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Identification of *Bacillus anthracis* by *rpoB* Sequence Analysis and Multiplex PCR

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Comparative sequence analysis was performed upon *Bacillus anthracis* and its closest relatives, *B. cereus* and *B. thuringiensis*. Portions of *rpoB* DNA from 10 strains of *B. anthracis*, 16 of *B. cereus*, 10 of *B. thuringiensis*, 1 of *B. mycoides*, and 1 of *B. megaterium* were amplified and sequenced. The determined *rpoB* sequences (318 bp) of the 10 *B. anthracis* strains, including five Korean isolates, were identical to those of Ames, Florida, Kruger B, and Western NA strains. Strains of the "*B. cereus* group" were separated into two subgroups, in which the *B. anthracis* strains formed a separate clade in the phylogenetic tree. However, *B. cereus* and *B. thuringiensis* could not be differentiated. Sequence analysis confirmed the five Korean isolates as *B. anthracis*. Based on the *rpoB* sequences determined in the present study, multiplex PCR generating either *B. anthracis*-specific amplicons (359 and 208 bp) or *cap* DNA (291 bp) in a virulence plasmid could be used for the rapid differential detection and identification of virulent *B. anthracis*.

Bacillus anthracis is a large, gram-positive, aerobic, spore-forming bacillus. Its endospores do not divide, have no measurable metabolism, and are resistant to drying, heat, UV light, gamma radiation, and many disinfectants. In some cases, spores can remain dormant for decades. *B. anthracis* causes a zoonotic disease, anthrax. It also causes acute and often lethal disease in humans, such as cutaneous, intestinal, and pulmonary anthrax. For a long time, this species has attracted attention because of its hardiness, dormancy, and thus its potential use as a biological weapon (12, 13). In October 2001, *B. anthracis* spores were used to attack human populations in Florida, New Jersey, New York, and Washington, D.C. (12), which heightened public awareness and concern about anthrax.

B. anthracis infections are confirmed mainly by conventional microbiological methods, i.e., Gram staining, capsule staining, colony morphology, and biochemical characteristics (4, 18). However, because of its clinical importance and its implication concerning public security, suspected specimens are usually referred to public health laboratories for definitive identification, epidemiologic study, and susceptibility testing (28). Therefore, not only precise but also rapid identification of isolated *Bacillus* species is needed. In addition, it is also important to know whether detected or isolated *B. anthracis* strains contain virulence plasmids or not because the virulence of *B. anthracis* is related to encapsulating and toxin-encoding plasmids.

Given this situation, genotype analysis would seem to be most appropriate for the precise differential identification of virulent *B. anthracis*. However, genotype analysis is not straightforward for several reasons. Phylogenetically, *B. anthracis* is considered a member of the "*B. cereus* group," which also includes *B. cereus*, *B. thuringiensis*, and *B. mycoides* (18). Moreover, *B. anthracis* is genotypically differentiated from its close relatives, *B. cereus* and *B. thuringiensis*, only by the presence of toxin-encoding plasmids (19), and the genomes of these three species show high levels of similarity. For example, this group share almost identical 16S ribosomal DNA sequences (1), and for this reason were suggested to be one species based on multilocus enzyme electrophoresis (MLEE) (11). Moreover, the genome of *B. anthracis* has 11 rRNA operons, which show sequence polymorphisms at 10 positions (27). Analysis of other chromosomal genes such as *gyrB* (9, 35) and the 16S-23S ribosomal intergenic spacer (2), which are usually used for bacterial genotyping or phylogenetic analysis also failed to discriminate *B. anthracis* from *B. cereus* and *B. thuringiensis*. Furthermore, it seems to be even more difficult to differentiate them by plasmid gene analysis, because of plasmid transfer among the closest species. For example, genes in the plasmid of *B. anthracis* have been successfully expressed in other bacteria (30) and been reported in other *Bacillus* species (22). It is important to note that pXO2 can be lost naturally (32). Due to the natural competence of *B. thuringiensis* and *B. cereus*, the horizontal transfer of plasmids has been reported (8, 26, 35). The findings presented above show why the detection and identification of *B. anthracis* from clinical or environmental samples must be performed precisely and why *B. anthracis*-specific chromosomal markers should be developed to differentiate *B. anthracis* from its closest relatives (23).

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The *rpoB* gene, encoding the RNA polymerase β-subunit, has been used as a marker for bacterial identification and for phylogenetic study (5, 6, 14, 16, 17, 20, 25). Recently, the *rpoB* gene was used for the real-time PCR detection of *B. anthracis* (23); however, false-positive results were observed. According to Ellerbrok et al. (7), *B. cereus* and *B. megaterium* strains were also detected by real-time *rpoB* PCR and, therefore, a more reliable detection and identification method is required for *B. anthracis* chromosomal DNA.

In the present study, partial *rpoB* sequences (318 bp), which are located downstream of those used for real-time PCR (23) and which contain a region related to rifampin resistance, Rif^r (21, 33), were compared for the genotyping of *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. megaterium*. Subsequently, we undertook to identify five Korean isolates based on their *rpoB* sequences and to develop a simple multiplex PCR method that can be used for the rapid and differential detection of virulent *B. anthracis*.

MATERIALS AND METHODS

Bacterial strains, DNA extraction, and PCR amplification. Thirty-seven strains belonging to five species (*B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. megaterium*) were analyzed in the present study (Table 1). *B. anthracis* reference strains (ATCC 14185, ATCC 14186, ATCC 14578, Sterne, and Pasteur no. 2 Army strains) and five Korean isolates (GJ-1, GJ-2, BC, CN, and HS), which were isolated from the blood of infected humans and cows, were provided by I. J. Kim (School of Medicine, Dongguk University), W. Kim (Chung-Ang University College of Medicine), and J. M. Kim (National Veterinary Research and Quarantine Service). Although ATCC 14578 (Vollum strain) has both pXO1 and pXO2 originally, the pXO1-cured strain was used in the present study. The *rpoB* sequences of *B. anthracis* Florida isolate A2012, Ames, Kruger B, and Western NA strains were obtained from the public database GenBank or from the website of The Institute for Genomic Research (www.tigr.org). Total DNAs were extracted from cultured colonies by using the bead beater-phenol extraction method (14, 16) and used as a template for PCR. A primer pair, BA-RF (5′-GAC GAT CAT YTW GGA AAC CG-3′) and BA-RR (5′-GGN GTY TCR ATY GGA CAC AT-3′), was used to amplify a portion of *rpoB* DNA (359-bp) containing the *rif*^{r} region (14). Template DNA (ca. 50 ng) and 20 pmol of each primer were added to a PCR mixture tube (AccuPower PCR PreMix; Bioneer, Daejeon, Korea) containing 1 U of *Taq* DNA polymerase, a 250 μM concentration of deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 1.5 mM $MgCl_2$, and gel loading dye (16). The final volume was adjusted to 20 μl with distilled water, and the reaction mixture was then amplified for 30 cycles. Each cycle consisted of 30 s at 95°C for denaturation, 30 s at 45°C for annealing, and 1 min at 72°C for extension, and this was followed by a final extension at 72°C for 5 min (model 9700 ThermoCycler; Perkin-Elmer Cetus). Amplified PCR products were purified for sequencing by using a Qiaex II gel extraction kit (Qiagen, Hilden, Germany).

Nucleotide sequencing. Sequences of the purified PCR products were determined directly with forward and reverse primers by using an Applied Biosystems automated sequencer (model 377) and a BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom). For the sequencing reaction, 30 ng of purified PCR product, 2.5 pmol of each primer, and 4 μl of BigDye terminator RR mix (Perkin-Elmer Applied Biosystems; part no. 4303153) were mixed and adjusted with distilled water to a final volume of 10 μl. The reaction was run with 5% (vol/vol) dimethyl sulfoxide for 30 cycles of 15 s at 95°C, 5 s at 50°C, and 4 min at 60°C. Both strands were sequenced as a cross-check.

Sequence analysis and phylogenetic analysis. Alignment of the *rpoB* sequences (Fig. 1) was accomplished by using the MegAlign program in DNA STAR (Madison, Wis.). Amino acid sequences were also deduced by the MegAlign program. A phylogenetic tree was inferred from the *rpoB* nucleotide sequences by the neighbor-joining method described in PAUP (29) and by using *B. mycoides* and *B. megaterium* as outgroups to root the tree. Branch supporting values were evaluated with 1,000 bootstrap replications.

***B. anthracis*-specific PCR.** *B. anthracis*-specific forward primer (Ba-SF, 5′-TTC GTC CTG TTA TTG CAG-3′) was designed based on the aligned *rpoB* sequences (Fig. 1). This specific primer was utilized with BA-RF and BA-RR for the specific amplification of *B. anthracis* DNA by multiplex PCR. Multiplex PCR was performed as described above but with an extension time of 30 s. To test the specificity of the multiplex PCR, DNAs or cell suspensions of other *Bacillus* species and of the *B. cereus* group members examined in the present study were also applied as templates. PCR products were analyzed by electrophoresis in a 3% agarose gel.

The multiplex PCR targeting *rpoB* DNA to identify *B. anthracis* was performed simultaneously with the *cap* PCR, which is a molecular detection method based on the virulence plasmid (pXO2) by using a Cap-S and Cap-R primer set (7). Virulent *B. anthracis* strains, which can make capsule by *cap* in pXO2, will show three different bands. One is *Bacillus* genus specific (359 bp), the second is *B. anthracis* specific (208 bp), and the third is virulence plasmid DNA (291 bp).

TABLE 1. *B. anthracis, B. cereus, B. thuringiensis, B. mycoides,* and *B. megaterium* strains used in this study

Bacillus sp.	Strain no.[a]	Accession no.
B. anthracis Sterne		AY169510
B. anthracis Pasteur no. 2 Army		AY169511
B. anthracis	ATCC 14185	AY169512
B. anthracis	GJ-1[c]	AY169513
B. anthracis	GJ-2[c]	AY169514
B. anthracis Ames[b]		NC003997[b]
B. anthracis Florida[b]	A2012	NC003995[b]
B. anthracis Kruger B[b]		NC004126[b]
B. anthracis Western NA[b]		
B. cereus	ATCC 9634	AY169515
B. cereus	IMSNU 11011	AY169516
B. cereus	IMSNU 11012	AY169517
B. cereus	IMSNU 11013	AY169518
B. cereus	IMSNU 13043	AY169519
B. cereus	IMSNU 13044	AY169520
B. cereus	IMSNU 13045	AY169521
B. cereus	IMSNU 13046	AY169522
B. cereus	IMSNU 13047	AY169523
B. cereus	IMSNU 12076	AY169524
B. cereus	IMSNU 12077	AY169525
B. cereus	IMSNU 12078	AY169526
B. cereus	IMSNU 12079	AY169527
B. cereus	KCTC 1012	AY169528
B. cereus	KCTC 1014	AY169529
B. thuringiensis	KCTC 1507	AY169530
B. thuringiensis	KCTC 1509	AY169531
B. thuringiensis	IMSNU 12089	AY169532
B. thuringiensis subsp. *berliner*	IMSNU 12095	AY169533
B. thuringiensis subsp. *dendrolimus*	IMSNU 12096	AY169534
B. thuringiensis subsp. *entomocidus*	IMSNU 12097	AY169535
B. thuringiensis subsp. *finitimus*	IMSNU 12098	AY169536
B. thuringiensis subsp. *indiana*	IMSNU 12099	AY169537
B. thuringiensis subsp. *kurstaki*	IMSNU 10051	AY169538
B. thuringiensis subsp. *pakistani*	IMSNU 12092	AY169539
B. mycoides	KCCM 40260	AY169540
B. megaterium	KCTC 3007	AY169541

[a] ATCC, American Type Culture Collection; IMSNU, Institute of Microbiology Seoul National University; KCTC, Korean Collection for Type Cultures; KCCM, Korean Culture Center of Microorganisms.

[b] *B. anthracis* strains that for which the *rpoB* sequences were obtained from the public database GenBank or from the website of The Institute for Genomic Research (www.tigr.org).

[c] *B. anthracis* strains isolated from human (GJ-1) and cow (GJ-2) sources in Korea.

RESULTS

***rpoB* sequence analysis.** The *rpoB* sequences (318 bp), determined unambiguously in the present study, showed >95.3% similarity between the strains of *B. cereus*, *B. thuringiensis*, and *B. anthracis*. After we excluded the most divergent strain, *B. cereus* IMSNU 13043, the least similarity increased to 96.9%.

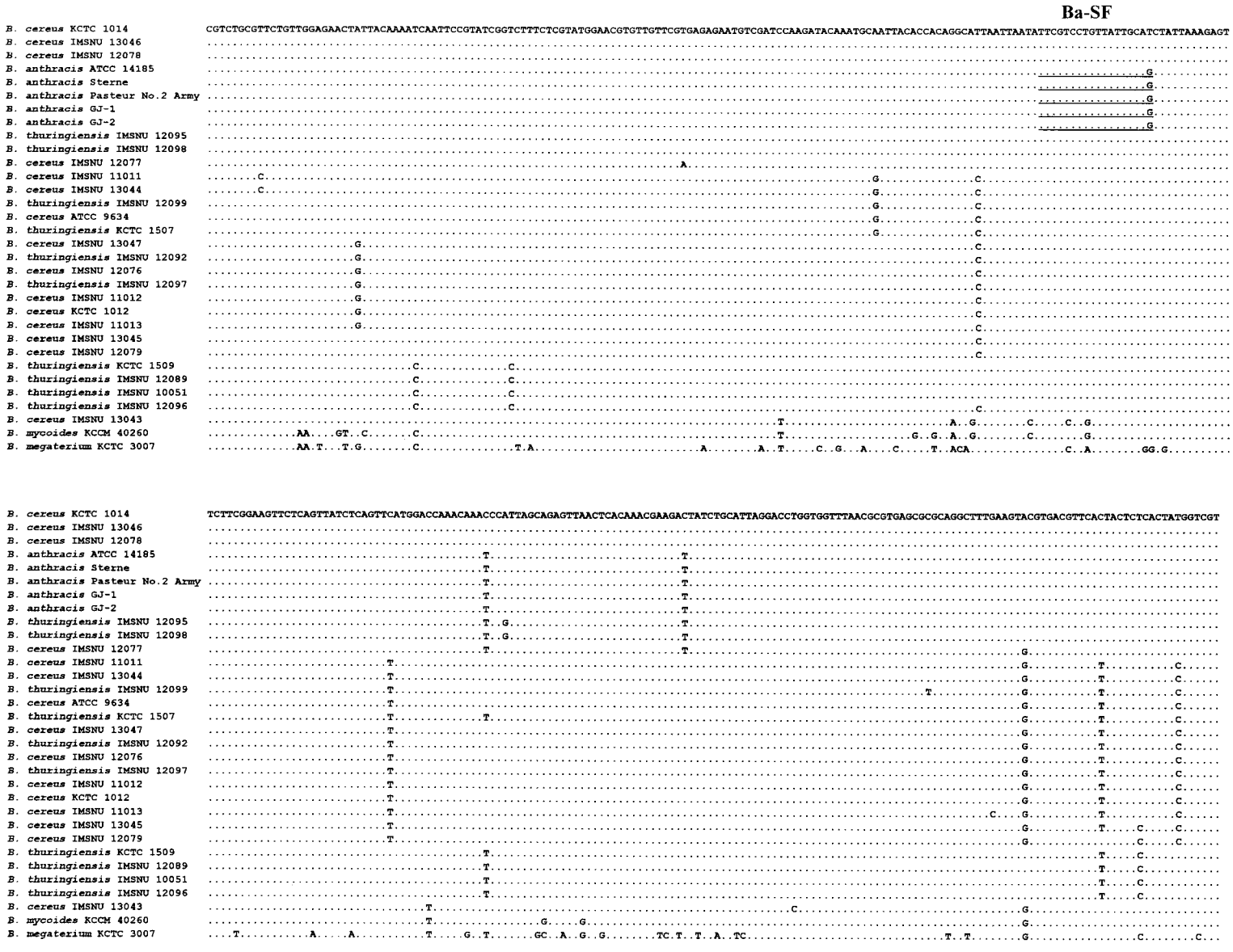

FIG. 1. *rpoB* nucleotide sequences (318 bp) from 31 strains of *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. megaterium*. Nucleotides identical to those of *B. cereus* KCTC 1014 are indicated by dots. The sequences of the five Korean isolates (GJ-1, GJ-2, BC, CN, and HS) were identical to those of the other *B. anthracis* strains. The underlined sequences indicate *B. anthracis*-specific forward primer (Ba-SF).

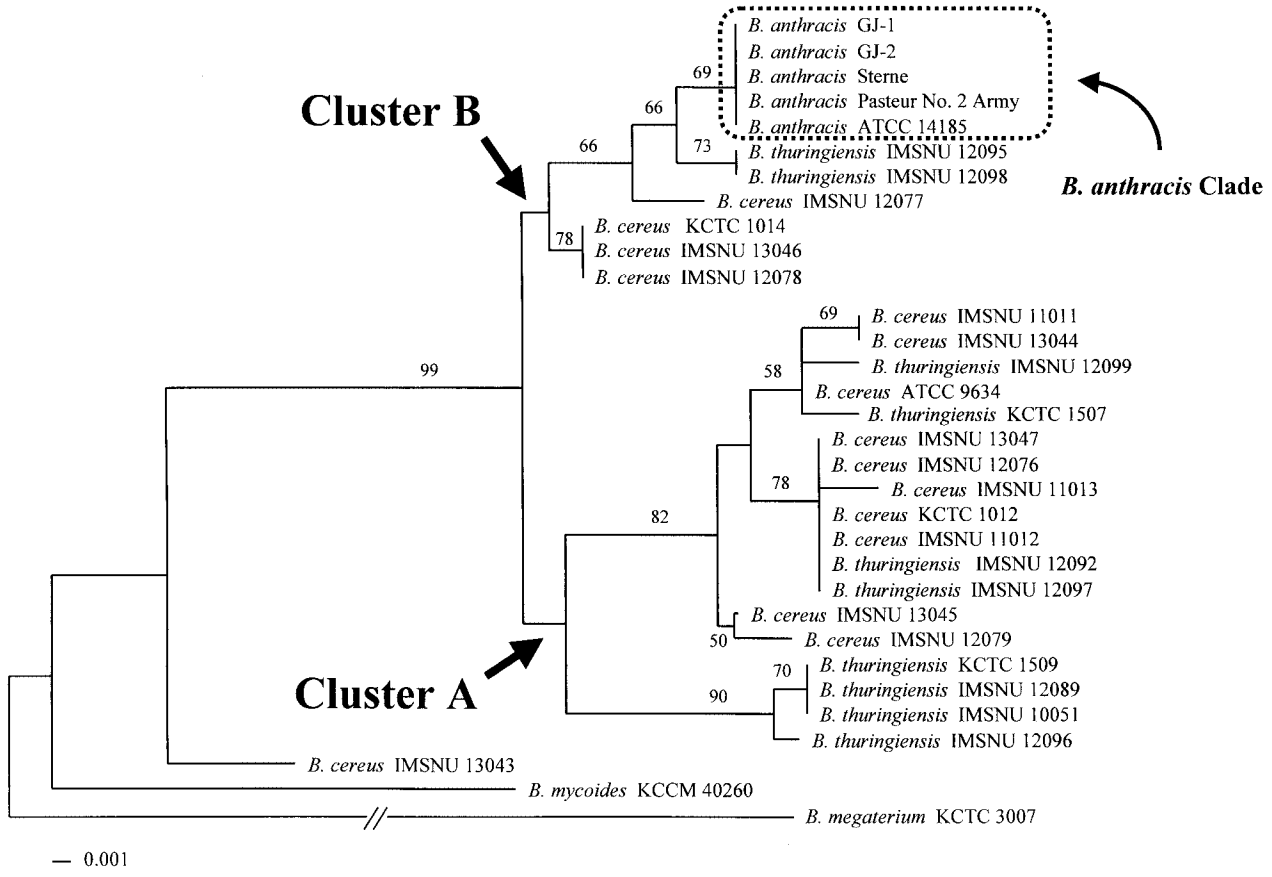

FIG. 2. Phylogenetic relationships of the 34 strains of the *Bacillus cereus* group inferred from partial *rpoB* DNA sequences. The tree was constructed by the neighbor-joining method in PAUP (29). *B. mycoides* and *B. megaterium* were used as outgroups to root the tree. The bootstrap values presented at corresponding branches were determined from 1,000 replications; those with values of <50% are not indicated. Clusters A and B identified in the present study are indicated by arrows and the *B. anthracis* clade is indicated by the box.

Five *B. anthracis* reference strains (ATCC 14185, ATCC 14186, ATCC 14578, Sterne, and Pasteur no. 2 Army strains) had the same *rpoB* sequence as the four whole-genome sequenced strains, namely, the Ames, Florida, Kruger B, and Western NA strains. The five Korean isolates also had sequences identical to those of the reference strains. Thus, the amplification and sequencing of a portion of the *rpoB* DNAs from the five Korean isolates confirmed them as *B. anthracis* (Fig. 1). It is interesting that no variation was observed in the *rpoB* DNA sequence of the 14 *B. anthracis* strains analyzed in the present study. *B. mycoides* KCCM 40260 and *B. megaterium* KCTC 3007 showed 93.1 to 96.2% and 83.6 to 85.8% similarities with three species (*B. anthracis*, *B. cereus*, and *B. thuringiensis*) of the *B. cereus*, respectively. The sequence similarity between *B. mycoides* and *B. megaterium* was 86.5%.

It was noteworthy that the *B. anthracis* was found to differ from *B. cereus* and *B. thuringiensis* at one amino acid, $S_{442} \rightarrow A_{442}$. This amino acid originates from one nonsynonymous nucleotide change (i.e., TCT → GCT) and was used in the designation of the *B. anthracis*-specific forward primer, Ba-SF (Fig. 1) (see below). The deduced amino acids of *B. cereus* and *B. thuringiensis* were identical, with one exception. *B. cereus* IMSNU 11013 differed from the other strains at one site: $E_{487} \rightarrow Q_{487}$. *B. mycoides* KCCM 40260 also had a single unique amino acid change, i.e., $G_{398} \rightarrow R_{398}$, and *B. megaterium* showed differences at five amino acid positions versus the consensus. However, no rifampin resistance-related amino acid substitution was found.

Phylogenetic analysis. The phylogenetic tree inferred from the *rpoB* sequences showed two main clusters, i.e., clusters A and B, in the *B. cereus* group (Fig. 2). Previous studies that used MLEE separated strains of the *B. cereus* group into two subgroups (9, 11, 34). However, because of the incongruent strains studied, it is unclear whether the two main clusters observed in the present study correspond to the subgroups described by these earlier studies.

The *B. anthracis* clade of cluster B was distinctly separated (Fig. 2), as found in an amplified fragment length polymorphism (AFLP) study (31), although it could not be differentiated from several *B. cereus* strains in a population study by using MLEE (11). This demonstrates the improved discriminatory power of the *rpoB* sequence versus enzyme mobility study. By referring to the phylogenetic tree, five Korean isolates, including GJ-1 and -2, were easily identified (Fig. 2). However, *rpoB* phylogeny showed no clear distinction between *B. cereus* and *B. thuringiensis*, which is also consistent with the results of previous studies by using MLEE- and AFLP-based methods (9, 10, 11, 32, 34).

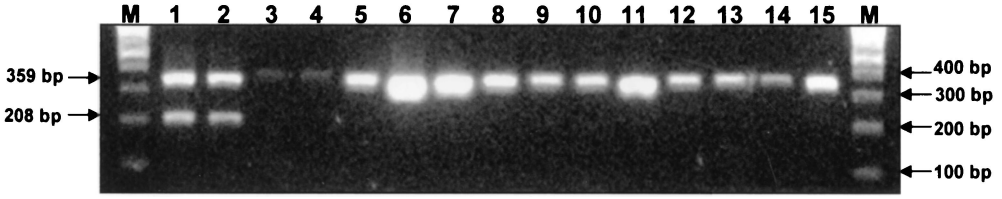

FIG. 3. Specific identification of *B. anthracis* by multiplex PCR. Two amplicons were observed for *B. anthracis* (359 and 208 bp; lanes 1 and 2) and only one larger amplicon (359 bp) for the other *Bacillus* species (lanes 3 to 15). Lanes: M, 100-bp ladder marker; 1, *B. anthracis* ATCC 14185; 2, *B. anthracis* Korean isolate; 3, *B. thuringiensis* IMSNU 12095; 4, *B. cereus* IMSNU 12077; 5, *B. cereus* IMSNU 13046; 6, *B. cereus* IMSNU 11011; 7, *B. cereus* IMSNU 11013; 8, *B. thuringiensis* IMSNU 12089; 9, *B. cereus* IMSNU 13043; 10, *B. mycoides* KCCM 40260; 11, B. *megaterium* KCTC 3007; 12, *B. licheniformis* KCTC 1918; 13, *B. sphaericus* KCTC 3346; 14, *B. pumilus* KCTC 3348; 15, *B. subtilis* KCTC 3040. In addition, 30 other *Bacillus* species (*B. fastidiosus*, *B. thermoglucosidadius*, *B. psychrophilus*, *B. azotoformans*, *B. marinus*, *B. flexus*, *B. simplex*, *B. pasteurii*, *B. niacini*, *B. pallidus*, *B. halophilus*, *B. thermoevolans*, *B. cohni*, *B. smithii*, *B. firmus*, *B. atrophaeus*, *B. mojavensis*, *B. vallismortis*, *B. globisporus*, *B. insolitus*, *B. lentus*, *B. badius*, *B. ehimensis*, *B. amyloliquefaciens*, *B. benzoevorans*, *B. fusiformis, B. macrocanus*, *B. psychrosaccharolytica*, *B. coagulans*, and *B. circulans*) in KCTC and IMSNU were also tested (data not shown).

The tight *B. anthracis* clade in the *rpoB* tree also suggests that *B. anthracis* is very homogeneous and is among the most monomorphic species known (11, 24, 31). According to the *rpoB* tree, *B. anthracis* appears to be genetically separated from the other members of the *B. cereus* group, such as *B. cereus* and *B. thuringiensis*, although the number of strains of *B. cereus* and *B. thuringiensis* included in the present study was limited. Two *B. thuringiensis* strains were found to be most closely related to the *B. anthracis* clade (Fig. 2), and four *B. cereus* strains were placed at the basal position of cluster B. In cluster A, four *B. thuringiensis* strains—KCTC 1509, IMSNU 12089, IMSNU 10051, and IMSNU 12096—constituted a distinct group that was supported by a bootstrap value of 90%. However, the other three *B. thuringiensis* strains were mixed with *B. cereus* strains.

Based on *rpoB* phylogeny, the evolution of *B. anthracis*, *B. thuringiensis*, and *B. cereus* has been very complicated, which supports the results of MLEE and AFLP studies (11, 31). A previous report suggested that *B. cereus* might be an ancestral species (11). However, because the *B. thuringiensis* clade was located at the basal position in cluster A, its ancestral species was not obvious in the present study. Although *B. cereus* IMSNU 13043 was found to be more divergent than the other strains and, therefore, could be said to be ancestral, this is only a single strain and may be abnormal. Thus, as indicated in a previous report (11), research involving the multilocus sequencing of more strains is needed to elucidate the evolution of the *B. cereus* group.

Identification of *B. anthracis*. When three primers (BA-RF, BA-RR, and Ba-SF) were used simultaneously, two amplicons (359 and 208 bp) were observed from *B. anthracis* but only one amplicon (359 bp) from the nonanthrax *Bacillus* species (Fig. 3). All *B. anthracis* strains produced two bands as expected. However, a smaller amplicon specific for *B. anthracis* was not detected in more than 30 other *Bacillus* species tested (Fig. 3). Although the nucleotide sequence of *B. megaterium* is identical to that of *B. anthracis* at the 3′ terminus of the specific primer, the target DNA was not amplified due to an overall dissimilarity in the primer region (Fig. 1).

When multiplex PCR, targeting *rpoB* DNA to identify *B. anthracis*, was simultaneously performed with the *cap* PCR, only two amplicons corresponding to *rpoB* DNA were generated by the Sterne strain, which does not contain pXO2 plasmid, and by the two ATCC strains, ATCC 14185 and ATCC 14186 (Fig. 4). However, three bands, including *cap* DNA (291 bp), were observed from ATCC 14578 (Vollum strain), which possesses pXO2. Multiplex PCR also indicated that the Korean isolates are virulent *B. anthracis*, which demonstrates that the developed multiplex method can be used both to identify *B. anthracis* and to indicate virulence.

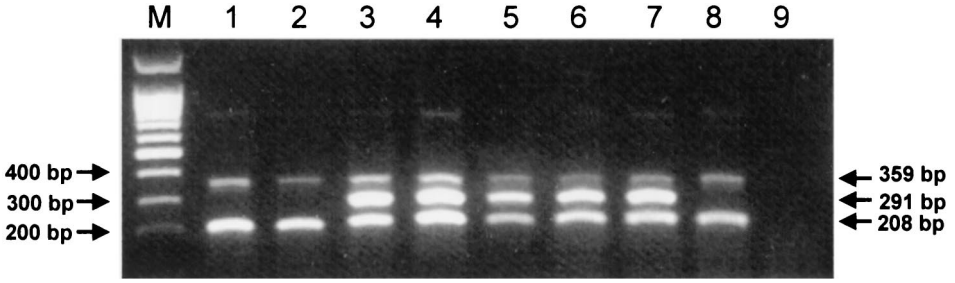

FIG. 4. Multiplex PCR amplification of *rpoB* (359 and 208 bp) and the *cap* (291 bp) gene DNA, which are located in the chromosome and the pXO2 plasmid of *B. anthracis*, respectively. Lanes: M, 100-bp ladder marker; 1, *B. anthracis* ATCC 14185; 2, *B. anthracis* ATCC 14186; 3, *B. anthracis* ATCC 14578 (Vollum strain); 4 to 7, Korean isolates of *B. anthracis* GJ-2, BC, CN, and HS, respectively; 8, *B. anthracis* Sterne strain; 9, negative control.

DISCUSSION

Although clinical *B. anthracis* isolates causing fatal human infection can be referred for definitive identification, preliminary results should be reported as quickly as possible by clinical laboratories. Because large-scale public screening may be necessary after proven public exposure, strain-specific identification and the rapid and precise identification of *B. anthracis* is needed. In this respect, clinical microbiological laboratories should play an important role in early detection and identification. Routine culture and biochemical testing methods performed in clinical laboratories are useful but are not sufficient for the definitive identification of *B. anthracis*. Thus, specimens should be referred to a reference laboratory for epidemiological study and further specific analysis, such as detailed biochemical analysis and genotyping. However, rapid identification and diagnosis by molecular techniques are available and have become essential in clinical laboratories. PCR can be used as a preliminary method for detecting *B. anthracis* DNA as an adjunct to bacterial culture. Moreover, PCR may be extended to the large-scale exposure screening for anthrax spores. Previously, *gyrB* and virulence-related genes in the plasmid were used in PCR to detect or identify *B. anthracis*. However, obstacles remain to be overcome. Specifically, nonvirulent strains that do not contain virulence plasmids or a number of *Bacillus* species phenotypically similar to *B. anthracis* may produce false-positive results (6, 24). Actually, sequences of several chromosomal genes such as *gyrB* (9, 36) and the 16S-23S ribosomal intergenic spacer (2) are not sufficient to discriminate *B. anthracis* from *B. cereus* and *B. thuringiensis*.

It might be difficult to find proper chromosomal markers for *B. anthracis*. Thus far, most molecular methods used to detect *B. anthracis* have been based on the genes, such as *pag* and *cap* (7), of plasmids; on randomly amplified polymorphic DNA by using SG-850 (3); or on variable-number tandem repeat regions (15). Randomly amplified polymorphic DNA and variable-number tandem repeat-based methods could be useful for epidemiologic study, but they are time-consuming or labor-intensive and lack specificity (23). In addition, due to the horizontal transfer of plasmids, a more specific chromosomal marker is needed.

In the present study, we have shown that *B. anthracis* and its closest relatives can be differentiated by comparative sequence analysis of a portion of *rpoB*. Another region, located upstream, was used previously for the real-time PCR detection of *B. anthracis* (23). However, it is likely that information on the corresponding sequences of other *Bacillus* species is insufficient and, therefore, false-positive results are possible in real-time PCR (7). To ensure the clear differentiation of *B. cereus* group strains based on the *rpoB* sequences, we compared a highly conserved region containing *rif*^{r} in more than 30 *Bacillus* species (data not shown) in addition to 34 strains of five species in Table 1.

Although the four species, which are collectively termed the *B. cereus* group, are genetically very similar (11), *B. anthracis* can be distinguished by using target sequences. Unfortunately, because of the many regulations concerning the acquisition of clinical and environmental isolates or DNAs of *B. anthracis*, we were unable to add strains to the present study. However, in view of the fact that all of the *rpoB* sequences compared were found to be identical and that previous molecular analysis results suggest that *B. anthracis* is extremely monomorphic, we believe that the sequence analysis used in the present study can be used to practically differentiate *B. anthracis* from other *Bacillus* species. Thus, we were able to verify the five Korean isolates as *B. anthracis.*

We also used *B. anthracis*-specific multiplex PCR to differentiate it from other *Bacillus* species. Multiplex PCR can provide critical criteria, which can be used to rule out *Bacillus* species other than *B. anthracis*, by generating two different patterns of PCR product (Fig. 3). Moreover, the combination of *rpoB* PCR and *cap* PCR is a more useful application. When *rpoB* PCR is combined with molecular detection methods based on pXO1 and pXO2, which determine the virulence of *B. anthracis* (7), the combined method appears to offer the more rapid detection and identification of *B. anthracis* and also determines virulence. Therefore, multiplex PCR may be used as a simple and basic tool for clinical microbiologists.

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REFERENCES

1. **Ash, C., J. A. E. Farrow, M. Dorsch, E. Stackebrandt, and M. D. Collins.** 1991. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bacteriol. **41:**343–346.
2. **Bourque, S. N., J. R. Valero, M. C. Lavoie, and R. C. Levesque.** 1995. Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. Appl. Environ. Microbiol. **61:**1623–1626.
3. **Daffonchio, D., S. Borin, G. Frova, R. Gallo, E. Mori, R. Fani, and C. Sorlini.** 1999. A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. Appl. Environ. Microbiol. **65:**1298–1303.
4. **Dixon, T. C., M. Meselson, J. Guillemin, and P. C. Hanna.** 1999. Anthrax. N. Engl. J. Med. **341:**815–826.
5. **Drancourt, M., A. Carlioz, and D. Raoult.** 2001. *rpoB* sequence analysis of cultured *Tropheryma whippelii*. J. Clin. Microbiol. **39:**2425–2430.
6. **Drancourt, M., and D. Raoult.** 2002. *rpoB* gene sequence-based identification of *Staphylococcus* species. J. Clin. Microbiol. **40:**1333–1338.
7. **Ellerbrok, H., H. Nattermann, M. Özel, L. Beutin, B. Appel, and G. Pauli.** 2002. Rapid and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-time PCR. FEMS Microbiol. Lett. **214:**51–59.
8. **Gonzales, J. M. J., B. S. Brown, and B. C. Carlton.** 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. Proc. Natl. Acad. Sci. USA **79:**6951–6955.
9. **Harrell, L., G. L. Andersen, and K. H. Wilson.** 1995. Genetic variability of *Bacillus anthracis* and related species. J. Clin. Microbiol. **33:**1847–1850.
10. **Helgason, E., D. A. Caugant, I. Olsen, and A.-B. KolstØ.** 2000. Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with periodontitis and other human infections. J. Clin. Microbiol. **38:**1615–1622.
11. **Helgason, E., L. A. (swsl)Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A.-B. KolstØ.** 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*: one species on the basis of genetic evidence. Appl. Environ. Microbiol. **66:**2627–2630.
12. **Ingleby, T. V., T. O'Toole, D. A. Henderson, J. G. Barlett, M. S. Ascher, E. Eitzen, A. M. Friedlander, J. Gergerding, J. Hauer, J. McDade, M. T. Osterholm, G. Parker, T. M. Perl, P. K. Russel, and K. Tonat.** 2002. Anthrax as a biological weapon, 2002. JAMA **287:**2236–2252.
13. **Jackson, P. J., M. E. Hugh-Jones, D. M. Adair, G. Green, K. K. Hill, C. R. Kuske, L. M. Grinberg, F. A. Abramova, and P. Keim.** 1998. PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: the presence of multiple *Bacillus anthracis* strains in different victims. Proc. Natl. Acad. Sci. USA **95:**1224–1229.
14. **Kim, B.-J., S.-H. Lee, M.-A. Lyu, S.-J. Kim, G.-H. Bai, S.-S. Kim, G.-T. Chae, E.-C. Kim, C.-Y. Cha, and Y.-H. Kook.** 1999. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). J. Clin. Microbiol. **37:**1714–1720.
15. **Kim, W., Y. Hong, J. Yoo, W. Lee, C. Choi, and S. Chung.** 2001. Genetic

relationships of *Bacillus anthracis* and closely related species based on variable-number tandem repeat analysis and BOX-PCR genomic fingerprinting. FEMS Microbiol. Lett. **207:**21–27.
16. **Ko, K. S., H. K. Lee, M.-Y. Park, K.-H. Lee, Y.-J. Yun, S.-Y. Woo, H. Miyamoto, and Y.-H. Kook.** 2002. Application of RNA polymerase β-subunit gene (*rpoB*) sequences for the molecular differentiation of *Legionella* species. J. Clin. Microbiol. **40:**2653–2658.
17. **Lee, S.-H., B.-J. Kim, J.-H. Kim, K.-H. Park, S.-J. Kim, and Y.-Y. Kook.** 2000. Differentiation of *Borrelia burgdorferi* sensu lato on the basis of RNA polymerase gene (*rpoB*) sequences. J. Clin. Microbiol. **38:**2557–2562.
18. **Logan, N. A., and P. C. B. Turnbull.** 1999. *Bacillus* and recently derived genera, p. 357–369. *In* P. R. Murray et al. (ed.), Manual of clinical Microbiology, 7th ed. American Society for Microbiology, Washington, D.C.
19. **Mock, M., and A. Fouet.** 2001. Anthrax. Annu. Rev. Microbiol. **55:**647–671.
20. **Mollet, C. M. Drancourt, and D. Raoult.** 1997. *rpoB* sequence analysis as a novel basis for bacterial identification. Mol. Microbiol. **26:**1005–1011.
21. **Nicholson, W. I., and H. Maughan.** 2002. The spectrum of spontaneous rifampin resistance mutations in the *rpoB* gene of *Bacillus subtilis* 168 spores differs from that of vegetative cells and resembles that of *Mycobacterium tuberculosis*. J. Bacteriol. **184:**4936–4940.
22. **Pannucci, J., R. T. Okinaka, R. Sabin, and C. R. Kuske.** 2002. *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. J. Bacteriol. **184:**134–141.
23. **Qi, Y., G. Patra, X. Liang, L. E. Williams, S. Rose, R. J. Redkar, and V. G. Del Vecchio.** 2001. Utilization of the *rpoB* gene as a specific chromosomal marker for real-time PCR detection of *Bacillus anthracis*. Appl. Environ. Microbiol. **67:**3720–3727.
24. **Read, T. D., S. L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J. D. Busch, K. L. Smith, J. M. Schupp, D. Solomon, P. Keim, and C. M. Fraser.** 2002. Comparative genome sequencing for discovery of novel polymorphism in *Bacillus anthracis*. Science **296:**2028–2033.
25. **Renesto, P., J. Gouvernet, M. Drancourt, V. Roux, and D. Raoult.** 2001. Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. J. Clin. Microbiol. **39:**430–437.
26. **Sabelnikov, A. G., and L. V. Ulyashova.** 1990. Plasmid transformation of *Bacillus cereus* on cellophane membrane. FEMS Microbiol. Lett. **72:**123–126.
27. **Sacchi, C. T., A. M. Whitney, L. W. Mayer, R. Morey, A. Steigerwalt, A. Boras, R. W. Weyant, and T. Popovic.** 2002. Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. Emerg. Infect. Dis. **8:**1117–1123.
28. **Swartz, M. N.** 2001. Recognition and management of anthrax: an update. N. Engl. J. Med. **345:**1621–1626.
29. **Swofford, D. L.** 1999. PAUP*: phylogenetic analysis by using parsimony (* and other methods), version 4. Sinauer Associates, Sunderland, Mass.
30. **Thwaite, J. E., L. W. J. Baillie, N. M. Carter, K. Stephenson, M. Rees, C. R. Harwood, and P. T. Emmerson.** 2002. Optimization of the cell wall microenvironment allows increased production of recombinant *Bacillus anthracis* protective antigen from *B. subtilis*. Appl. Environ. Microbiol. **68:**227–234.
31. **Ticknor, L. O., A.-B. Kolstø, K. K. Hill, P. Keim, M. T. Laker, M. Tonks, and P. J. Jackson.** 2001. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. Appl. Environ. Microbiol. **67:**4863–4873.
32. **Turnbull, P. C., R. A. Huston, M. J. Ward, M. N. Jones, C. P. Quinn, N. J. Finnie, C. J. Duggleby, J. M. Kramer, and J. Melling.** 1992. *Bacillus anthracis* but not always anthrax. J. Appl. Bacteriol. **72:**21–28.
33. **Vogler, A. J., J. D. Busch, S. Percy-Fine, C. Tipton-Hunton, K. L. Smith, and P. Keim.** 2002. Molecular analysis of rifampin resistance in *Bacillus anthracis* and *Bacillus cereus*. Antimicrob. Agents Chemother. **46:**511–513.
34. **Vilas-Boas, G., V. Sanchis, D. Lereclus, M. V. F. Lemos, and D. Bourguet.** 2002. Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. Appl. Environ. Microbiol. **68:**1414–1424.
35. **Wicks, A., N. Jayaswal, D. Lereclus, and L. Andrup.** 1998. Characterization of plasmid pAW63, a second self-transformissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. Microbiology **144:**1263–1270.
36. **Yamada, S., E. Ohashi, N. Agata, and K. Venkateswaran.** 1999. Cloning and nucleotide sequence analysis of *gyrB* of *Bacillus cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis* and their application to the detection of *B. cereus* in rice. Appl. Environ. Microbiol. **65:**1483–1490.