoI produced two bands like the digestion of CBD 63 plasmid, but the smaller band is slightly more clearly separated from the larger band, and it is more intense. There was one large band sometimes seen in the NotI digestion that migrated much more slowly, migrating to the same location as the large band seen in the NotI, PvuI, and XhoI digestions of CBD 118; it appears to be either a nicked or torn plasmid.

On Southern blots made from enzyme-digested plasmids, the labeled probes hybridized to bands of similar sizes for CBD 63 and 119 although it was not always clear which of two closely migrating bands hybridized with the probe (Fig. 3). When we probed the Southern blots made from CBD 118 digested DNA for specific genes, the oligonucleotide probes hybridized with different-size bands than seen with CBD 63. For example, in all of the digestions with EcoNI, KpnI, MluI, and NotI, the *capC* oligonucleotide probes hybridized with the faster, smaller, more intense bands of CBD 118 and not with the larger bands as was seen with both CBD 63 and 119 (Fig. 3). The larger bands seen in the PvuI and XhoI digestions did not consistently hybridize with the *capC* probe, suggesting that not all of the plasmid copies in the reaction tubes were fully cut when these two digestions were performed.

Using different oligonucleotide primer combinations, we were able to sequence 100% of the 10 genes and most (97.3%)
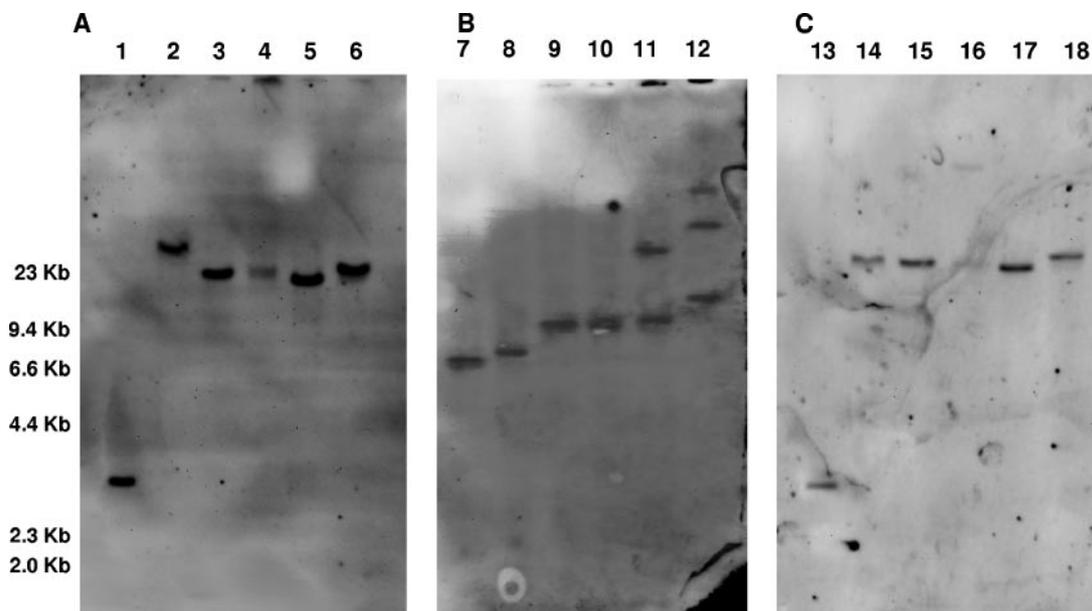
FIG. 3. Hybridization of Southern blotted membranes of digested plasmid DNA with *capC* oligonucleotide probe. (A) *B. anthracis* CBD 63; (B) CBD 118; (C) CBD 119. Lanes 1, 7, and 13, plasmid DNA digested with EcoNI; lanes 2, 8, and 14, plasmid DNA digested with KpnI; lanes 3, 9, and 15, plasmid DNA digested with MluI; lanes 4, 10, and 16, plasmid DNA digested with NotI; lanes 5, 11, and 17, plasmid DNA digested with PvuI; lanes 6, 12, and 18, plasmid DNA digested with XhoI.

of the capsule promoter from CBD 118 and 119 and the two *B. anthracis* strains (CBD 63 and 131). When the sequences were assembled and compared against published sequences in the NCBI library, the sequences of the nine genes (*acpA*, *capA*, *capB*, *capC*, *capR*, IS*1627*, ORF 48, ORF 61, and *repA*) from CBD 118 and 119 had 100% nucleotide identity and 100% amino acid identity with four *B. anthracis* pX02 sequences in GenBank, *B. anthracis* Pasteur (accession number NC_002146.1), *B. anthracis* Ames (accession number AE017335.3), *B. anthracis* A2012 (accession number AE011191.1), and *B. anthracis* (accession number AF188935.1) (01/06).

The only differences were seen in the *capD* and capsule promoter sequences. The sequences for the *capD* genes from CBD 118 and 119 had a single nucleotide substitution (A→G) at position 53138 (based upon GenBank accession number NC_002146.1) when compared to *B. anthracis* (accession number AF188935.1) and *B. anthracis* Pasteur (accession number NC_002146.1 (Fig. 4) (01/06). This substitution is also seen in CBD 63, CBD 131, *B. anthracis* A2012 (accession number

AE011191.1), and *B. anthracis* Ames (accession number AE017335.3). However, the amino acid sequences encoded by *capD* are identical. The capsule operon promoters of both CBD 118 and 119 had identical single base pair substitutions at two different locations (Fig. 5). At position 57837 (based upon NC_002146.1), CBD 118 and 119 show one base pair substitution (A→G). This differs from AE011191.1 and AE017335, yet matches the other *B. anthracis* sequences. The second base pair substitution is at position 58137 (based upon the GenBank sequence of NC_002146.1), where CBD 118 and 119 have a cysteine (C) matching CBD 63 and 131 instead of a thymine (T) as seen in the other four sequences (NC_002146.1, AF188935.1, AE011191.1, and AE017335) (Fig. 5) (01/06).

DISCUSSION

Here we report the presence of 10 genes, including the capsule operon of the *B. anthracis* plasmid pX02, on two different large plasmids in a *B. circulans* and a *Bacillus* species
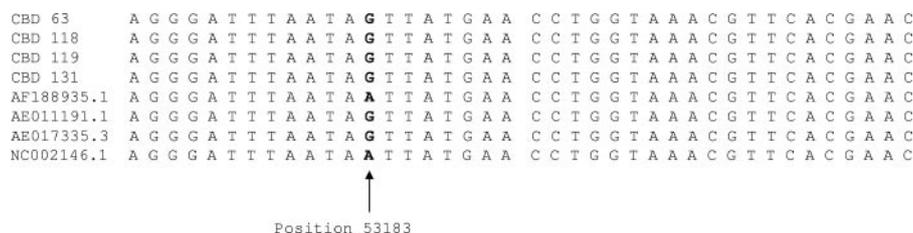

FIG. 4. Partial sequence of the *capD* gene showing a single base pair difference. At position 53183 (based upon the GenBank sequence of NC_002146.1), CBD 118 and 119 match four of the six sequences of the pX02 of *B. anthracis* but are different from the other two GenBank sequences (A→G) (AF0188935.1 and NC_002146.1). CBD 63 is *B. anthracis* Pasteur strain, CBD 118 is *B. circulans*, CBD 119 is a *Bacillus* sp., and CBD 131 is *B. anthracis*. Strains designated AF188935.1 (*B. anthracis*), AE011191.1 (*B. anthracis* A2012), AE017335.3 (*B. anthracis* Ames), and NC_002146.1 (*B. anthracis* Pasteur) are the sequences available in GenBank for the *B. anthracis* pX02 plasmid.

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CBD 63      T G A A T A T T A G G T T T T T C C T G A T   A T A A C A A T T G A T A A T A A T G
CBD 118     T G A A T A T T A G G T T T T T C C T G A T   A T A A C A A T T G A T A A T A A T G
CBD 119     T G A A T A T T A G G T T T T T C C T G A T   A T A A C A A T T G A T A A T A A T G
CBD 131     T G A A T A T T A G G T T T T T C C T G A T   A T A A C A A T T G A T A A T A A T G
AF188935    T G A A T A T T A G G T T T T T C C T G A T   A T A A C A A T T G A T A A T A A T G
AE011191.1  T G A A T A T T A G G T T T T T C C T A A T   A T A A C A A T T G A T A A T A A T G
AE017335    T G A A T A T T A G G T T T T T C C T A A T   A T A A C A A T T G A T A A T A A T G
NC002146.1  T G A A T A T T A G G T T T T T C C T G A T   A T A A C A A T T G A T A A T A A T G

                                          Position 57837


CBD 63      T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T C C
CBD 118     T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T C C
CBD 119     T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T C C
CBD 131     T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T C C
AF188935    T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T T C
AE011191.1  T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T T C
AE017335    T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T T C
NC002146.1  T T T T T A C A A A A A A A G A A A A A T   A C T A T T T A G A T T C T G A T T C

                                                            Position 58137
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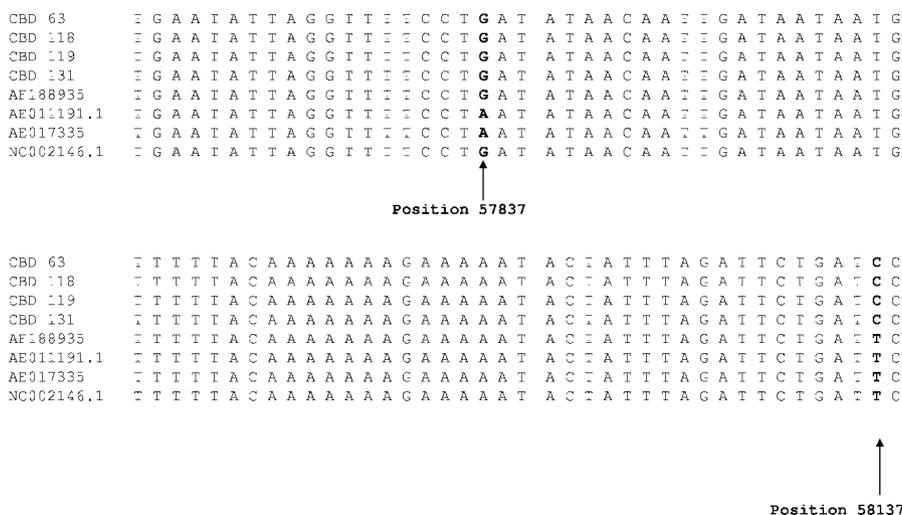
FIG. 5. Two partial sequences of the capsule operon promoter showing a single base pair difference at each location. At position 57837 (based upon the GenBank sequence of NC_002146.1), CBD 118 and 119 match four of the six sequences of the pX02 of *B. anthracis* and differ from the other two sequences (A→G). At position 58137 (based on the GenBank sequence of NC_002146.1 CBD 118 and 119 have a cysteine (C) matching CBD 63 and 131 instead of a thymine (T) as the other sequences from GenBank have. CBD 63 is *B. anthracis* Pasteur strain, CBD 118 is *B. circulans*, CBD 119 is a *Bacillus* sp., and CBD 131 is *B. anthracis*. AF188935 (*B. anthracis*), AE011191.1 (*B. anthracis* A2012), AE017335 (*B. anthracis* Ames), and NC_002146.1 (*B. anthracis* Pasteur) are the sequences available on GenBank for the *B. anthracis* pX02 plasmid.

distinct from but closely related to *B. luciferensis*. To our knowledge, this is the first time large plasmids with these genes have been identified in *Bacillus* species not of the *B. cereus* group. We have shown that a large plasmid in *B. circulans* (CBD 118) is different from the pX02 plasmid by restriction enzyme pattern using a total of five restriction enzymes, yet this plasmid carried all of the genes targeted in this study. It is possible that there is more than one large plasmid present in CBD 118. This would explain the high number of large DNA bands seen in the digestions and also the very different intensities seen in the DNA bands. One predominant plasmid of high copy number may be masking the presence of a second plasmid. This could also be why the NotI, PvuI, and XhoI digestions produced a large band that is seen migrating much more slowly than other large DNA bands. However, the hybridization studies suggest that the high-copy-number plasmid with the distinctive restriction digestion pattern carries the different pX02 genes. This finding implies that there may be other large plasmids that carry the pX02 genes.

We have also shown that the plasmid found in CBD 119, the *Bacillus* isolate not yet fully identified, has a similar restriction enzyme pattern as pX02 in our *B. anthracis* Pasteur strain. This plasmid appears to have two EcoNI restriction sites and one KpnI restriction site more than the *B. anthracis* isolate. Yet, our control *B. anthracis* also appeared to lose an EcoNI, a KpnI, and a PvuI restriction site and to gain an XhoI site, suggesting that the large plasmid in CBD 119 may still in fact be pX02. This may not be surprising, since pX02 has previously been identified in *B. cereus* isolates, but this is the first time that a plasmid carrying the pX02 virulence genes has been found in a *Bacillus* isolate that is not related to *B. anthracis* or *B. cereus*. Further work will determine the magnitude of similarity this plasmid has to pX02. We plan to look for more pX02 genes in this plasmid and to eventually fully sequence the two plasmids. Other studies to be undertaken are to explore why the capsule

is not expressed although the capsule genes are present and to determine if there is any interaction between these plasmids and the chromosome. Animal studies of these two isolates and the plasmids described above would be additional areas of exploration.

The prevalence of these plasmids in other species of *Bacillus* is not known. Nor is it known if these plasmids are restricted to the *Bacillus* genus. We can only speculate as to how or when the two *Bacillus* isolates obtained the two plasmids, although other researchers report that horizontal transfer of plasmids occurs in *Bacillus* spp. by conjugation and transduction (2, 24). This raises the possibility that the plasmids could be mobile in other *Bacillus* species and suggests that more individual isolates of different *Bacillus* species may also carry the pX02 plasmid or like plasmids. The facts that the sequences of the 10 genes had 100% nucleotide and amino acid identity over the entire lengths of the genes with 9 of the *B. anthracis* pX02 genes and that the nucleotide difference in *capD* did not result in different amino acids imply not only that the genes are functional but also that the plasmids have been recently introduced into these two isolates. Mobility of the two plasmids has not yet been studied.

The DFA results were intriguing since they suggest that the *B. circulans* isolate (CBD 118) expressed an epitope similar to the *B. anthracis* capsule epitope that is recognized by the test antibody. However, after further culturing, retests using the same reagents with the same and different lot numbers were negative, implying that the ability to express the same epitope was impaired. The Department of Health personnel who performed this test on the isolates are very experienced, and they repeated the assessment at the initial testing. In addition, other personnel also examined the slides and agreed that the DFA test was positive. Therefore, we have full confidence that the test was performed correctly and that the epitope was initially expressed. This suggests a number of possibilities: (i) an envi-

ronmental change or lack of host-specific factor inhibited capsule expression, (ii) the organism can only express the capsule under specific conditions, (iii) the organism may have a deficiency in the secretion of the capsule, or (iv) regulation of capsule production may differ from *B. anthracis*.

It is possible that the temperatures and the media used were not optimal for the strain to produce the capsule. CBD 118 and 119 both grew luxuriantly at 30 to 35°C, and, although temperature tolerance was tested, the normal temperatures for culture incubation were not above 37°C. We used TSA and BUG, both supplemented with sodium bicarbonate, before performing India ink mounts and did not examine other media. The FDOH personnel used bicarbonate-supplemented media and horse serum when performing the antibody tests for the capsule. The incubator temperature used was 35 ± 2°C. Therefore, it is possible that the temperature was higher in the initial test and lower in subsequent tests. Other researchers have found that isolates that do not carry the pX01 plasmid needed incubation in 20% CO_2 in order to express the capsule (12). In fact, their strains did not express the capsule when incubated in 5% CO_2 even when sodium bicarbonate was added to the media. However, our *B. anthracis* strain (CBD 63) carries only pX02 and expressed the capsule, as seen by both gross examination of colonies and microscopic inspection of India ink mounts, regardless of whether or not the strain was incubated with 5% or 20% CO_2. Even at 20% CO_2, CBD 118 did not produce any capsule material that we could detect microscopically. Further examination of this organism's capsule operon and its regulation may help us understand what has occurred. In addition, if growth parameters and other conditions can be adjusted and result in capsule formation, we will attempt to directly analyze the capsule material. It is possible that CBD 118 is able to change the epitope or switch from one isomer to another. *B. anthracis* produces only the D isomer of glutamic acid (30), but perhaps *B. circulans* cannot normally do this easily and may switch to a mixture or to the L form. This would explain why the DFA results changed from positive to negative, but it does not explain why India ink did not detect a capsule since this test is not based on a specific molecular epitope. The bacterial cell may produce the capsule but may have difficulty in secreting the material to the exterior of the cell. The first positive DFA tests may have been performed on colonies that had lysed cells where the capsule components were released into the environment. Lastly, it may be noteworthy that we had difficulty in sequencing the full promoter sequence of the capsule operon, especially since there was a difference in the nucleotide sequence in both isolates. Perhaps the full promoter sequence is not present. This may explain why the capsule was not expressed in CBD 119, but not why it appeared to be expressed in CBD 118 initially.

The capsule of CBD 119 seen with India ink has not yet been analyzed. Because the antibody tests have been consistently negative for this isolate, the capsule could be composed of different material from that of *B. anthracis*, even though the *B. anthracis* capsule operon is present. There may be competition between the production and secretion of the native (*Bacillus* sp.) capsule material and the "foreign" (*B. anthracis*) capsule material. This will also be addressed in future studies.

While culturing is the "gold standard" for the isolation and identification of *B. anthracis*, PCR targeting for the virulence genes on pX01 and pX02 is normally employed as one of the first steps in the LRN protocols for the rapid presumptive detection of *B. anthracis* in a credible-threat environmental or clinical sample. For example, the genes in the capsule operon have long been successfully exploited for identifying the pX02 plasmid in such testing. However, the Tampa laboratory still identified 19 isolates that were positive for the chromosomal marker and the pX02 plasmid by the LRN protocol and considered the specimens to carry *B. anthracis* until further testing was performed. In addition, the FDOH laboratory has had specimens that tested positive for the pX02 target and negative for the other targets (chromosomal and pX01) although the exact number of these types of specimens is not known. In our collection of 300 soil, environmental, and clinical isolates of different *Bacillus* species of the *B. cereus* group (not including *B. anthracis*), we have 73 isolates that are positive for only Ba813 and 17 isolates with both Ba813 and pX02. It is also unknown how many other species of *Bacillus* in the environment have large plasmids that carry these virulence genes or other genes normally found in pX02. To our knowledge, a systematic search for this plasmid or genes in the general soil bacterial population has not been undertaken.

The LRN protocol uses three targets—the first on the chromosome, the second on pX01, and the third on pX02—to determine the presence of *B. anthracis* in environmental samples. After a colony is confirmed to be *B. anthracis* by gamma phage assay and DFA, PCR assays are used to confirm the presence of the two plasmids. For detection and identification purposes, the three targets (whether individually present or absent) are equally important. We do not suggest that the LRN discontinue testing for the pX02 plasmid because cases in which a Sterne or Pasteur strain was used would be missed. If there were a real biothreat event, the LRN would attempt to characterize fully the strain of *B. anthracis* involved and determine the danger to exposed persons. Yet the fact that 11 sequences that we analyzed are not limited to pX02 or to the *B. cereus* group raises questions about the usefulness of these sequences for detection. Perhaps other sequences carried on pX02 would be better targets for the molecular detection of *B. anthracis*, targets that are truly unique to this pathogen. Whether there is a sequence on pX02 that is limited to *B. anthracis* remains to be seen and will be further evaluated as we compare the plasmids from these two isolates to pX02. In conclusion, the presence of these capsule genes in two soil *Bacillus* species other than *B. anthracis* alerts us to the necessity of identifying additional genes or sequences that will signal the presence of *B. anthracis* in forensic, clinical, and environmental samples.

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