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Generation of a Specific Marker to Discriminate *Bacillus anthracis* from Other Bacteria of the *Bacillus cereus* Group

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Abstract *Bacillus anthracis* is a soil pathogen capable of causing anthrax that is closely related to several environmental species, including *B. cereus*, *B. mycoides*, and *B. thuringiensis*. DNA homology studies showed that *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. thuringiensis* are closely related, with a high sequence homology. To establish a method to specifically detect *B. anthracis* in situations such as environmental contamination, we initially performed RAPD-PCR with a 10-mer random primer and confirmed the presence of specific PCR bands only in *B. anthracis* species. One region specific for *B. anthracis* was cloned and sequenced, and an internal primer set was designed to amplify a 241-bp DNA fragment within the sequenced region. The PCR system involving these specific primer sets has practical applications. Using lyses methods to prepare the samples for PCR, it was possible to quickly amplify the 241-bp DNA segment from samples containing only a few bacteria. Thus, the PCR detection method developed in this study is expected to facilitate the monitoring of environmental *B. anthracis* contamination.

Keywords: *Bacillus anthracis*, *Bacillus cereus*, group bacteria, discrimination, identification, RAPD-PCR, specific marker

Bacillus anthracis is a soil microorganism and the causative agent of anthrax, a lethal disease in humans and animals [3, 25]. It is critically important to speedily detect this species, because the bacteria are very resistant to environmental stresses and can be spread widely through their ability to form spores [11, 20, 26].

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B. anthracis is closely related to several environmental species, especially the *Bacillus cereus* group including *B. cereus*, *B. mycoides*, and *B. thuringiensis* [17, 21]. *B. anthracis* also shares many phenotypic and antigenic properties with these closely related species, and it is not always easy to distinguish by using the common biochemical or serological tests available in most clinical laboratories [10, 27].

Recently, DNA-based methods have been applied to identify and type many microorganisms [2, 4, 13–16, 19, 22, 24], but it is very difficult to identify *B. anthracis* species among other similar species, because of their high genetic homology [1, 6, 7, 18, 27]: There is no difference in their rRNA sequences [1, 8] and, although there are repeated sequences and variable number tandem repeats (VNTR) among *B. anthracis* species, similar sequences exist also in other *B. cereus*, *B. mycoides*, and *B. thuringiensis* species [1, 8, 9, 21, 22]. Recently, protein-based methods have been applied to identify many microorganisms; nevertheless, it is still very difficult to identify *B. anthracis* species among other similar species [5, 12, 25, 26, 28].

The only established difference among the *B. cereus* group is the presence of genes coding for toxins, protective antigen, lethal factor, and edema factor, which are usually present in plasmids [17]. When these plasmids are lost, it is very difficult to discriminate group members from each other and, consequently, some studies erroneously considered them as the same species [1, 8].

Conventional tests, which are available only in specialized reference and research laboratories, include detection of toxin (pXO1) and capsule (pXO2) contained on virulent plasmids [17, 27]. However, avirulent *B. anthracis* strains that lack these plasmids (pXO1$^-$ and pXO2$^-$) have been found [17, 27] and appear to be very similar to *B. cereus*

and other related species when bacteriophage susceptibility, motility, and hemolysis are tested [27].

Therefore, when *B. anthracis* is encountered in clinical specimens, quicker and more reliable tests are needed to confirm its identity. The development of suitable genomic markers rather than plasmid markers of particular value would be to distinguish *B. anthracis* from other species of the *B. cereus* group. The randomly amplified polymorphic DNA (RAPD) fingerprinting technique has been proposed as a tool for generating taxon-specific markers with different specificities [9, 18].

We modified the RAPD-PCR method by adding a cloning step. We initially performed RAPD-PCR with an 8-mer random primer and confirmed the presence of specific PCR bands only in *B. anthracis* species. One specific product was cloned and sequenced, and internal oligonucleotide primers were then designed to amplify a 241-bp DNA fragment within the sequenced region that is specific for *B. anthracis* species. Our RAPD-PCR-based method overcame the disadvantages of conventional tests. The method is faster and easier to implement than other methods, because it detects a single band (241-bp) present only in *B. anthracis*. Furthermore, it can detect all *B. anthracis* species independent of virulence, since the assay is based at the genomic level, but not at the plasmid DNA level. Therefore, this PCR system could be used as a rapid, reliable, reproducible, and highly specific screening technique to discriminate *B. anthracis* from other related species in the environment.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used in this study are listed in Table 1. All strains were inoculated into 100 ml of brain heart infusion broth and grown by shaking at 200 rpm for 8 h at 37°C [25].

Isolation of DNA

To isolate total bacterial DNA, cells were suspended in TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0) containing lysozyme (10 mg/ml), sodium dodecyl sulfate (SDS), and proteinase K [4]. After incubation at 55°C, the DNA preparation was treated with RNase,

Table 1. Bacterial strains used in this study.

Numbers	Strains	Sources	Plasmids
1	*Bacillus anthracis*	Kyungjoo	pxO1$^+$, pxO2$^+$
2	*Bacillus anthracis*	Hongsung	pxO1$^+$, pxO2$^+$
3	*Bacillus anthracis*	ATCC 14578	pxO1$^-$, pxO2$^+$
4	*Bacillus anthracis*	ATCC 14185	pxO1$^+$, pxO2$^-$
5	*Bacillus anthracis*	Pasteur No2	pxO1$^-$, pxO2$^+$
6	*Bacillus anthracis*	Sterne, 34F2	pxO1$^+$, pxO2$^-$
7	*Bacillus anthracis*	Bongcheon	pxO1$^-$, pxO2$^+$
8	*Bacillus subtilis*	ATCC6633	
9	*Bacillus amyloliquefaciens*	ATCC 23350	
10	*Bacillus licheniformis*	ATCC 27811	
11	*Bacillus brevis*	IMSNU*12063*	
12	*Bacillus megaterium*	ATCC 14581	
13	*Bacillus pacificus*	ATCC 25089	
14	*Bacillus pumilus*	JCM2508	
15	*Bacillus stearothermophilus*	ATCC 12980	
16	*Bacillus cereus*	KCTC 1661	
17	*Bacillus cereus*	KCCM 11204	
18	*Bacillus cereus*	KCCM 11341	
19	*Bacillus cereus*	KCTC 1092	
20	*Bacillus cereus*	KCTC 1014	
21	*Bacillus cereus*	KCCM 11773	
22	*Bacillus cereus*	KCCM 12142	
23	*Bacillus thuringiensis*	KCTC 1509	
24	*Bacillus thuringiensis*	KCTC 7452	
25	*Bacillus thuringiensis*	KCCM 40030	
26	*Bacillus thuringiensis*	KCTC 1507	
27	*Bacillus thuringiensis*	KCCM 11613	
28	*Bacillus thuringiensis* subsp. *kurstaki*	KCCM 11429	
29	*Bacillus mycoides*	KCCM 40260	

extracted three times with phenol-chloroform, precipitated with ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

RAPD-PCR

Each PCR mixture (100 μl) contained 150 ng of DNA template, 200 μM dNTPs, 3.0 mM Mg^{2+}, 100 pmole of random 10-mer primer, and 2.5 U of *Taq* DNA polymerase. The following thermal cycling conditions were used: an initial denaturation step (94°C for 5 min) and 30 cycles consisting of denaturation at 94°C for 15 s, annealing at 40°C for 30 s, and primer extension at 72°C for 60 s. Samples were overlaid with 100 μl of mineral oil to minimize evaporation during thermal cycling. When the PCR was completed, the samples were frozen at −20°C to remove the mineral oil without losing the sample. Once the samples were thawed, they were analyzed by electrophoresis.

Preparation of DNA, PCR, and Sequencing

Following Seakem GTG (FMC) agarose gel electrophoresis in 1× Tris-acetate-EDTA or 1× Tris-borate-EDTA running buffer, the 710-bp RAPD-PCR product was excised and purified using a QIAquick purification kit (Qiagen, Hilden, Germany), when necessary. Sequencing reactions were performed on an ABI PRISM 3100 sequencer (Perkin-Elmer, U.S.A.) with fluorescein dye-deoxy terminator-labeled oligonucleotide primers, corresponding to the primers used in the PCR step, using a Bigdye-terminator Cycle Sequencing kit (Perkin-Elmer, U.S.A.). DNA sequences were analyzed and assembled using sequence analysis software (Perkin-Elmer, U.S.A.).

PCR with *B. anthracis*-Specific Primer Set

The *B. anthracis*-specific primer set was designed within the cloned and sequenced region by Primer 3 (Whitehead Institute). The following *B. anthracis*-specific primer set was used in this study: 5'-CTGCTACTATTGTAGGAGGA-3' and 5'-CGTAACAAGAGGAAAGAGCA-3'.

Each PCR mixture (100 μl) contained 150 ng of DNA template, 200 μM dNTPs, 1.5 mM Mg^{2+}, *B. anthracis*-specific primer set at a concentration of 100 pmole, and 2.5 U of *Taq* DNA polymerase. The following thermal cycling conditions were used: an initial denaturation step (94°C for 5 min) and 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and primer extension at 72°C for 1 min. Samples were overlaid with 100 μl of mineral oil to minimize evaporation during thermal cycling. When the PCR was completed, the samples were frozen at −20°C to remove the mineral oil without losing the sample. Once the samples were thawed, they were analyzed by electrophoresis.

RESULTS

RAPD-PCR Fingerprinting

Fingerprinting of *B. cereus* group bacteria was performed using a RAPD-PCR method with a random 10-mer primer. We identified bands at 550-bp and 710-bp regions that are specific to *B. anthracis* when compared with other *Bacillus* subgroups (Fig. 1). The PCR conditions were optimized by testing various annealing temperatures (35 to 65°C) and Mg^{2+} concentrations (0.5 to 5.0 mM) to provide the best possible fingerprint. Ultimately, 40°C annealing temperature and 3 mM Mg^{2+} concentration were selected. Differences were observed between *B. anthracis* and the other members of the *B. cereus* group as well as among the *B. anthracis* strain types (Figs. 1 and 2). The profiles of the RAPD-PCR generated DNA fragments for these species and other strains revealed some bands common to *B. anthracis* and also different band patterns (Fig. 1).

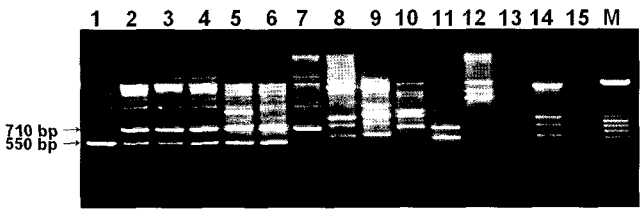

Fig. 1. RAPD-PCR fingerprinting of *B. anthracis* and *Bacillus* group strains performed with a random 10-mer primer. PCR products were separated by electrophoresis on a 1.5% agarose gel at 2.5 V/cm.
Lanes 1 through 7: *Bacillus anthracis* Kyungjoo, Hongsung, ATCC 14578, ATCC 14185, Pasteur No. 2, Sterne, 34F2, and Bongcheon, respectively. Lane 8, *Bacillus subtilis* ATCC 6633; lane 9, *Bacillus amyloliquefaciens* ATCC 23350; lane 10, *Bacillus licheniformis* ATCC 27811; lane 11, *Bacillus brevis* IMSNU*12063*; lane 12, *Bacillus megaterium* ATCC 14581; lane 13, *Bacillus pacificus* ATCC 25089; lane 14, *Bacillus pumilus* JCM2508; lane 15, *Bacillus stearothermophilus* ATCC 12980.

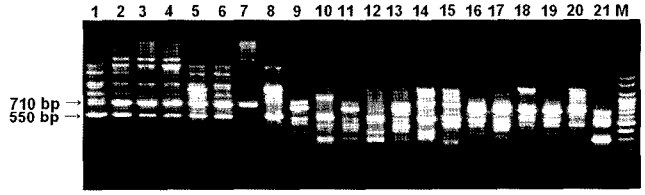

Fig. 2. RAPD-PCR fingerprinting of *B. cereus* group strains performed with a random primer. PCR products were separated by electrophoresis on a 1.5% agarose gel at 2.5 V/cm.
Lanes 1 through 7: *Bacillus anthracis* Kyungjoo, Hongsung, ATCC 14578, ATCC 14185, Pasteur No. 2, Sterne, 34F2, and Bongcheon, respectively. Lanes 8 through 14: *Bacillus cereus* KCTC 1661, *Bacillus cereus* KCCM 11204, *Bacillus cereus* KCCM 11341, *Bacillus cereus* KCTC 1092, *Bacillus cereus* KCTC 1014, *Bacillus cereus* KCCM 11773, and *Bacillus cereus* KCCM 12142, respectively. Lanes 15 through 20: *Bacillus thuringiensis* KCTC 1509, *Bacillus thuringiensis* KCTC 7452, *Bacillus thuringiensis* KCCM 40030, *Bacillus thuringiensis* KCTC 1507, *Bacillus thuringiensis* KCCM 11613, and *Bacillus thuringiensis* subsp. *kurstaki* KCCM 11429, respectively. Lane 21, *Bacillus mycoides* KCCM 40260.

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1 TGAAACGGGTGATACTATTACTGGTGTGACTACTACAGCTGCTACTATTGTAGGAGGAGC
61 TGTCCTAGATGGAGGAACTCCTATCGCAATCGAATTTACAATAACGCAAATTTAGTTATC
121 TTTTTAGAGGTTTCATTAAAAGAAACCTCTAATTTTAAAAACTGAAATTAATTTTAGTAG
181 CAACTACATAATACAGTGCATATACTAGTATAGAATAATTGGTATTTAATACTCACTCTC
241 AAAAAGAGCACTTATATGGTGCTCTTTCCTCTTGTTACGAAATACAAATCTTTGTATAAA
301 ACTGTACCTCTCTAAAAACCATACATACAATATGATATGAACCCTTTTTTCTAAATAAGT
361 CTTGGTCATAAAGCGCCTTCCTCCAAAGGCGCTCTTTAATTTTCAAATAACATTTTCAAG
421 TAATTACGAGAGCGGATTTTATTATGTTGTACAAGCACTACTTCGCTCTATTAAATAGAC
481 TATATAGACATCTAAAAGAAAGGAGTTTTAAATTCATATGGCTGATTACTTTTATAAAGA
541 TGGTAAAAAGTATTATAAAAATCAATCGCATTCGGACCGTCAAAAAAATAACTGTTTTGT
601 TGAGACTCATACAATTGGTGGTTCAGGAGAAAATTTAAATGGAAATATACCTACATCTAT
661 TGACCTTCTAAATACCACTCCACAAACAGTATTTGAAGATTTCACCAACA
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Fig. 3. Sequence of the cloned region isolated from RAPD-PCR analysis of *B. anthracis*, with the NCBI GenBank accession number of AF360750.
The underlined sequence indicates the 241-bp *B. anthracis*-specific marker region. Boxed sequences denote the *B. anthracis*-specific marker primer.

PCR and Sequencing

Since it was not easy to distinguish *B. anthracis* within the *B. cereus* group by comparing RAPD-PCR band patterns alone (Fig. 2), we further introduced a cloning step and a second PCR amplification. We cloned and sequenced the PCR band at 710 bp that was specific to *B. anthracis* (Fig. 3). We then designed internal primers within the 710-bp cloned region. This *B. anthracis*-specific primer set yielded a 241-bp PCR product, which is a *B. anthracis*-specific marker (Fig. 3).

B. anthracis-Specific PCR Primer Set

PCR performed after using the *B. anthracis*-specific primer set gave a single 241-bp band pattern only in *B. anthracis* (Figs. 4 and 5). Using this technique, *B. anthracis* strains could readily be distinguished from other *B. cereus* group strains, including *B. anthracis* isolates that lacked one or both of the virulence plasmids. These results demonstrate that the PCR fingerprint obtained for *B. anthracis* is independent of virulence status and distinct from the fingerprints of other group members.

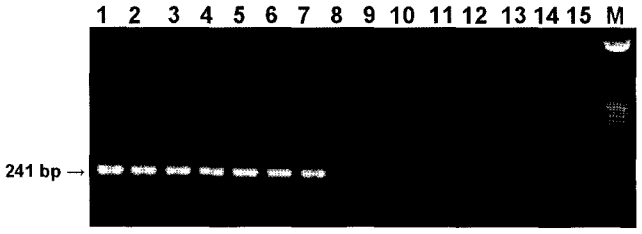

Fig. 4. PCR analysis of *B. anthracis* and *Bacillus* group strains using the *B. anthracis*-specific primer set. PCR products were separated by electrophoresis on a 1.5% agarose gel at 2.5 V/cm.
Lanes 1 through 7: *Bacillus anthracis* Kyungjoo, Hongsung, ATCC 14578, ATCC 14185, Pasteur No. 2, Sterne, 34F2, and Bongcheon, respectively. Lane 8, *Bacillus subtilis* ATCC 6633; lane 9, *Bacillus amyloliquefaciens* ATCC 23350; lane 10, *Bacillus licheniformis* ATCC 27811; lane 11, *Bacillus brevis* IMSNU*12063*; lane 12, *Bacillus megaterium* ATCC 14581; lane 13, *Bacillus pacificus* ATCC 25089; lane 14, *Bacillus pumilus* JCM2508; lane 15, *Bacillus stearothermophilus* ATCC 12980.

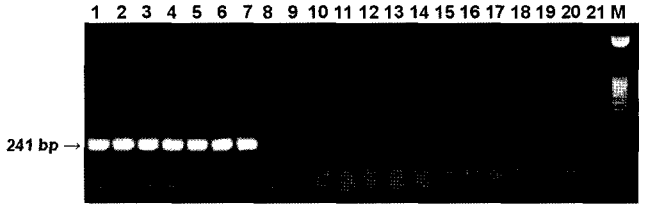

Fig. 5. PCR analysis of *B. anthracis* and *B. cereus* group strains performed with the *B. anthracis*-specific primer set. PCR products were separated by electrophoresis on a 1.5% agarose gel at 2.5 V/cm.
Lanes 1 through 7: *Bacillus anthracis* Kyungjoo, Hongsung, ATCC 14578, ATCC 14185, Pasteur No. 2, Sterne, 34F2, and Bongcheon, respectively. Lanes 8 through 14: *Bacillus cereus* KCTC 1661, *Bacillus cereus* KCCM 11204, *Bacillus cereus* KCCM 11341, *Bacillus cereus* KCTC 1092, *Bacillus cereus* KCTC 1014, *Bacillus cereus* KCCM 11773, and *Bacillus cereus* KCCM 12142, respectively. Lanes 15 through 20: *Bacillus thuringiensis* KCTC 1509, *Bacillus thuringiensis* KCTC 7452, *Bacillus thuringiensis* KCCM 40030, *Bacillus thuringiensis* KCTC 1507, *Bacillus thuringiensis* KCCM 11613, and *Bacillus thuringiensis* subsp. *kurstaki* KCCM 11429, respectively. Lane 21, *Bacillus mycoides* KCCM 40260.

DISCUSSION

In this study, we established a PCR system that can detect *B. anthracis* using a *B. anthracis*-specific primer set (Figs. 4 and 5) designed against genomic DNA. Therefore, it is possible to detect all *B. anthracis* strains, irrespective of whether they harbor pXO1 or pXO2. The PCR system was found to be specific, giving rise to an amplified 241-bp DNA product only for *B. anthracis* (Figs. 4 and 5). More importantly, it is quite possible with this system to make a clear diagnosis within a day from a very small amount of bacteria. The system in many regards is also more reliable than the conventional methods for the detection of *B. anthracis* [1, 8, 23]. Until now, the methods available to identify and distinguish *B. anthracis* from the *B. cereus* group had definite limitations [1, 4, 8, 23, 27]. Currently, a large number of phenotypic characteristics common within the group indicate that further identification of *B. anthracis* is based on the detection of virulence factors, such as capsule and toxin production [17]. However, the existence of avirulent "anthrax-like" organisms in the environment that lack the genes for toxin and/or capsule production can easily compromise such techniques [22, 27]. Therefore, a more sensitive and specific approach for identification and differentiation is required. This new PCR system overcomes this identification problem since the primer set is based on genomic DNA, but not plasmid DNA.

We also examined the RAPD-PCR band patterns. *B. anthracis*-like members of the *B. cereus* group produce fingerprints very similar to those of conventional *B. anthracis* strains. These isolates may represent a link between the members of the group and a common ancestor. All of the *B. cereus* group isolates analyzed with the random primer had several bands in common with *B. anthracis*, suggesting

some conservation of chromosomal organization [21]. In addition, differences in RAPD-PCR band patterns may represent phenotypic differences that could be investigated further by sequence analysis.

B. anthracis is a soil bacterium that is able to survive in spore form for extended periods of time. Surveillance of contamination in certain soils, such as in stock farms, is needed. In this context, the PCR system developed in this study would facilitate the monitoring of bacterial contamination through simple detection of the *B. anthracis*-specific 241-bp band in soil samples.

The GenBank accession number of the sequence reported in this paper is AF360750.

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