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Simultaneous Detection and Identification of *Bacillus cereus* Group Bacteria Using Multiplex PCR

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Abstract *Bacillus cereus* group bacteria share a significant degree of genetic similarity. Thus, to differentiate and identify the *Bacillus cereus* group efficiently, a multiplex PCR method using the *gyrB* and *groEL* genes as diagnostic markers is suggested for simultaneous detection. The assay yielded a 400 bp amplicon for the *groEL* gene from all the *B. cereus* group bacteria, and a 253 bp amplicon from *B. anthracis*, 475 bp amplicon from *B. cereus*, 299 bp amplicon from *B. thuringiensis*, and 604 bp amplicon from *B. mycoides* for the *gyrB* gene. No nonspecific amplicons were observed with the DNA from 29 other pathogenic bacteria. The specificity and sensitivity of the *B. cereus* group identification using this multiplex PCR assay were evaluated with different kinds of food samples. In conclusion, the proposed multiplex PCR is a reliable, simple, rapid, and efficient method for the simultaneous identification of *B. cereus* group bacteria from food samples in a single tube.

Keywords: *Bacillus anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis*, multiplex PCR

Bacillus cereus group bacteria are Gram-positive, rod-shaped, and spore-forming environmental organisms that are highly related genetically. The *B. cereus* group includes at least four species: *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, and *Bacillus mycoides* [4]. Since these strains are potentially enterotoxic to humans or insects, the ability to rapidly detect the *B. cereus* group is crucial. *B. cereus* is often found in soil, and can be isolated from raw milk and dairy foods [1]. *B. cereus* also causes foodborne diseases associated with several proteins, resulting in diarrhea in higher animals [4, 20]. *B. anthracis* is the etiological agent of anthrax, a fatal disease found primarily among herbivores. The *B. anthracis* spores are also highly resistant to ultraviolet, ionizing radiation, heat, pressure, and various chemical agents [16, 17]. *B. anthracis* can be distinguished from other group members based on the existence of toxin-encoding pXO1 and capsule-encoding pXO2 plasmids [14]. Yet, since plasmids are less stable than chromosomal DNA, and isolates lacking either or both plasmids have been found in nature, chromosomal markers are crucial for the accurate identification of *B. anthracis* among *B. cereus* group bacteria in outbreaks [19]. *B. thuringiensis* is an insect pathogen used for crop protection and is closely related to *B. cereus* in the genome sequence [6, 12], making it difficult to discriminate *B. thuringiensis* from *B. cereus* when the plasmid containing the *cry* gene is lost [5]. Finally, *B. mycoides* has been identified as a plant growth-promoting bacterium associated with conifer roots [13].

The taxonomy of the *B. cereus* group is disputable, and several researchers have proposed that these closely related *Bacillus* species should be combined as the *B. cereus* group [4, 6, 8, 17]. The identification and differentiation of *B. cereus* group bacteria are difficult owing to the genetic and phenotypic similarities among them [2, 18]. Several approaches to differentiate *B. cereus* group members have already been suggested, including whole-genome DNA hybridization [2], sequence analysis of the 16S-23S operons [7], the *gyrB-gyrA* intergenic spacer region [7], multilocus enzyme electrophoresis [3], pulsed-field gel electrophoresis analysis [3], amplified fragment length polymorphism [11], virulence factors [18], arbitrary PCR [8], and PCR-restriction fragment length polymorphism [4]. However, a more rapid and simple method is still needed to distinguish between members of the *B. cereus* group. A multiplex PCR is an efficient method of differentiating and identifying multiple samples in a single tube [9, 15]. Accordingly, the purpose of this study was to develop a simultaneous identification and differentiation method for *B. cereus* group bacteria in a single tube using a multiplex PCR.

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Table 1. Bacterial strains used in this study.

Organism	Strain
Bacillus subtilis	ATCC 6633, ATCC 6051
Bacillus cereus	ATCC 14579, ATCC 10876, ATCC 11778
Bacillus thuringiensis	ATCC 10792, ATCC 35646, KCCM 41613
Bacillus mycoides	ATCC 6462
Bacillus anthracis	ATCC 14578
Staphylococcus aureus	ATCC 6538, ATCC 25923, ATCC 29737
Staphylococcus haemolyticus	ATCC 29970
Staphylococcus epidermidis	ATCC 14990
Shigella flexneri	ATCC 12022
Shigella sonnei	ATCC 25931
Shigella boydii	ATCC 8700
Yersinia enterocolitica	ATCC 23715
Salmonella typhimurium	ATCC 14028
Escherichia coli O157:H7	ATCC 43894
Escherichia coli	ATCC 11775, ATCC 23736, ATCC 25922, ATCC 27325
Vibrio parahaemolyticus	ATCC 17802
Campylobacter jejuni	ATCC 33560
Listeria monocytogenes	ATCC 19111, ATCC 19113

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The 29 bacterial strains tested in this study are shown in Table 1. All the reference strains, except for *B. anthracis*, were collected from the American Type Culture Collection (ATCC) and Korean Culture Center of Microorganisms (KCCM). The genomic DNA of *B. anthracis* was obtained from the Korea Center for Disease Control (KCDC). All the strains, except for the *B. cereus* group, were cultivated at 37°C overnight with shaking in appropriate media, whereas the *B. cereus* group was grown in a BHI broth (Difco Laboratories, Detroit, MI, U.S.A.) for 24 h at 30°C.

Oligonucleotide Primers

Specific oligonucleotide primers were designed for *B. anthracis*, *B. thuringiensis*, *B. cereus*, and *B. mycoides* based on the *gyrB* sequence (GenBank accession numbers AY265467, AY461778, AY265483, and AY265514), whereas the primers for the *B. cereus* group bacteria were designed from the *groEL* sequence (GenBank accession numbers NC004722, NC007530, and NC005957) using Vector NTI (Invitrogen, Carlsbad, CA, U.S.A.). All the primers were synthesized by the Bionics Corp. (Seoul, Korea). The sequences of the primers used in this study are shown in Table 2.

Multiplex PCR Conditions

The genomic DNA used in the PCR assays was extracted using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the concentration of the extracted DNA measured using a UV-spectrophotometer (Model UV-1700, Shimadzu, Tokyo, Japan). The multiplex PCR was performed with a thermocycler (Model PC 808, ASTEC, Fukuoka, Japan). The total reaction volume of 25 μl of the PCR mixture contained 1×PCR

Table 2. Sequences of oligonucleotide primers used in this study.

Species	Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)
B. cereus group	BCGSH-1F	GTG CGA ACC CAA TGG GTC TTC	*groEL*	400
	BCGSH-1R	CCT TGT TGT ACC ACT TGC TC		
B. anthracis	BASH-2F	GGT AGA TTA GCA GAT TGC TCT TCA AAA GA	*gyrB*	253
	BASH-2R	ACG AGC TTT CTC AAT ATC AAA ATC TCC GC		
B. thuringiensis	BTJH-1F	GCT TAC CAG GGA AAT TGG CAG	*gyrB*	299
	BTJH-R	ATC AAC GTC GGC GTC GG		
B. cereus	BCJH-F	TCA TGA AGA GCC TGT GTA CG	*gyrB*	475
	BCJH-1R	CGA CGT GTC AAT TCA CGC GC		
B. mycoides	BMSH-F	TTT TAA GAC TGC TCT AAC ACG TGT AAT	*gyrB*	604
	BMSH-R	TTC AAT AGC AAA ATC CCC ACC AAT		

buffer ($MgCl_2$ plus), 0.1 mM dNTP, different concentrations of the primers, 25 ng template DNA, and 1 U Takara Ex *Taq* polymerase (TaKaRa, Shiga, Japan). The reaction parameters were an initial denaturation at 94°C for 5 min, 30 cycles of amplification with denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, an extension at 72°C for 30 sec, and final extension of the incompletely synthesized DNA at 72°C for 5 min. The PCR amplicons were analyzed by agarose gel electrophoresis, where 5 μl of the PCR products was loaded onto 2.5% agarose gels containing 0.5 μg ethidium bromide and subjected to electrophoresis for 25 min at 100 V in a 0.5×TAE buffer. The PCR products were then visualized under UV-irradiation and photographed using a digital camera (Model COOLPIX 4300, Nikon, Tokyo, Japan).

Detection of *B. cereus* Group from Food Samples

Twenty-five g of each food sample (boiled rice, lettuce, crown daisy, spinach, and spring onion) was homogenized with 225 ml of a phosphate-buffered saline (PBS) solution for 1 min using a stomacher (Stomacher Lab Blender 400, Seward Laboratories, London, U.K.), and then 100 μl of the mixture was spread onto a *Bacillus cereus* agar base (Acumedia Manufacturers Inc., Lansing, MI, U.S.A.), a medium used for the isolation and presumptive identification of *Bacillus cereus*, and incubated at 30°C for 24 h. Thereafter, colonies were selected and grown in a BHI broth.

Cloning and Sequencing of *gyrB* Gene

To compare the *gyrB* gene homology within the *B. cereus* group, species-specific primers were used for each *B. cereus* group member. The amplified *gyrB* gene fragments obtained from *B. cereus* ATCC 14579, *B. thuringiensis* ATCC 35646, *B. anthracis* ATCC 14578, and *B. mycoides* ATCC 6462 were inserted into a Perfect-T cloning kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The PCR fragments were then ligated into a

Table 3. Evaluation of specificity of five different PCR primer pairs for *B. cereus* group.

Strain	Amplicon size (bp)				
	BCGSH (400)	BASH (253)	BTJH (299)	BCJH (475)	BMSH (604)
Bacillus subtilis ATCC 6633	–	–	–	–	–
Bacillus subtilis ATCC 6051	–	–	–	–	–
Bacillus cereus ATCC 14579	+	–	–	+	–
Bacillus cereus ATCC 10876	+	–	–	+	–
Bacillus cereus ATCC 11778	+	–	–	+	–
Bacillus thuringiensis ATCC 35646	+	–	+	–	–
Bacillus thuringiensis ATCC 10792	+	–	+	–	–
Bacillus thuringiensis KCCM 41613	+	–	+	–	–
Bacillus mycoides ATCC 6462	+	–	–	–	+
Bacillus anthracis ATCC 14578	+	+	–	–	–
Staphylococcus aureus ATCC 6538	–	–	–	–	–
Staphylococcus aureus ATCC 29737	–	–	–	–	–
Staphylococcus aureus ATCC 25923	–	–	–	–	–
Staphylococcus haemolyticus ATCC 29970	–	–	–	–	–
Staphylococcus epidermidis ATCC 14990	–	–	–	–	–
Shigella flexneri ATCC 12022	–	–	–	–	–
Shigella sonnei ATCC 25931	–	–	–	–	–
Shigella boydii ATCC 8700	–	–	–	–	–
Yersinia enterocolitica ATCC 23715	–	–	–	–	–
Salmonella typhimurium ATCC 14028	–	–	–	–	–
Escherichia coli O157:H7 ATCC 43894	–	–	–	–	–
Escherichia coli ATCC 27325	–	–	–	–	–
Escherichia coli ATCC 23736	–	–	–	–	–
Escherichia coli ATCC 25922	–	–	–	–	–
Escherichia coli ATCC 11775	–	–	–	–	–
Vibrio parahaemolyticus ATCC 17802	–	–	–	–	–
Campylobacter jejuni ATCC 33560	–	–	–	–	–
Listeria monocytogenes ATCC 19113	–	–	–	–	–
Listeria monocytogenes ATCC 19111	–	–	–	–	–

BCGSH primers targeting *B. cereus* group, BASH primers targeting *Bacillus anthracis*, BTJH primers targeting *Bacillus thuringiensis*, BCJH primers targeting *Bacillus cereus*, BMSH primers targeting *Bacillus mycoides*. + Specific product; – no product.

pMD18 vector (TaKaRa) and transformed into *Escherichia coli* DH5α. Selected *E. coli* containing the recombinant plasmid were then cultivated and the PCR products sequenced twice using an ABIPRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, U.S.A.). The DNA sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) program.

RESULTS AND DISCUSSION

Sensitivity of Primer Pairs and Specificity of Multiplex PCR

In the present study, five primer sets were selected to simultaneously detect 4 different species of the *B. cereus* group using a multiplex PCR. The 4 species-specific primers were synthesized using the *gyrB* gene, plus one specific primer for the *B. cereus* group was constructed from the *groEL* gene. To verify and evaluate the specificity of the primers used, a single PCR was performed with DNA templates prepared from the 29 different bacterial type strains, including positive control strains, as shown in Table 3. The primers BCGSH-1F and -1R produced a 400 bp amplicon from the *B. cereus* group, primers BASH-2F and -2R yielded a 253 bp amplicon from *B. anthracis*, primers BTJH-1F and -R produced a 299 bp amplicon from *B. thuringiensis*, primers BCJH-F and 1R yielded a 475 bp amplicon from *B. cereus*, and primers BMSH-F and -R produced a 604 bp amplicon from *B. mycoides* (Fig. 1). To avoid nonspecific amplification, the multiplex PCR conditions were optimized with respect to the primer concentrations [15]. Following careful comparison of various reactions, the primer concentrations for *B. anthracis*, *B. thuringiensis*, *B. cereus*, *B. mycoides*, and the *B. cereus* group were set up as 80 nM, 32 nM, 40 nM, 100 nM, and 48 nM, respectively. Thus, when a multiplex PCR was performed with templates from *B. anthracis*, *B. thuringiensis*, *B. cereus*, and *B. mycoides* strains, only amplicons of the expected size were produced (Fig. 1). To confirm this primer specificity, the PCR products were cloned and sequenced. The sequenced results showed that the *gyrB* gene sequence had a high homology among the *B. cereus* group bacteria, as shown in Table 4. In particular, *B. cereus* and *B. thuringiensis* exhibited a relatively high homology among the *B. cereus* group bacteria. Nonetheless, despite this high homology, the primers used in this study were still able to distinguish *B. cereus* group bacteria with specificity.

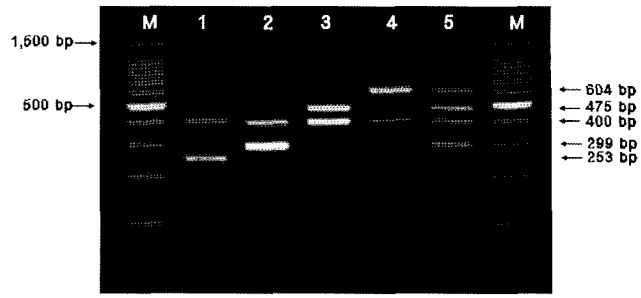

Fig. 1. Multiplex PCR products from genomic DNA of the *B. cereus* group.
Lane M, 100-bp molecular size marker; lane 1, *B. anthracis* ATCC 14578; lane 2, *B. thuringiensis* ATCC 35646; lane 3, *B. cereus* ATCC 14579; lane 4, *B. mycoides* ATCC 6462; lane 5, *B. anthracis* ATCC 14578, *B. thuringiensis* ATCC 35646, *B. cereus* ATCC 14579, and *B. mycoides* ATCC 6462.

The *groEL* gene has already been used in a PCR to detect the *B. cereus* group [4]. However, the current study constructed a new *B. cereus* group primer set based on the *groEL* gene to enable *B. cereus* group bacteria with different sizes of PCR product to be distinguished from other *Bacillus* species in a multiplex PCR.

Multiplex PCR for Detection of *B. cereus* Group from Food Samples

The specificity and sensitivity of the *B. cereus* group identification using the multiplex PCR assay were evaluated with different types of food sample. A total of 47 strains that had been isolated from *Bacillus* selection media were monitored using the multiplex PCR. The 27 *Bacillus* isolates from boiled rice were identified as 4 strains of *B. thuringiensis* and 23 strains of *B. cereus* (Fig. 2, lanes 1–27). Among the four kinds of vegetables, twelve strains of *B. cereus*, six strains of *B. thuringiensis*, and two strains of *B. cereus* group bacteria were detected (Fig. 2, lanes 28–47). Although two isolates from lettuce (Fig. 2, lanes 28, 31) were assumed to be from the *B. cereus* group, the strains could not be identified in this study, but are thought to be *B. pseudomycoides*, *B. weihenstephanensis*, or *B.*

Table 4. Sequence similarity of each PCR product to *Bacillus cereus* group bacteria.

PCR product		% Similarity of *gyrB*			
Bacterium	Strain	*B. cereus*	*B. thuringiensis*	*B. anthracis*	*B. mycoides*
B. cereus	ATCC 14579	100	99	90	89
B. thuringiensis	ATCC 35646	95	100	86	93
B. anthracis	ATCC 14578	95	99	100	87
B. mycoides	ATCC 6462	91	93	87	100

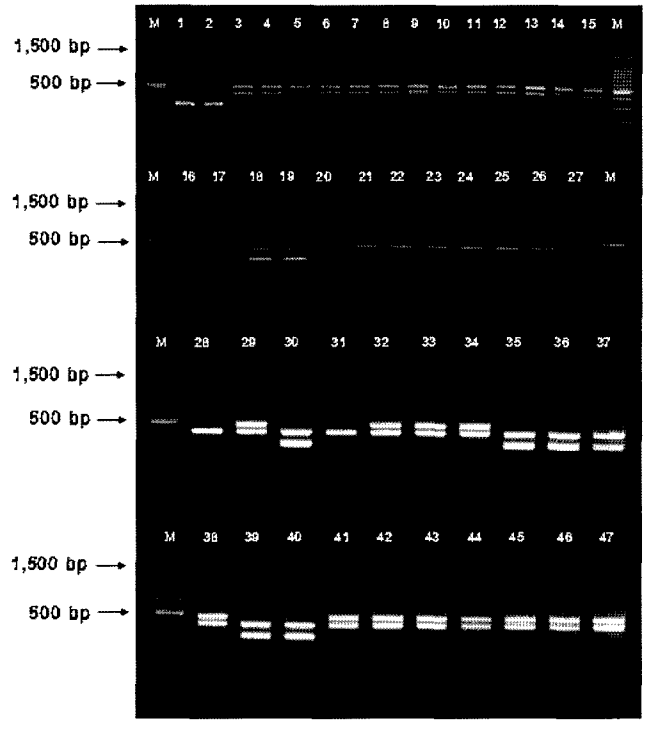

Fig. 2. Detection of *B. cereus* and *B. thuringiensis* in contaminated food samples when using multiplex PCR.
Lane M, 100-bp molecular size marker; lanes 1 to 27, boiled rice; lanes 28 to 33, lettuce; lanes 34 to 36, crown daisy; lane 37, spinach; lanes 38 to 47, spring onion.

medusa from the *B. cereus* group. However, as no genomic data are available, these species have not been analyzed.

Recently, *B. anthracis* was distinguished from *B. cereus* and *B. thuringiensis* using a selective chromogenic agar medium based on morphology and chemical reagents [10]. Moreover, a microarray analysis of *B. cereus* group bacteria was reported based on virulence factor genes [18]. However, these methods are too expensive and time-consuming for the rapid detection of *Bacillus cereus* group bacteria.

In contrast, the multiplex PCR described in the present study is an efficient and rapid method for identifying and differentiating *B. cereus* group bacteria from food samples in a single reaction.

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