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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

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As 16S rRNA sequence analysis has proven inadequate for the differentiation of *Bacillus cereus* from closely related species, we employed the gyrase B gene (*gyrB*) as a molecular diagnostic marker. The *gyrB* genes of *B. cereus* JCM 2152^T, *Bacillus thuringiensis* IAM 12077^T, *Bacillus mycoides* ATCC 6462^T, and *Bacillus anthracis* Pasteur #2H were cloned and sequenced. Oligonucleotide PCR primer sets were designed from within *gyrB* sequences of the respective bacteria for the specific amplification and differentiation of *B. cereus*, *B. thuringiensis*, and *B. anthracis*. The results from the amplification of *gyrB* sequences correlated well with results obtained with the 16S rDNA-based hybridization study but not with the results of their phenotypic characterization. Some of the reference strains of both *B. cereus* (three serovars) and *B. thuringiensis* (two serovars) were not positive in PCR amplification assays with *gyrB* primers. However, complete sequencing of 1.2-kb *gyrB* fragments of these reference strains showed that these serovars had, in fact, lower homology than their originally designated species. We developed and tested a procedure for the specific detection of the target organism in boiled rice that entailed 15 h of preenrichment followed by PCR amplification of the *B. cereus*-specific fragment. This method enabled us to detect an initial inoculum of 0.24 CFU of *B. cereus* cells per g of boiled rice food homogenate without extracting DNA. However, a simple two-step filtration step is required to remove PCR inhibitory substances.

Bacillus cereus is a gram-positive, spore-forming, facultatively anaerobic bacterium. Differentiation of *B. cereus* from its closely related microorganisms depends upon the absence of toxin crystals (from *B. thuringiensis*), hemolytic activity (from *B. anthracis*), and nonrhizoid growth and motility (from *B. mycoides*). *B. cereus* produces emetic toxin and enterotoxins (2, 16, 18, 28, 29, 33). Strains of *B. thuringiensis* are also reported to produce enterotoxins (1, 3, 14, 23), and molecular characterization revealed that the enterotoxin-encoding gene isolated from *B. thuringiensis* was similar to that of *B. cereus* (3).

The nucleotide sequence of 16S rRNAs of the *B. cereus* group exhibited very high levels of sequence similarity (>99%) that were consistent with the close relationships shown by previous DNA hybridization studies (37). The 16S rRNA sequences of *B. mycoides* and *B. thuringiensis* differed from each other and from the sequences of *B. anthracis* and *B. cereus* by 0 to 9 nucleotides (5). Likewise, Ash and Collins (4) reported that the 23S rRNA gene sequences of *B. anthracis* and an emetic strain *B. cereus* were found to be identical. The lesser variations noted in the spacer regions between the 16S and 23S rRNAs do not seem to be sufficient to allow the design of a species-specific oligonucleotide probe for the *B. cereus-B. thuringiensis-B. anthracis* group (9). Specific DNA probes based on variable region VI of 16S rRNAs of *B. cereus* and *B. thuringiensis* were designed by te Giffel et al. (39) and used in hybridization experiments, but screening of isolates with this probe from various sources and outbreaks is necessary to validate their claim. Single-strand conformation polymorphism of the PCR products did not allow species discrimination within the *B. cereus* group (8). Virulence factors (22, 35), restriction fragment length polymorphism (25), pulsed-field gel electrophoresis, analysis of intergenic spacer regions (20), and the arbitrary PCR (12, 21) differentiated *B. anthracis* from *B. cereus* but failed to differentiate *B. cereus* from *B. thuringiensis*. Because of the indistinguishable phenotypic and genotypic characteristics of these organisms, Ash et al. (6) proposed considering *B. thuringiensis*, *B. mycoides*, and *B. anthracis* as subspecies of *B. cereus*.

Since no universal probe is available to differentiate *B. cereus* from other related species, we have studied the possibility of using *gyrB* genes that encode the subunit B protein of DNA gyrase (topoisomerase type II) as targets of highly specific probes (50). In this study, 1.2-kb fragments of the *gyrB* genes of *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycoides* were amplified, cloned, and sequenced. We designed suitable PCR primer sets that could amplify the *gyrB* fragments of *B. cereus*, *B. anthracis*, and *B. thuringiensis* to specifically identify the organism irrespective of its phenotypes, serotypes, and virulence factors. A protocol for the direct detection of *B. cereus* from boiled rice without extracting DNA is also described.

MATERIALS AND METHODS

Bacterial strains. Microorganisms included in this study were purchased from various culture collections or were gifts from the Nagoya Public Health Research Institute and Mie University, Nagoya, Japan. Strains isolated from various aquatic environments and foods are also included. All microorganisms were grown in nutrient broth (Nissui, Tokyo, Japan) at 35°C for 24 h before use. Presumptive colonies appearing on the NGKG agar (Nissui) were picked, and biochemical characterization of the isolates was carried out as described elsewhere (28, 38, 45).

DNA isolation. Chromosomal DNA of overnight-grown cultures was extracted by phenol-chloroform solvents and ethanol precipitation (36). The dried DNA was then dissolved in Tris-EDTA (TE) buffer (pH 7.5) and used as the DNA

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FIG. 1. Nucleotide sequence alignment of *gyrB* of various *Bacillus* species. Nucleotides identical to those of *B. cereus* are indicated by dots. Nucleotide positions for various primers appear on a black field. Primers BC1 and BC2r are *B. cereus* specific; primers BT1 and BT2r are *B. thuringiensis* specific; and primers BA1 and BA2r are *B. anthracis* specific.

template. The purity of the DNA was checked by agarose gel electrophoresis, and the DNA concentration was measured with a spectrophotometer.

Cloning and sequencing of the *gyrB* gene. Primers (UP-1 and UP-2r) within the known DNA sequence (50) were added to the PCR mixture at a concentration of 1 μM, and the solution was subjected to 30 cycles of PCR (43). The amplified *gyrB* fragments from *B. cereus* JCM 2152^T, *B. thuringiensis* IAM 1077^T, *B. anthracis* (Pasteur #2H), and *B. mycoides* ATCC 6462^T were cloned in pGEM-ZF$^+$ (Promega, Madison, Wis.) by conventional recombinant methods (36). Expansion of the probes was carried out as documented previously (36). After ligation of the PCR fragments into the vector, *Escherichia coli* cells were transformed with the ligation mixture by calcium chloride-mediated transformation. After transformation, the transformants were cultured under conditions that promote growth. Plasmids were recovered from a transformant by lysis and purification by an alkaline method. The purified intact plasmid was then utilized as a probe. The identity of the fragment was verified by sequencing from both ends by the dideoxy chain termination method with a Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) and with an ABI 373A automatic sequencer as described by the manufacturer (Perkin-Elmer Corp., Foster City, Calif.). DNA sequences were determined from both strands by extension from vector-specific (T7 and SP6 primers from pGEM-ZF$^+$) priming sites and by primer walking.

Oligonucleotide primers. Various oligonucleotide primers based on the *gyrB* nucleotide sequence data of the *Bacillus* species were synthesized according to the instructions of the manufacturer (Beckman, Fullerton, Calif.).

PCR assay. Whole bacterial cells without extracting DNA were used as templates. In this case, the freshly grown cells (overnight incubation at 35°C) on nutrient agar plates were used. If the bacterial cells were grown in liquid medium, the bacterial cells were harvested by centrifugation and washed once with phosphate-buffered saline (PBS; 0.1 M, pH 7.5), and appropriate counts of bacterial cells were used. In some cases, purified DNA was used as a template for PCR amplification.

PCR assays were performed in a DNA Thermal Cycler (Perkin-Elmer). Reaction volumes of 100 μl contained 100 ng of genomic DNA, deoxynucleoside triphosphates at a concentration of 200 μM each, and primers at 1 μM each in reaction buffer (Tris-HCl, 100 mM; $MgCl_2$, 15 mM; KCl, 500 mM; pH 8.3). The 1.2-kb *gyrB* gene was amplified as described elsewhere (50). The amplification of various *Bacillus* species-specific (*B. cereus*, 365 bp; *B. thuringiensis*, 368 bp; and *B. anthracis*, 245 bp) fragments was performed by using PCR for 30 cycles, each consisting of 1 min at 94°C, 1.5 min at 58°C, and 2.5 min at 72°C, with a final extension step at 72°C for 7 min. After DNA amplification, PCR fragments were analyzed by submarine gel electrophoresis, stained, and visualized under UV illumination (44). Suitable molecular size markers were included in each gel.

Hybridization with 16S rDNA probes. Hybridizations were performed with 16S rDNA probes that are specific to *B. cereus* (TTA AGA ACT TGC TCT TAT) and *B. thuringiensis* (TTG AGA GCT TGC TCT CAA) as described by te Giffel et al. (39). The purified 16S fragment was transferred onto a nylon membrane (Hybond-N; Amersham) by Southern blotting. The blotted membranes were neutralized in 0.2 M Tris-HCl (pH 7.5) in 0.3 M NaCl–0.03 M sodium citrate and air dried. Prehybridization and hybridization were performed in 0.5 M sodium dodecyl sulfate and 1% bovine serum albumin. After 30 min of prehybridization at 45°C, the commercially available probe, which had been 3′ end DIG labeled (Boehringer Mannheim, Foster City, Calif.), was added, and signals were detected according to the manufacturer's instruction with DIG DNA labeling kits (Boehringer Mannheim).

PCR assay sensitivity for the detection of artificially contaminated *B. cereus* in boiled rice. A 25-g sample of boiled rice was homogenized for 1 min with a homogenizer (SH-001; Elmex, Tokyo, Japan) in 225 ml of nutrient broth to produce a uniform food homogenate for all experiments. *B. cereus* JCM 2152^T was grown in nutrient broth overnight at 35°C and serially diluted with the food homogenate as the diluent to final concentrations ranging from 0 to 10^9 CFU per g. These artificially contaminated food homogenate microcosms (10 ml each) were incubated at 35°C. Subsampling (1 ml) was carried out after incubations of 0, 6, and 15 h (overnight); cells were then centrifuged (4°C; 10,000 × *g* for 10 min) and resuspended in 1 ml of sterile PBS.

Suitable controls such as buffer, media, PCR mixtures, and *B. cereus* DNA were employed to check any false-positive or false-negative reactions. Appropriate dilutions of test sample prepared at various intervals in nutrient broth were spread plated onto standard plate count agar (Nissui) for total viable counts and on NGKG agar (Nissui) for the enumeration of the *B. cereus* population.

To remove any PCR-inhibitory substances from food, samples drawn from the microcosms were subjected to two-step filtration as described previously (44), with some modifications. Briefly, a 400-μl sample of a sterile-PBS-washed sample was passed through a 5-μm-diameter Ultrafree filter tube (UFC3 0GV; Millipore, Bedford, Mass.) and centrifuged (4°C; 10,000 × *g* for 10 min). The 5-μm-

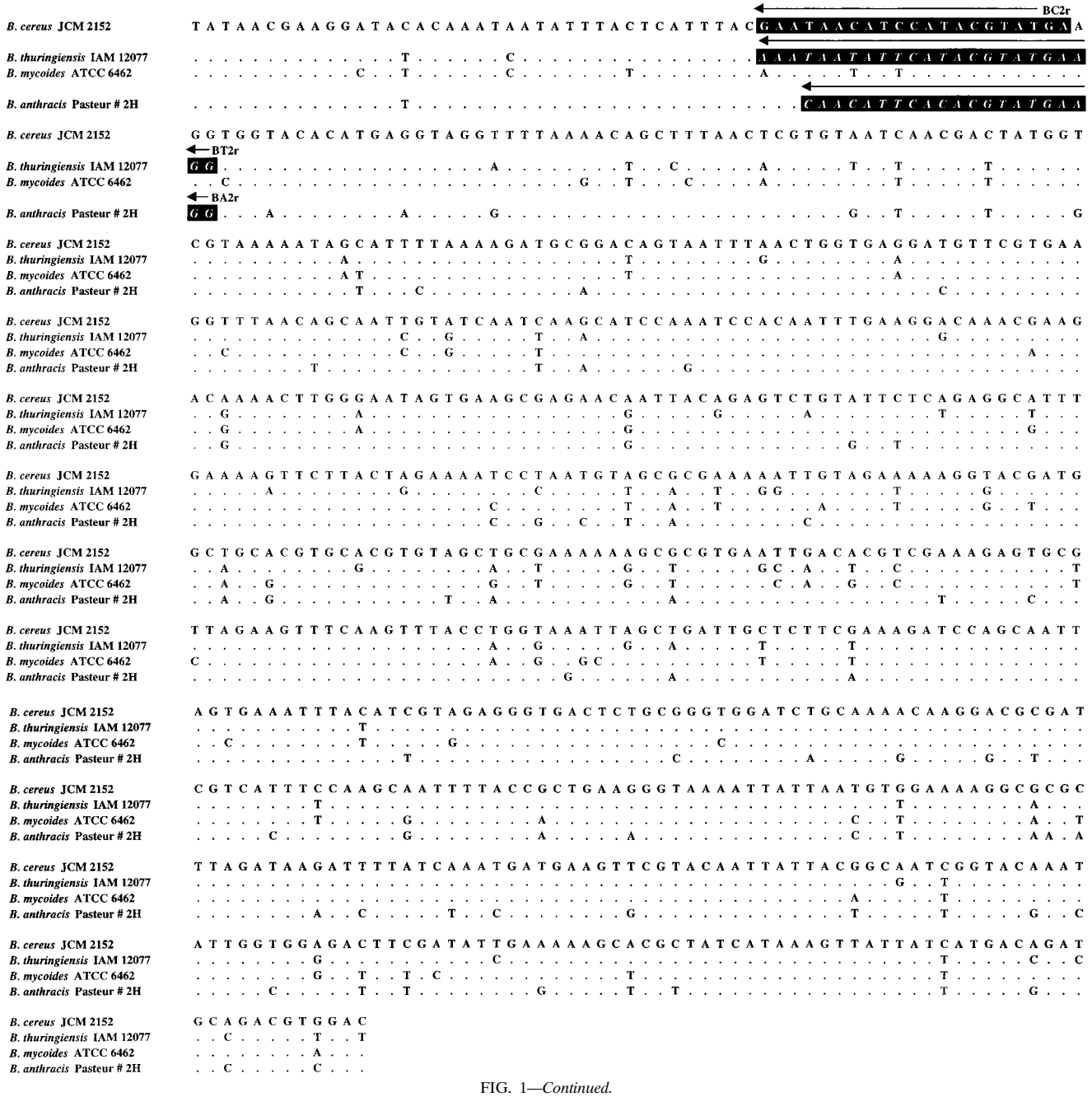

FIG. 1—*Continued.*

diameter-tube filtrate was then passed through 0.2-μm-diameter Ultrafree centrifuge tube (SE3P009E4; Millipore) and centrifuged (4°C; 10,000 × *g* for 10 min) to remove bacteria. The material trapped on the 0.2-μm (pore size) filter was then resuspended in 50 μl of sterile PBS and boiled before 10 μl of supernatant was used as a template for the PCR assay.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here will appear in the GenBank nucleotide sequence database with the indicated accession numbers: *B. cereus* JCM 2152^T (AF090330), *B. thuringiensis* IAM 12077^T (AF090331), *B. anthracis* Pasteur #2H (AF090333), and *B. mycoides* ATCC 6462^T (AF090332).

RESULTS

***gyrB* sequence of *Bacillus* species.** Complete sequences of the 1.2-kb *gyrB* fragments of *B. cereus* JCM 2152^T, *B. thuringiensis* IAM 12077^T, *B. anthracis* Pasteur #2H, and *B. mycoides*

TABLE 1. Similarities among *gyrB* genes of various *Bacillus* species

Bacterium	Strain	% Similarity to *gyrB* of:			
		B. cereus	*B. thuringiensis*	*B. anthracis*	*B. mycoides*
B. cereus	JCM 2152^T		96.8[b]	98.8[b]	95.5[b]
B. thuringiensis	IAM 12077^T	90.2[a]		96.5[b]	96.8[b]
B. anthracis	Pasteur #2H	90.7[a]	87.4[a]		95.3[b]
B. mycoides	ATCC 6462^T	88.8[a]	91.0[a]	86.6[a]	

[a] Percentage of similarity in *gyrB* nucleic acid sequences.
[b] Percentage of similarity in *gyrB* amino acid sequences.

TABLE 2. Base-pair substitutions in *gyrB* genes of various *Bacillus* species

Bacterium	Strain	No. of base-pair substitutions in:			
		B. cereus	*B. thuringiensis*	*B. anthracis*	*B. mycoides*
B. cereus	JCM 2152^T		2[b]	2[b]	6[b]
B. thuringiensis	IAM 12077^T	121[a]		4[b]	4[b]
B. anthracis	Pasteur #2H	113[a]	153[a]		8[b]
B. mycoides	ATCC 6462^T	136[a]	111[a]	162[a]	

[a] Base-pair substitutions in *gyrB* nucleic acid sequences.
[b] Base-pair substitutions in *gyrB* amino acid sequences.

TABLE 3. Base-pair substitutions and similarities in 16S rDNA nucleotide sequences for various *Bacillus* species

Bacterium	Strain	Accession no.[a]	No. of base-pair substitutions or % similarity in:			
			B. cereus	*B. thuringiensis*	*B. anthracis*	*B. mycoides*
B. cereus	ATCC 14579^T	D16266		99.6[c]	100[c]	99.4[c]
B. thuringiensis	ATCC 12077^T	X55062	5[b]		99.6[c]	99.3[c]
B. anthracis	Sterne	X55059	0[b]	5[b]		99.4[c]
B. mycoides	ATCC 6462^T	Z84592	8[b]	10[b]	8[b]	

[a] Taken from GenBank public database.
[b] Base-pair substitutions in 16S rDNA nucleic acid sequences.
[c] Percentage of similarity in 16S rDNA nucleic acid sequences.

ATCC 6462^T were determined and aligned (Fig. 1). The percentages of similarity (Table 1) and numbers of base-pair substitutions (Table 2) in *gyrB* nucleotide sequences and translated protein sequences for all four of these *Bacillus* species are given. Likewise, the percentages of similarity and numbers of base-pair substitutions in 16S rDNA nucleotide sequences are depicted in Table 3. The similarity in the *gyrB* sequences of *B. cereus* and *B. thuringiensis* was 90.2% versus 99.6% for the 16S rDNA sequences. Alignment of the amino acid sequences of the *gyrB* proteins (Fig. 2) translated from the nucleotide sequences showed that only 2 of the 121 substitutions caused amino acid substitutions. The amino acid sequence similarity between the gyrase subunit B proteins of *B. cereus* and *B. thuringiensis* was 96.8% (Table 1). The frequency of base substitutions in the published 16S rRNA was lower than that in *gyrB*. For example, between the sequences of *B. cereus* and *B. thuringiensis* 121 base substitutions were observed in *gyrB*, while only 5 base substitutions were observed in 16S rRNA. It is interesting to note that no substitution was observed between the sequences of *B. cereus* ATCC 14579^T and *B. anthracis* serotype Sterne.

Designing *B. cereus*-, *B. thuringiensis*-, and *B. anthracis*-specific PCR primer sets. Oligonucleotide primers that are universal and specific to various *Bacillus* species were synthesized based on the nucleotide sequences of *gyrB* (Table 4). A forward primer with a nucleotide position of 175 to 195 (BC1) and an antisense primer with a position of 519 to 539 were synthesized (BC2r). When these primers were used to generate 365-bp PCR products, *B. cereus* could be differentiated from *B. anthracis* and type strains of *B. thuringiensis* IAM 12077^T and *B. mycoides* ATCC 6462^T. Likewise, a 368-bp fragment specific to *B. thuringiensis* (made by using BC1 and BT2r) and a 245-bp amplicon specific to *B. anthracis* (made by using BA1 and BA2r) were amplified with the appropriate primer sets.

Specificity of PCR primers in the detection of *Bacillus* species. *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides* strains received from various culture collections and identified as different serogroups or isolated from numerous food and environmental specimens were tested with *gyrB* primer sets specific for these *Bacillus* species.

PCR results in the differentiation of these *Bacillus* species for the type strains and various serogroups are given in Table 5. Type strains of all four *Bacillus* species showed species-specific positive amplification. However, *B. cereus*-specific signal was not observed for some *B. cereus* serotypes (H5, H6, H7, H16, and H17). In addition, amplification of 365-bp fragment

FIG. 2. Amino acid sequence alignment of the *gyrB* products of various *Bacillus* species. Amino acids identical to those of *B. cereus* are indicated by dots.

TABLE 4. *gyrB* PCR primers in the differentiation of *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides*

Specificity	Primer	Oligonucleotide sequence	No. of bp[a]
Universal primers[b]	UP-1	GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA	41
	UP-2r	AGC AGG ATA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT	44
B. cereus	BC1	ATT GGT GAC ACC GAT CAA ACA	21
	BC2r	TCA TAC GTA TGG ATG TTA TTC	21
B. thuringiensis	BT1	ATC GGT GAT ACA GAT AAG ACT	21
	BT2r	CCT TCA TAC GTA TGA ATA TTA TTT	24
B. anthracis	BA1	AAT CGT AAT ATT AAA CTG ACG	21
	BA2r	CCT TCA TAC GTG TGA ATG TTG	21

[a] bp, base pairs.
[b] Universal primers were as described by Yamamoto and Harayama (50).

that is specific to *B. cereus* was noted for *B. thuringiensis* serotypes kurstaki (HD-1) and aizawai. When tested with various PCR primer sets that were established in this study, serotypes H5, H6, H7, and H17 showed a positive signal for *B. anthracis*, and serotype H16 exhibited an amplicon specific to *B. thuringiensis*.

The 1.2-kb *gyrB* nucleotide sequences of *B. cereus* serotypes H2, H6, and H16 and *B. thuringiensis* serotype kurstaki (HD-1) were determined. By comparing the *gyrB* nucleotide sequences of these strains along with type strain sequences, both H2 and kurstaki (HD-1) showed high similarity with *B. cereus* JCM 2152^T (98.4%). Likewise, a high similarity value was noted between *B. anthracis* and H6 (97.9%), as well as between *B. thuringiensis* and H16 (93.0%).

A total of 104 *Bacillus* strains isolated from various environments, including food and clinical sources, consisting of various phenotypes, serotypes, and toxigenic properties were tested for PCR assay (Table 6). *B. cereus* isolates obtained from IFO and IAM culture collections were identified perfectly as *B. cereus*. However, among 50 *B. cereus* strains isolated from foods, 4 and 2% of the strains produced amplicons specific to *B. thuringiensis* and *B. anthracis*, respectively. Likewise, 6 of 20 *B. cereus* isolates from various environmental sources were identified as *B. anthracis*. Eight of ten environmental isolates that were identified as *B. thuringiensis* on the basis of crystal protein were recognized as *B. cereus*. One of nine *B. mycoides* isolates obtained from the ATCC showed a positive signal for *B. cereus*.

Comparison of *gyrB* PCR and 16S rDNA hybridization techniques in the differentiation of *Bacillus* species. Specific DNA probes based on variable region VI of 16S rRNAs of *B. cereus* and *B. thuringiensis* were reported to be useful for differentiating these species (39). Type strains, serotypes, and wild-type strains that were identified as *Bacillus* species on the basis of biochemical and serological classifications were subjected to Southern hybridization by using 16S rDNA probes and *gyrB* PCR with various *Bacillus* species primer sets (Table 7). Among the type strains tested, the hybridization signal showed good correlation with its species by *gyrB* PCR except for *B. mycoides* ATCC 6462^T, where a positive signal was noted when *B. cereus*-specific 16S rDNA probes were used. te Giffel et al. (39) also reported such a false-positive signal for *B. mycoides*. On the other hand, *gyrB* PCR did not produce any false-positive amplicon and was able to differentiate *B. cereus* from *B. mycoides*. In contrast, serotypes kurstaki and aizawai of *B. thuringiensis* were identified as *B. cereus* by conceding positive signals for *B. cereus* in both hybridization and PCR techniques. This indicated that *B. thuringiensis* serotype kurstaki and aizawai are, in fact, *B. cereus* and not *B. thuringiensis*. However, *B. cereus* serotype H16 showed a positive signal for *B. cereus* in hybridization experiments but showed an amplicon specific for *B. thuringiensis* by the *gyrB* PCR technique. To clarify these findings, we tried to procure H16 serotype from other culture collections, but we did not succeed in acquiring the strain. Also, *B. cereus* serotypes H5, H6, H7, and H17, which showed positive amplification for *B. anthracis*, did not yield any *B. cereus*-specific hybridization signal. Complete sequencing of 1.2-kb *gyrB* fragments of these strains supported our claim that these strains were indeed misidentified.

Sensitivity of the PCR primers in the detection of *Bacillus* species. To evaluate the sensitivity of the PCR assay, a dilution series of genomic DNA from various *Bacillus* species was pre-

TABLE 5. Specificity of *gyrB* PCR primers in the differentiation of *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides*

Bacterial strain and serotype[a]	Amplification of a specific PCR fragment[b] that identified the strain as:		
	B. cereus	*B. thuringiensis*	*B. anthracis*
B. cereus			
JCM 2152^T	+	−	−
H1	+	−	−
H2	+	−	−
H3	+	−	−
H4	+	−	−
H5	−	−	+
H6	−	−	+
H7	−	−	+
H8	+	−	−
H9	+	−	−
H10	+	−	−
H11	+	−	−
H12	+	−	−
H13	+	−	−
H14	+	−	−
H15	+	−	−
H16	−	+	−
H17	−	−	+
H18	+	−	−
B. thuringiensis			
berliner (IAM 12077^T)	−	+	−
kurstaki (HD-1)	+	−	−
galleriae	−	+	−
aizawai	+	−	−
israelensis	−	+	−
B. anthracis Pasteur #2H	−	−	+
B. mycoides ATCC 6462^T	−	−	−

[a] JCM, Japan Collection of Microorganisms; IAM, Institute of Applied Microorganisms; ATCC, American Type Culture Collection; and ^{T}, type strain.
[b] PCR primer sets BC1-BC2r, BT1-BT2r, and BA1-BA2r were used to amplify *B. cereus*-, *B. thuringiensis*-, and *B. anthracis*-specific fragments, respectively.

TABLE 6. Differentiation of *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides* wild strains by using *gyrB* PCR primers

Bacterium	Source[a]	No. of strains tested	% of strains that were identified by PCR as:		
			B. cereus[b]	*B. thuringiensis*[c]	*B. anthracis*[d]
B. cereus	IFO	10	100		
B. cereus	IAM	5	100		
B. cereus	Food[e]	50	94	4	2
B. cereus	Environment[f]	20	70		30
B. thuringiensis	Environment[e]	10	80	20	
B. mycoides	ATCC	9	11.1		

[a] For abbreviation explanations, see Table 5.
[b] BC1 and BC2r primers were used.
[c] BT1 and BT2r primers were used.
[d] BA1 and BA2r primers were used.
[e] Obtained from Nagoya Public Health Research Institute, Nagoya, Japan.
[f] Obtained from Mie University, Nagoya, Japan.

pared in TE buffer, and aliquots were used as templates for PCR amplification. Aliquots containing a picogram level of genomic DNA were successfully detected after amplification with primer-specific PCR primer sets. A dilution series of freshly cultured cells of *Bacillus* species showed that the primer set employed in this study amplified species-specific PCR fragments when 2 to 5 CFU of bacterial cell per reaction tube was used.

Detection of *B. cereus* in the artificially contaminated boiled rice. The sensitivity of the PCR assay for detecting artificially contaminated *B. cereus* in cooked boiled rice is presented in Table 8. Absence of *B. cereus*-like organisms in the test sample was confirmed by both the conventional enrichment method and by PCR assay. When the food homogenate was incubated for 15 h in nutrient broth at 37°C, an initial inoculum of 0.24 CFU of *B. cereus* per g of food homogenate amplified the desired PCR product (Fig. 3). At time zero, 2.4×10^4 CFU of *B. cereus* per g of boiled-rice homogenate did not yield any PCR products. The detection of *B. cereus* directly from the food sample was possible by the combination of a 15-h enrichment period in nutrient broth and PCR assay even without DNA extraction.

DISCUSSION

Enterotoxigenic *B. cereus*, which causes acute gastroenteritis after the consumption of contaminated foods, has been known to produce the emetic and the diarrheal toxin types (18). *B. thuringiensis* can be distinguished from *B. cereus* only by the production of toxin crystals and can be detected by simple microscopy or by hybridization with specific probes for the delta endotoxin gene (10). However, this character is plasmid borne and transmissible to *B. cereus* by conjugation (13, 15). *B. cereus* strains can grow at temperatures between 4 and 37°C (40, 42), and psychrotrophic *B. cereus* strains can produce enterotoxin both aerobically and anaerobically (17). Good diagnostic tools are thus required to ensure the hygienic quality of susceptible food items. Although some PCR methods are developed to detect *B. cereus* targeting the toxigenic properties (30), the production of *B. cereus*-type enterotoxins in *Bacillus* species, including *B. thuringiensis* (23), raised doubts about the validity of these diagnostic probes in specifically identifying *B. cereus*. Thus, identifying *B. cereus* irrespective of its virulence factors is necessary from the viewpoint of public health. We therefore wanted to develop a rapid and reliable method for differentiating *B. cereus* from *B. thuringiensis*.

As 16S rDNA sequences have very high similarity between *B. cereus* and *B. thuringiensis*, we were looking for appropriate molecular taxonomic markers. Yamamoto and Harayama (50) suggested that genes that are not spread horizontally among different bacterial species may be used to trace the evolutionary record of host bacteria. Based on the information that the average substitution rate for 16S rRNA is 1% per 50 million years and that the rate for synonymous sites of protein-coding DNA is 0.7 to 0.8% per million years (31), Yamamoto and Harayama (50) proposed the *gyrB* gene as a substitute for 16S rRNA as a molecular taxonomic marker for bacterial species. The *gyrB* gene is essential for DNA replication, a housekeeping activity; it is also single copied and has conserved regions for the development of PCR primers. We have designed PCR primers by exploiting differences in *gyrB* genes to differentiate *V. parahaemolyticus* from *V. alginolyticus*, a pair that exhibited 99.7% similarity in their 16S rDNA nucleotide sequences (43). Likewise, the similarity of 1.2-kb *gyrB* sequences of *B. cereus* and *B. thuringiensis* was not high (90.7%); thus, we were able to design a suitable PCR primer set for differentiating these two organisms by amplifying a *B. cereus*-specific 365-bp amplicon. The type strains of both *B. cereus* and *B. thuringiensis* responded specifically with the developed PCR primer set by giving a positive signal for *B. cereus* and a negative signal for *B. thuringiensis*. Also, these PCR primers, which are specific for *B. cereus*, did not yield any amplicon for the *B. anthracis* strain and the *B. mycoides* type strain.

The *gyrB* PCR primer set designed in this study detected 2 to 5 CFU of *B. cereus* cells per reaction tube or correspondingly low levels (10 pg) of extracted DNA. The sensitivity accords

TABLE 7. Comparison of *gyrB* PCR and 16S rDNA hybridization probe techniques in the differentiation of *B. cereus* and *B. thuringiensis*

Bacterial strain or serotype	Hybridization signal positive for:		Amplification of *gyrB* PCR fragment that is specific to:		
	B. cereus[a]	*B. thuringiensis*[b]	*B. cereus*[c]	*B. thuringiensis*[d]	*B. anthracis*[e]
B. cereus					
JCM 2152^T	+	−	+	−	−
H5	−	−	−	−	+
H6	−	−	−	−	+
H7	−	−	−	−	+
H16	+	−	−	+	−
H17	−	−	−	−	+
Nagoya 126[f]	−	+	−	+	−
Nagoya 127[f]	−	+	−	+	−
B. thuringiensis					
berliner (IAM 12077^T)	−	+	−	+	−
kurstaki (HD-1)	+	−	+	−	−
galleriae	−	+	−	+	−
aizawai	+	−	+	−	−
israelensis	−	+	−	+	−
B. anthracis Pasteur #2H	−	−	−	−	+
B. mycoides ATCC 6462^T	+	−	−	−	−

[a] *B. cereus*-specific 16S rDNA-based probe (39).
[b] *B. thuringiensis*-specific 16S rDNA-based probe (39).
[c] BC1-BC2r primer set was used.
[d] BT1-BT2r primer set was used.
[e] BA1-BA2r primer set was used. Ba primer set was also used (35).
[f] Food isolate obtained from Nagoya Public Health Research Institute.

TABLE 8. Influence of preenrichment incubation time and bacterial population in the amplification of PCR product specific to *B. cereus*

Sample or parameter	Preenrichment incubation in nutrient broth (h)	PCR band amplified when initial inoculum level was in the range[a] or CFU per g of cooked-rice homogenate[b]:						
		2.4×10^4	2.4×10^3	2.4×10^2	2.4×10^1	2.4	0.24	0
Food homogenate	0	−	−	−	−	−	−	−
	6	+	+	+	−	−	−	−
	15	+	+	+	+	+	+	−
Population	0	2.4×10^4	2.4×10^3	2.4×10^2	2.4×10^1	2.4	0.24	0
	6	9.0×10^6	4.6×10^5	4.0×10^4	9.7×10^3	1.5×10^3	2.0×10^2	0
	15	3.6×10^7	2.3×10^7	3.8×10^7	4.3×10^7	6.8×10^7	7.7×10^7	0

[a] −, 365-bp amplified product specific to *B. cereus* is not seen; +, 365-bp amplified product is seen.
[b] Since cooked rice was used as a sample, no culturable food microflora was observed, and the counts given are for *B. cereus*.

with that described for PCR with other bacteria, being between 1 and 20 CFU (27, 32, 41, 47) or between 1 and 100 pg for DNA extracted from the bacterial population (24, 49). Increased sensitivity may be achieved by Southern blot analyses as reported earlier (7). The *gyrB* primer set recognized all strains identified as *B. cereus* and its group by conventional methods, but it did not recognize the other bacteria tested. In addition, some serotypes that were designated in culture collections as *B. cereus* or *B. thuringiensis* were differentiated and their species were identified. Specialized laboratories use elaborate techniques for confirming the *B. cereus* group and their strain identity, techniques such as toxin antigen detection, detection of virulent plasmids, the use of crystal protein, etc. Since the *gyrB* PCR result is in agreement with 16S rDNA-based hybridization probe technique, this simple PCR method is thus a powerful tool for the confirmation and differentiation of the *B. cereus* species in clinical, food, and environmental samples.

Two major limitations to using PCR as a diagnostic tool are that false-positive reactions can occur from DNA contamination and that false-negative reactions can occur from a number of substances found in samples that inhibit PCR (44, 47). We have included suitable negative and positive controls to overcome these limitations when and where necessary. Sensitivity of detection in food samples has, however, been low because only small samples (10 to 100 μl) can be analyzed, since many sorts of food contain substances that are PCR inhibitory. Such PCR-inhibitory substances were reported in many clinical samples, such as urine, blood, sputum, fecal specimens, food, and environmental samples (26, 46, 48). However, with bacteria in food, the sensitivity of the PCR was far lower than that with bacteria in saline. With bacteria in food, the lower detection limit was higher than the number of CFU per unit volume of food, a result which is usually found with processed food. Previous studies suggested the extraction of DNA (27) or the application of chemicals (11). A simple two-step filtration procedure that we reported previously successfully removed PCR-inhibitory products during this study (44). However, a simple preenrichment in a nonspecific medium was necessary to permit the proliferation of target bacteria. A similar phenomenon was documented for the detection of *V. parahaemolyticus* from shrimp (43).

The findings reported here describe a rapid, sensitive, specific, and reliable method for the detection of *B. cereus* in boiled rice. The fact that this technique allows detection of the genetic potential and permits differentiation from related species may make it useful as both a screening test and a confirmatory test. The data provided from this test could yield additional information that will be useful to epidemiological studies.

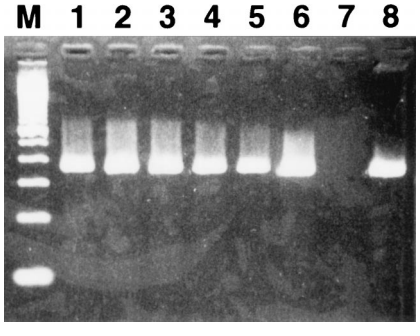

FIG. 3. Detection of *B. cereus* in artificially contaminated cooked rice by the BC-1 and BC-2 primers (365-bp product). *B. cereus* cells grown overnight in nutrient broth were serially diluted in cooked rice homogenate (see details in Materials and Methods) to obtain appropriate dilutions. Lanes: M, 100-bp DNA ladder; 1 to 6, initial inocula of 2.4×10^4 (lane 1), 2.4×10^3 (lane 2), 2.4×10^2 (lane 3), 2.4×10^1 (lane 4), 2.4 (lane 5), and 0.24 (lane 6) CFU of *B. cereus* added per g of rice homogenate and incubated overnight at 35°C; 7, cooked rice homogenate that was not spiked with *B. cereus* (negative control) incubated overnight at 35°C; and 8, 2.4×10^4 CFU of *B. cereus* cells per ml prepared in PCR mixture (positive control).

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