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Utilization of the *rpoB* Gene as a Specific Chromosomal Marker for Real-Time PCR Detection of *Bacillus anthracis*

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The potential use of *Bacillus anthracis* as a weapon of mass destruction poses a threat to humans, domesticated animals, and wildlife and necessitates the need for a rapid and highly specific detection assay. We have developed a real-time PCR-based assay for the specific detection of *B. anthracis* by taking advantage of the unique nucleotide sequence of the *B. anthracis rpoB* gene. Variable region 1 of the *rpoB* gene was sequenced from 36 *Bacillus* strains, including 16 *B. anthracis* strains and 20 other related bacilli, and four nucleotides specific for *B. anthracis* were identified. PCR primers were selected so that two *B. anthracis*-specific nucleotides were at their 3′ ends, whereas the remaining bases were specific to the probe region. This format permitted the PCR reactions to be performed on a LightCycler via fluorescence resonance energy transfer (FRET). The assay was found to be specific for 144 *B. anthracis* strains from different geographical locations and did not cross-react with other related bacilli (175 strains), with the exception of one strain. The PCR assay can be performed on isolated DNA as well as crude vegetative cell lysates in less than 1 h. Therefore, the *rpoB*-FRET assay could be used as a new chromosomal marker for rapid detection of *B. anthracis*.

Bacillus anthracis is a causal agent of anthrax, a serious and often fatal infection of livestock and humans. It is considered one of the most effective biological weapons of mass destruction because of its highly pathogenic nature and spore-forming capability and has attracted attention due to its potential use as a biological warfare agent (2). This bacterium can infect humans by cutaneous, gastrointestinal, or respiratory routes. The standard laboratory method of identification takes advantage of the lytic nature of the *B. anthracis*-specific gamma bacteriophage (9). Anthrax bacilli are often distinguished on the basis of time-consuming morphological or phenotypic characteristics, such as gram-positive staining, spore-forming capability, nonhemolytic reaction on sheep blood agar, sensitivity to penicillin, nonmotile nature, and inability to ferment salicin (11). *B. anthracis* is distinguished from the other members of the closely related *Bacillus cereus* group of bacteria by the presence of the toxin-encoding pXO1 (19, 24) and capsule-encoding pXO2 plasmids (14, 23, 34). Both plasmids are needed for virulence; thus, the absence of either plasmid results in attenuation.

B. anthracis, Bacillus thuringiensis, B. cereus, and *Bacillus mycoides*, are members of the *B. cereus* group of bacilli. These closely related bacteria are pathogens of mammals (*B. anthracis* and *B. cereus*) and insects (*B. thuringiensis*). The *B. cereus* group is one of the most taxonomically ambiguous group of bacilli (27). In fact, DNA-DNA hybridization (30) and pulsed-field gel electrophoresis (15) have shown great homology among *B. anthracis, B. thuringiensis*, and *B. cereus*. A recent multilocus enzyme electrophoresis study has concluded that the members of this group belong to one species (16).

Although specific assays are available for the detection of pathogenicity-related plasmids (18, 28), chromosomal markers in conjuction with plasmid markers should be used for complete genotyping of *B. anthracis* strains. Such a combined approach will provide insight into the chromosomal backbone or genetic background and indicate the pathogenic nature of the strain. Plasmids are more unstable than chromosomal DNA, and isolates lacking either or both plasmids have been found to exist in nature (33). Also, pXO2 has been successfully transferred into other bacilli, and toxin genes, such as *lef* and *cya*, have been expressed in heterologous systems (4, 5, 20). Thus, naturally occurring as well as genetically modified *B. anthracis* strains cannot be characterized without ambiguity. Moreover, chromosomal markers are stable targets for detection and are important for accurate identification of *B. anthracis* in outbreaks (26) as well as during the analysis of ancient samples (C. Redmond, M. J. Pearce, R. J. Manchee, and B. P. Berdal, Letter, Nature **393:**747–748).

Several chromosomal markers are currently available for *B. anthracis* detection, such as the *vrrA* gene (1, 17), Ba813 marker (25), and SG-850 marker (10). These marker assays suffer from being time consuming or labor intensive or having limited specificity. For instance, the SG-850 assay involves PCR amplification of the SG-749 locus, followed by enzymatic digestion with *Alu*I and gel analysis. The *vrr*A marker can group *B. anthracis* isolates into several categories based on the number of repeat units of this sequence, which requires post-PCR analysis (17). Recently some *B. cereus* and *B. thuringiensis* isolates have been found to contain the Ba813 marker (26); hence its use as a *B. anthracis*-specific target is questionable (29). The 16S rRNA gene also does not provide sufficient polymorphism to differentiate *B. anthracis* from closely related

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TABLE 1. *B. anthracis* strains used in the *rpoB*-FRET PCR assays

Strain	pXO1[a]	pXO2[a]	Origin	Strain	pXO1[a]	pXO2[a]	Origin
7700	−	−	Africa	A66	+	+	Europe
7702	+	−	Africa	A67	+	+	Europe
Sterne	+	−	Africa	A68	+	+	Europe
AC1	+	−	S. America	A69	+	+	Europe
AC2	+	−	S. America	A72	+	+	Europe
AC3	+	+	S. America	A73	−	+	Europe
Texas 0077	+	−	N. America	A74	−	−	Europe
Ames	+	+	N. America	001	+	+	Asia
Δ Ames	−	−	N. America	002	+	+	Asia
Vollum	+	+	Europe	044	+	−	Asia
4229	−	+	Europe	103	+	+	Asia
RA3	+	+	Europe	104	+	+	Asia
RA3R	+	−	Europe	105	+	+	Asia
RA4	+	+	Europe	108	+	+	Asia
7611	+	+	Europe	116	+	+	Asia
7611R	+	−	Europe	125	+	−	Asia
10517	+	+	Europe	126	+	+	Asia
6183	+	+	Europe	128	+	−	Asia
6042	+	+	Europe	129	+	+	Asia
BC 575	+	+	Europe	136	+	+	Asia
9066	+	+	Europe	137	+	+	Asia
7193	+	+	Europe	139	+	+	Asia
6681	+	+	Europe	140	+	+	Asia
8403	+	+	Europe	141	+	+	Asia
9240	+	+	Europe	142	+	−	Asia
8490	+	+	Europe	143	+	−	Asia
6291	+	+	Europe	144	+	+	Asia
A3	−	+	Europe	145	+	+	Asia
A4	−	+	Europe	146	+	+	Asia
A5	+	+	Europe	147	+	+	Asia
A6	−	+	Europe	148	+	+	Asia
A7	+	+	Europe	149	+	+	Asia
A8	+	+	Europe	150	+	+	Asia
A9	+	+	Europe	151	+	+	Asia
A10	+	+	Europe	152	+	+	Asia
A11	+	+	Europe	153	+	+	Asia
A12	+	+	Europe	154	+	+	Asia
A16	+	+	Europe	155	+	+	Asia
A18	−	+	Europe	156	+	+	Asia
A19	−	+	Europe	157	+	+	Asia
A22	+	+	Europe	158	+	+	Asia
A23	−	+	Europe	159	+	+	Asia
A24	+	+	Europe	160	+	+	Asia
A25	+	+	Europe	161	+	+	Asia
A28	+	+	Europe	162	+	+	Asia
A29	+	+	Europe	163	+	+	Asia
A30	+	+	Europe	164	+	−	Asia
A32	+	+	Europe	165	+	+	Asia
A33	+	+	Europe	166	+	+	Asia
A34	+	+	Europe	167	+	+	Asia
A35	+	+	Europe	168	+	+	Asia
A36	+	+	Europe	169	+	+	Asia
A37	+	+	Europe	170	+	+	Asia
A38	+	+	Europe	171	+	+	Asia
A39	+	+	Europe	172	+	+	Asia
A40	+	+	Europe	173	+	+	Asia
A41	+	+	Europe	174	+	+	Asia
A42	+	+	Europe	175	+	+	Asia
A43	+	+	Europe	176	+	+	Asia
A44	+	+	Europe	177	+	+	Asia
A45	+	+	Europe	178	+	+	Asia
A46	−	+	Europe	179	+	+	Asia
A47	+	+	Europe	180	+	+	Asia
A49	+	+	Europe	181	+	+	Asia
A58	−	−	Europe	182	+	+	Asia
A59	+	+	Europe	183	+	+	Asia
A60	−	+	Europe	Ba107	+	+	ND
A61	+	+	Europe	ΔUM-2311	−	−	ND
A62	+	+	Europe	ΔANR-1099	−	−	ND
A63	+	+	Europe	1014	−	+	ND
A64	+	+	Europe	ACB	+	+	ND
A65	+	+	Europe	0074	+	−	ND

[a] Plasmids were detected by PCR (28). +, detected; −, not detected; ND, no data.

TABLE 2. *rpoB Bacillus* species sequences analyzed in this study

Bacillus species	Strain ID	pXO1[a]	pXO2[a]	Derivation or location	Accession no.
B. anthracis	Vollum	−	+	Europe	AF205319
B. anthracis	Ames	+	+	N. America	AF205320
B. anthracis	ΔAmes	−	−	N. America	AF205321
B. anthracis	ΔUM-2311	−	−	Unknown	AF205322
B. anthracis	Sterne	+	−	Africa	AF205323
B. anthracis	Texas 0077	+	−	N. America	AF205324
B. anthracis	001	+	+	Asia	AF205325
B. anthracis	002	+	+	Asia	AF205326
B. anthracis	044	+	−	Asia	AF205327
B. anthracis	A58	−	−	Europe	AF205328
B. anthracis	A74	−	−	Europe	AF205329
B. anthracis	A7	+	+	Europe	AF205330
B. anthracis	AC1	+	−	S. America	AF205331
B. anthracis	7193	+	+	Europe	AF205333
B. anthracis	8403	+	+	Europe	AF205334
B. anthracis	RA3	+	+	Europe	AF205335
B. cereus	14579	−	−	ATCC[b] 14579	AF205336
B. cereus	229	−	−	Unknown	AF205337
B. cereus	27877	−	−	ATCC 27877	AF205338
B. cereus	49069	−	−	ATCC 49069	AF205339
B. cereus	776	−	−	ATCC 19637	AF205341
B. cereus	23261	−	−	ATCC 23261	AF205342
B. mycoides	6462	−	−	ATCC 6462	AF205343
B. thuringiensis	T07-005	−	−	IEBC[c] T07-005	AF205344
B. thuringiensis	T07-146	−	−	IEBC T07-146	AF205345
B. thuringiensis	T07-202	−	−	IEBC T07-202	AF205346
B. thuringiensis	10	−	−	Asia	AF205347
B. thuringiensis	35646	−	−	ATCC 35646	AF205348
B. thuringiensis	B8	−	−	Unknown	AF205349
Bacillus spp.	Ba813_11 (9594/3)	−	−	Europe	AF205350
Bacillus spp.	Ba813_12 (S8553/2)	−	−	Europe	AF205351
Bacillus spp.	Ba813_31 (IB/A)	−	−	Middle East	AF205352
Bacillus sp.	AX16	−	−	Unknown	AF205353
Bacillus sp.	N52	−	−	Unknown	AF205354
Bacillus sp.	V770	−	−	Unknown	AF205355
B. subtilis	6051	−	−	ATCC 6051	AF205356

[a] Ramisse et al., 1996 (28). +, detected; −, not detected; ND, no data.
[b] American Type Culture Collection, Manassas, Va.
[c] International Entomopathogenic *Bacillus* Centre Collection, Pasteur Institute, Paris, France.

bacilli (3). Thus, no absolutely specific chromosomal marker is presently available for the detection of *B. anthracis*.

The *rpoB* gene, which codes for the β-subunit of RNA polymerase, has served as a signature sequence for bacterial identification as well as a locus for phylogenetic analysis (21). Moreover, *rpoB* is a highly conserved housekeeping gene, and at least one copy is present in all bacteria because of its essential role in cellular metabolism. This gene, along with *rpoC*, which encodes for the β′-subunit, constitutes the catalytic center of the pentameric bacterial RNA polymerase (6). Due to its discriminatory power, the *rpoB* gene has been used to develop probes for specific detection and phylogenetic analysis of *Coxiella burnetti, Rickettsia*, and *Yersinia pestis* (12, 13, 22).

Bacterial strains and DNA preparation. A total of 144 *B. anthracis* strains from different geographical locations (Table 1), 29 *B. cereus* strains, 49 *B. thuringiensis* strains, 73 *Bacillus* spp. Ba813$^+$ strains (29), a strain each of *B. mycoides, B. subtilis*, and *B. megaterium*, and 22 unknown bacilli were used to test the specificity of the assay. Sixteen *B. anthracis* strains and a total of 20 other bacilli strains of *B. cereus* ($n = 6$), *B. thuringiensis* ($n = 6$), *B. mycoides* ($n = 1$), *B. subtilis* ($n = 1$), and other bacilli ($n = 6$) were used for the determination of variable region 1 of the *rpoB* gene sequence (Table 2). All strains used in this study were analyzed for plasmid content by a multiplex PCR assay (28). DNA was extracted by a method outlined by Schraft and Griffiths (32) with modifications as described elsewhere (8). For preparing crude vegetative cell lysates, a sterile toothpick was used to transfer a portion of a fresh colony into 300 μl of distilled water. The cell suspension was boiled at 100°C for 15 min and then centrifuged at 8,000 × *g* for 5 min. The supernatant was transferred to a fresh microcentrifuge tube and stored at −20°C until further use. For isolating the rifampin-resistant mutant, an individual fresh colony of the rifampin-sensitive *B. anthracis* 7700 was streaked out on a brain heart infusion agar containing 25 μg of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml and was incubated overnight at 37°C. Rifampin-resistant colonies were plated out a second time onto a plate containing 50 μg of rifampin/ml in order to confirm this phenotype.

Low-stringency PCR amplification and sequence analysis. The alignment of the amino acid sequences of the RNA polymerase β-subunits of *Bacillus subtilis* and *Escherichia coli* permitted the identification of two conserved regions. The conserved region found near the N terminus was RVIVSQ,

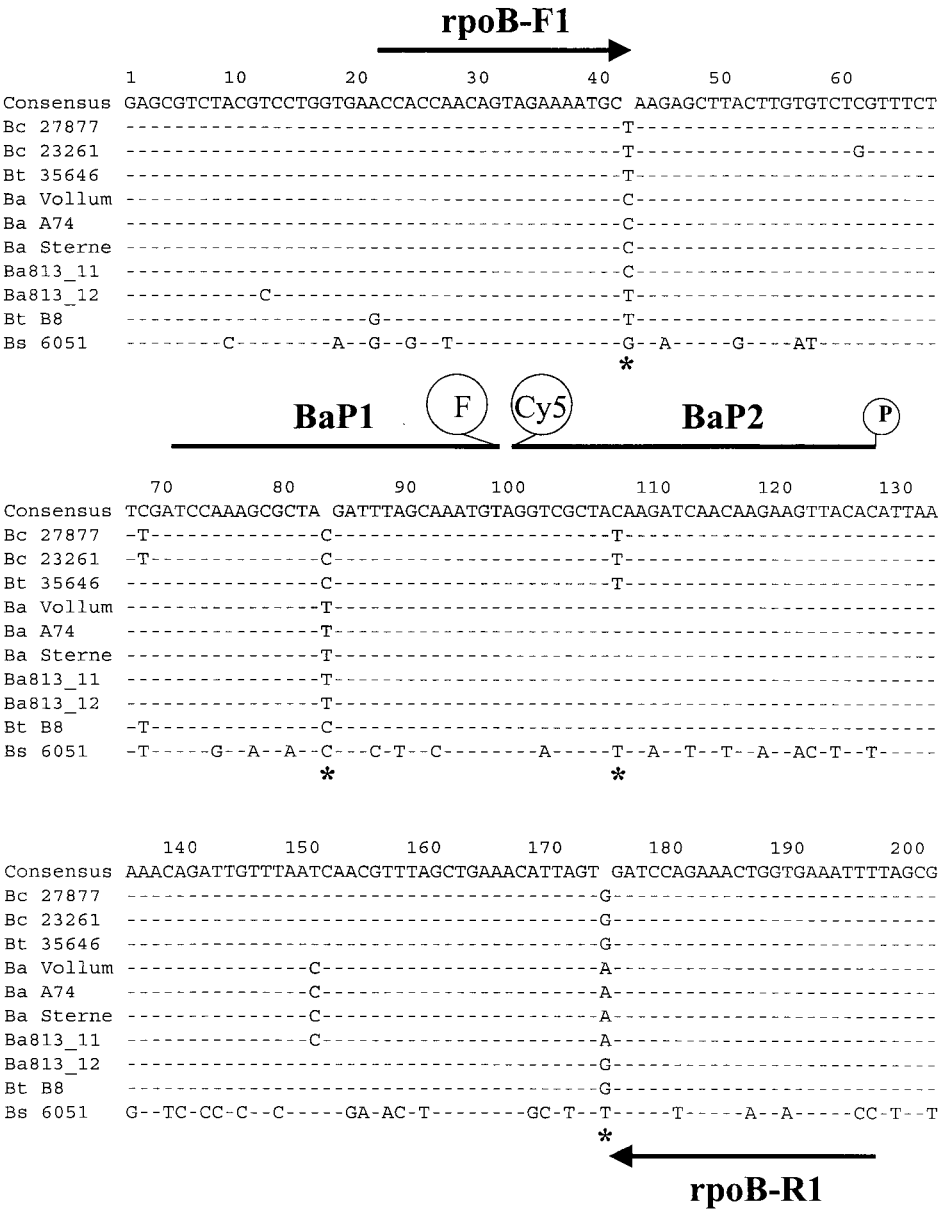

FIG. 1. Alignment of the nucleotide sequences from 10 representative *Bacillus* strains using Clustal W (32). The strains are the following: Bc 27877, *Bacillus cereus* 27877; Bc 23261, *Bacillus cereus* 23261; Bt 35646, *Bacillus thuringiensis* 35646; Ba Vollum, *Bacillus anthracis* Vollum; Ba A74, *Bacillus anthracis* A74; Ba Sterne, *Bacillus anthracis* Sterne; Ba813_11, *Bacillus* sp. strain Ba813_11; Ba813_12, *Bacillus* sp. strain Ba813_12; Bt B8, *Bacillus thuringiensis* BtB8; Bs 6051, *Bacillus subtilis* 6051. The locations of primers and probes are shown (F, fluorescein; Cy5, cyanine 5; P, phosphate group); the presence of an asterisk denotes a mismatch, a dash indicates identity with the consensus sequence, and nucleotide letters indicate positions showing polymorphism.

spanning amino acid residues 132 to 137 of *B. subtilis* and residues 143 to 148 of *E. coli* (6). The C terminus conserved region was DDIDHL, and it was found at positions 399 to 404 of *B. subtilis* and positions 443 to 448 of *E. coli*.

The sequences of primer rpoB1 (5′-CGTGTTATCGTTTC CCAGC-3′) and rpoB2 (5′-AAGATGATCGATATCATC TG-3′) were derived from the two conserved regions and correspond to nucleotides (nt) 1482 to 1500 and 2281 to 2300 of the *B. subtilis rpoB* gene (GenBank accession no. L24376). The PCR reaction mixture of 50 μl consisted of 10 mM Tris-HCl (pH 8.3), 75 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTPs (Boehringer Mannheim Corp., Indianapolis, Ind.), 1 μM (each) primers rpoB1 and rpoB2, 0.05 U of Ampli*Taq* DNA polymerase (Perkin Elmer Corp., Foster City, Calif.)/μl, and 100 ng of DNA template. Amplification was performed in a GeneAmp PCR System 2400 (Perkin-Elmer Corp., Norwalk, Conn.), and the cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 7 min. The amplicons were detected in 2% (wt/vol) SeaKem GTG agarose (FMC Bioproducts, Rockland, Maine) with 40 mM Tris-acetate–1mM EDTA (pH 8.3) as a running buffer and visualized by ethidium bromide staining.

Low-stringency amplification of the variable region of the

TABLE 3. Primers and probes used in the FRET-PCR assay

Primer or probe	Position[a]	T_m (°C)[b]	Sequence (5′→3′)[c]
rpoBF1 primer	(1821–1841)	64.6	CCA CCA ACA GTA GAA AAT GCC
rpoBR1 primer	(1973–1995)	64.2	AAA TTT CAC CAG TTT CTG GAT CT
BaP1 probe	(1871–1897)	74.4	TCC AAA GCG CTA TGA TTT AGC AAA TGT-F
BaP2 probe	(1899–1928)	74.1	Cy5-GGT CGC TAC AAG ATC AAC AAG AAG TTA CAC-P

[a] Based on the *B. subtilis rpoB* gene (GenBank accession no. L24376).
[b] Nearest neighbor method.
[c] F, fluorescein; Cy5, cyanine 5; P, phosphate.

rpoB gene from different *Bacillus* species and strains yielded the expected amplicons with a size of 819 bp. Bands of the expected size were excised from the gel, and the DNA was extracted using a QIAquick Gel Extraction kit (QIAGEN Inc., Valencia, Calif.). The PCR products were cloned into vector pCR 2.1 (Invitrogen Corp., Carlsbad, Calif.) and transformed into *E. coli*. Recombinant plasmids were prepared using the QIAGEN Plasmid Mini Kit. Three clones from each ligation reaction were sequenced in duplicate with the M13 forward and reverse primers using the Applied Biosystems model 373A automated sequencer and the BigDye terminator ready reaction kit (Perkin-Elmer Applied Biosystems). The nucleotide sequences were edited and assembled with the Sequencing Analysis 3.0 and AutoAssembler 3.1.2 programs, respectively; translation into amino acids was accomplished using the Sequence Navigator 3.0.1 program (Perkin-Elmer Applied Biosystems). These 36 sequences were aligned using the Clustal W program (32) from BioNavigator (eBioinformatics Pty Ltd: http://www.ebioinformatics.com/), and four bases specific for *B. anthracis* were identified. Figure 1 shows the alignment and nucleotide differences of 10 representative strains, including 3 strains of *B. anthracis*, 2 strains of *B. cereus*, 2 strains of *B. thuringiensis*, 2 $Ba813^+$ strains of *Bacillus* sp. (29), and a single strain of *B. subtilis*. Thus, nucleotides C (position 42), T (position 84), C (position 108), and A (position 174) are unique to *B. anthracis*, with the exception of Ba813_11 (Fig. 1). The region of the *rpoB* gene described in this study appears to be the only region within the *rpoB* gene that shows variation among different species of bacteria (6).

Nucleotide sequence accession number. The nucleotide sequences of the portion of the *rpoB* gene (variable region 1) described in this study were submitted to GenBank, and the accession numbers are listed in Table 2.

The translation of the nucleotide sequences showed that four bases specific for *B. anthracis* were in the third positions of the codons and did not change the amino acid sequence. The positions of the amino acids in the β-subunit were alanine at 251, tyrosine at 265, tyrosine at 273, and valine at 295. Thus, although there are differences in the nucleotide sequences, no differences were found in the primary sequence of the RpoB proteins for *B. anthracis, B. cereus*, and *B. thuringiensis*.

FRET-PCR assay. The primers rpoBF1 and rpoBR1 (Table 3; Fig. 1) were selected for high-stringency PCR amplification using Oligo 6 software (National Biosciences Inc., Plymouth, Minn.). The probes BaP1 (3′ end labeled with Fluorescein) and BaP2 (5′ end labeled with Cy5 and 3′ blocked with a phosphate group) were placed 1 bp apart within the PCR product (Fig. 1) and had T_ms (7) at least 10°C higher than those of the amplification primers (Table 3).

The PCR mixture (10 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 250 μg of bovine serum albumin/ml (Roche Molecular Biochemicals, Indianapolis, In), 1 μM (each) primers rpoBF1 and rpoBR1, 0.2 μM probe BaP1, 0.4 μM probe BaP2, 0.8U of DNA polymerase Klen*Taq*1 (Ab Peptides, St. Louis, Mo.), and 50 ng of DNA template or 2 μl of crude vegetative cell lysate. The amplification was performed on a Light-Cycler (Idaho Technology, Idaho Falls, Idaho), which is a rapid, forced-air thermocycler with an integrated fluorimeter for real-time monitoring of PCR reactions (35). The amplification was accomplished by initial denaturation at 95°C for 30 s, followed by 35 cycles of 95°C for 0 s, 63°C for 15 s, and 72°C for 5 s. Once the capillaries were placed in the thermocycler, amplification could be completed in less than 30 min. Detection of the amplification products is accomplished by hybridization of a pair of probes to the amplicons as they are formed, resulting in a fluorescence resonance energy transfer (FRET) (35). Fluorescence was measured once every cycle at the annealing step using the F2/F1 filter to monitor amplification in real time. F1 corresponds to the baseline fluorescein fluorescence, while F2 indicates FRET from fluorescein to Cy5, resulting in the ratio of Cy5/fluorescein fluorescence (F2/F1). The increase in fluorescence is proportional to the amount of PCR product generated (7, 35) and is displayed on the computer screen in the real-time mode. The reactions showing an increase in fluorescence by a minimum of 0.05 fluorescence units (y axis) were scored as positive amplification reactions. The PCR products were also visualized by 2% (wt/vol) gel electrophoresis.

The FRET assay was performed on 144 *B. anthracis* strains, harboring any combination of the two plasmids and isolated from different geographical locations (Table 1). All these strains tested positive in the FRET assay, since they displayed an increase in fluorescence as well as the presence of the expected PCR product by agarose gel analysis. Another 175 closely related strains, including the *B. cereus* group and $Ba813^+$ strains, were tested as negative controls to check the specificity of the assay. All related strains, with the exception of Ba813_11, were scored as negative because they did not exhibit an increase in fluorescence. Figure 2 shows the results of the FRET-PCR assay and the electrophoresis of the PCR amplicons using genomic DNA samples from representative strains. *B. cereus, B. thuringiensis, Bacillus* sp. strain Ba813_12, and *B. subtilis* did not show amplification. The *rpoB* FRET-PCR assay is extremely specific for *B. anthracis* because of the high-stringency PCR conditions coupled with the unique nature of the primers and probes. This specificity occurs at two different levels. The first is at the primer level, as seen in the case of *Bacillus* sp. strain Ba813_12. In this instance PCR products

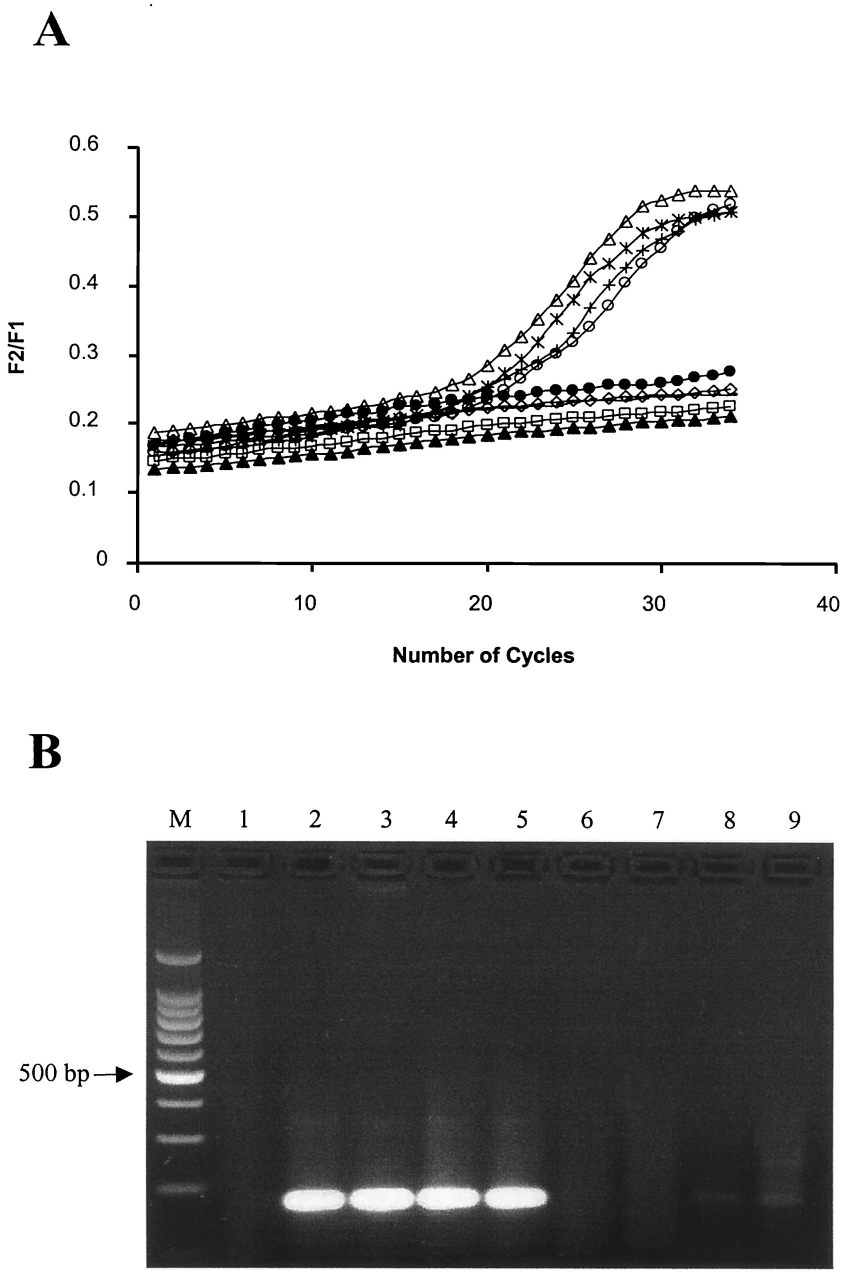

FIG. 2. Results of the FRET-PCR assay using genomic DNA. (A) Fluorescence ratio (F2/F1) is plotted against the number of PCR cycles. The samples are the following: 1, □, negative control (no DNA); 2, △, *Bacillus anthracis* A74; 3, ○, *Bacillus anthracis* Sterne; 4, ✱, *Bacillus* sp. strain Ba813_11; 5, +, *Bacillus anthracis* Vollum; 6, ●, *Bacillus cereus* 27877; 7, ◇, *Bacillus cereus* 23261; 8, −, *Bacillus* sp. strain Ba813_12; 9, ▲, *Bacillus thuringiensis* BtB8. (B) Gel electrophoresis of the PCR products. Lanes, M: 100 bp DNA ladder; 1, negative control (no DNA); samples 2 to 9 are the same as in panel A.

were not generated due to single base-pair difference at 3′ end of both primers, and as a result an increase in fluorescence was not observed in spite of 100% homology of the probe region with the *B. anthracis* sequence. The second level of specificity is at the probe level, since 100% base-pairing of probes with target sequence is required for FRET to occur. A single base-pair mismatch between either of the probe sequences with the target region stops the FRET process, indicating a negative result (unpublished data).

The amplicon derived from *Bacillus* sp. strain Ba813_11 was sequenced, and it was found to have a nucleotide sequence identical to that of *B. anthracis* (Fig. 1). Consequently, the FRET-PCR assay reported here cannot distinguish *Bacillus* sp. strain Ba813_11 from *B. anthracis* strains. The remaining 71 *Bacillus* spp. Ba813$^+$ strains have sequence identical to that of *Bacillus* sp. strain Ba813_12 in primer and probe binding regions, and *Bacillus* sp. strain Ba813_11 appears to be an exception. This strain, *Bacillus* sp. strain Ba813 (9594/3), was

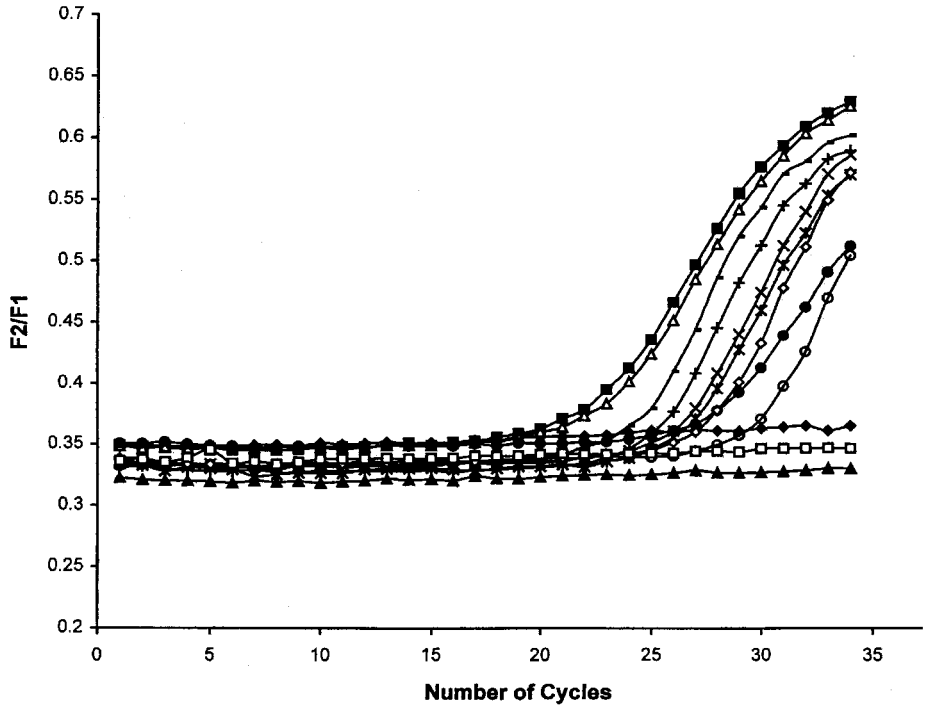

FIG. 3. Results of the FRET-PCR assay using crude vegetative cell lysates. The fluorescence ratio (F2/F1) is plotted against the number of PCR cycles. The sample are the following: ▲, negative control (no DNA); ■, *Bacillus anthracis* AC3; △, *Bacillus anthracis* 7702; ×, *Bacillus anthracis* ΔUM-2311; ✱, *Bacillus anthracis* A74; ●, *Bacillus anthracis* 0074; +, *Bacillus anthracis* Texas 0077; −, *Bacillus anthracis* ΔANR-1099; ○, *Bacillus anthracis* 7700; ◇, *Bacillus anthracis* A58; □, *Bacillus cereus* 14579; ♦, *Bacillus thuringiensis* 10792.

isolated from a station effluent in the Alps in 1997 and was designated a transitional strain because it could not be assigned to a particular species (26, 29). According to the SG-749 locus signature, *Bacillus* sp. strain Ba813_11 belongs to the *B. cereus* group (data not shown) and was shown to contain the $Ba813^{+}$ marker (26). In contrast to the phenotypic characteristics of *B. anthracis*, the *Bacillus* sp. strain Ba813_11 is hemolytic, motile, and resistant to penicillin, although it has an *rpoB* variable region 1 identical to that of *B. anthracis*.

The assay was not affected by the presence of exogenously added *E. coli* or mixed *Bacillus* species DNA (25 ng of *B. anthracis* DNA + 1,000 ng of exogenously added DNA) representing a mixed microbial community at the ratio of 1:40 (data not shown). The sensitivity of the FRET-PCR assay was examined using different concentrations of exogenously added DNA. Positive fluorescence signals and amplification, as shown by gel electrophoresis, were noticed even when as little as 1 pg of pure genomic DNA was used.

The FRET-PCR assay was also performed on crude vegetative cell lysates from *B. anthracis* and related bacilli. Figure 3 shows the results of the assay on selected strains. Only *B. anthracis* displayed an increase in fluorescence and the presence of the expected amplification product. The increase in fluorescence was observed after 22 to 30 cycles. The magnitude of increase in fluorescence is dependent on the quantity of template DNA or the copy number of the gene target in the reaction (35). It should be noted that the DNA amount was not normalized in the different cell lysates because the number of vegetative cells used in different samples was not identical. Thus, the assay can be directly used on freshly grown cultures for rapid identification of *B. anthracis* strains in less than 1 h. A rifampin-resistant colony exhibited positive results when tested by the FRET-PCR assay because the position of the mutation was found to be outside the *rpoB* target region, as is the case with *B. subtilis* (6). However, if new hotspots are found in the primer and/or probe binding sites, it may not be possible to use the assay for rifampin-resistant *B. anthracis* strains.

The FRET-PCR assay was able to clearly identify and distinguish *B. anthracis* from other closely related bacilli, signifying that the target of this assay is conserved in all strains of *B. anthracis* used in this study and that the detection of *B. anthracis* is independent of the plasmid content. These strains have been isolated from a wide variety of geographic locations, which gives us a reason to believe that this chromosomal marker will continue to be specific to *B. anthracis* even on further investigation. Extensive testing of strains of the *B. cereus* group has shown that the FRET-PCR assay is virtually free of cross-reactivity (99.4% specificity), with the exception of the case of *Bacillus* sp. strain Ba813_11. This assay can be used on endospore suspensions if PCR-amplifiable DNA is released from the spores.

The FRET-PCR assay has several advantages over standard molecular identification techniques. The amplification is monitored in real time, and reactions can be scored as positive or negative without time-consuming routine gel analysis. Moreover, the assay is rapid and highly sensitive when extracted DNA is used as a template for PCR. Using a DNA intercalating fluorescent dye such as SYBR Gold, the specificity of the reaction is evaluated at the end of the PCR amplification (18). The presence of contaminating DNA does not affect the results

of the assay, and hence it can be applied for detection of *B. anthracis* in epidemiological studies and suspected bioterrorist attacks and when analyzing ancient samples. Recent reevaluation of one of the *B. anthracis* strains (Zimbabwe) that was originally determined to be *rpoB* FRET positive has confirmed that it is in fact *rpoB* FRET negative. Moreover, using a newly described technique known as long-range repetitive-element polymorphism–PCR (8), we now have strong evidence suggesting that this strain needs to be regarded as a potential transitional *B. anthracis* strain. We are presently exploring this possibility, and in the meantime we have removed any mention of this particular strain from this report.

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