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A RAPD–PCR Method for the Rapid Detection of *Bacillus cereus*

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Distinction of *Bacillus cereus* from other closely related bacilli is challenging and new efficient methods are continually demanded. From our previous work on RAPD profiles of bacilli, we found a possibility that *B. cereus* strains could be distinguished from other bacilli. In this work, RAPD–PCR profiles of *B. cereus* strains were obtained using a 10-mer (S30) as a primer, and a *B. cereus* specific 0.91-kb band was produced from all tested strains. The RAPD–PCR procedure also successfully detected *B. cereus* from spiked *cheonggukjang* when *B. cereus* cells were present at more than 10^2/g sample.

Keywords: *Bacillus cereus*, food poisoning, rapid detection, RAPD–PCR

Bacillus cereus is one of the most common food-poisoning organisms and widely distributes in diverse environments such as soil, water, plant materials, and intestinal tracts of animals [5, 13]. Foods contaminated with *B. cereus* are often the cause of one of two different types of food poisoning; diarrhea or emesis [12, 13]. Each symptom is caused by heat-labile and heat-resistant toxin(s), respectively, produced by toxinogenic *B. cereus* strains. Traditionally, cultural methods using selective media are used for detection and identification of *B. cereus* cells from environmental samples including fermented foods. Selective media such as polymyxin–egg yolk–mannitol–bromothymol agar (PEMBA) or mannitol–egg yolk–polymyxin (MYP), however, often result in misidentifications among closely related species [4, 9]. Recently, PCR methods are quite often used for the identification purpose. Parts of toxin gene sequences have been utilized as primers for the detection of *B. cereus* [1, 3, 11]. Although various methods have been developed, any single method is far from satisfactory for the accurate identification of *B. cereus* strains from foods.

Cheonggukjang, Korean traditional fermented soyfood, is prepared by fermentation of soybean with bacilli [6, 7]. In traditional *cheonggukjang* preparation, boiled soybean is covered with rice straw, a rich source for bacilli spores including *B. cereus* [1]. Accordingly, *B. cereus* contamination is a major problem for *cheonggukjang* products. In this work, we report a RAPD–PCR protocol that can be used for the rapid detection of *B. cereus* from *cheonggukjang* and probably other fermented foods.

Bacillus strains were cultivated in Luria–Bertani (LB) broth at 37°C with shaking. Chromosomal DNA was prepared from overnight culture by the method of Martinez *et al.* [10]. Recovered cells after centrifugation (13,000 ×*g*, 3 min) were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 2% (w/v) SDS, and 400 µg/ml proteinase K (Takara, Shiga, Japan). After 30 min at 55°C, total DNA was obtained by sequential extractions using phenol and chloroform. The aqueous upper layer was transferred into a fresh tube and the same volume of isopropanol was added. DNA was precipitated by centrifugation (13,000 ×*g* for 20 min) at 4°C, washed with 70% (v/v) ethanol, and resuspended in 50 µl of water. DNA concentration was determined using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). RAPD–PCR reactions were performed in a 30 µl volume and each tube contained GoTaq green master mix (Promega, Madison, WI, USA), 10 pmol primer, S30 (5'-GTGATCGCAG-3'; Bionics, Seoul, Korea), and 1 µg template DNA. PCR was done using the GeneAmp 2400 PCR system (Perkin Elmer, Waltham, MA, USA) and amplification conditions included an initial denaturation step at 94°C for 5 min, 40 cycles of 94°C for 15 s, 35.5°C for 15 s, and 72°C for 2 min, and a final extension at 72°C for 4 min. Amplified products

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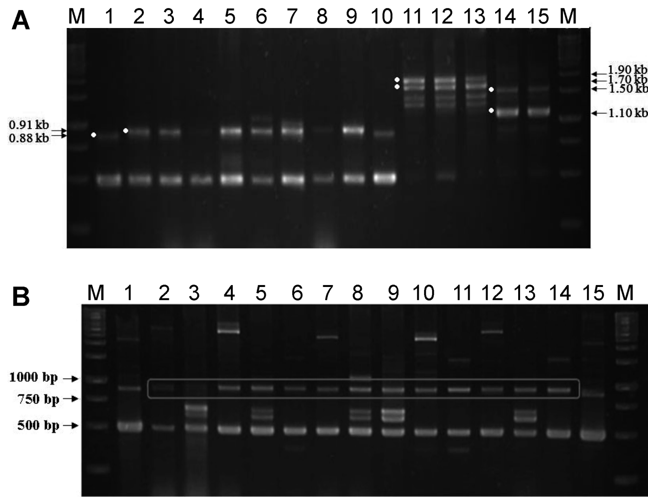
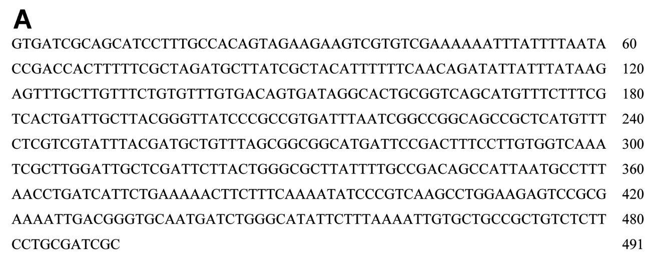


Fig. 1. RAPD−PCR profiles of *Bacillus* (**A**) and *B. cereus* (**B**) reference strains.

A. 1 and 10, *B. subtilis* 168; 2−9, *B. cereus* strains: 2, ATCC 9634; 3, ATCC 21768; 4, ATCC 14579; 5, ATCC 31429; 6, ATCC 27348; 7, ATCC 21366; 8, ATCC 21772; 9, ATCC 12480; 11−13, *B. licheniformis* strains: 11, ATCC 4527; 12, ATCC 21416; 13, ATCC 27811; 14−15, *B. amyloliquefaciens* strains: 14, ATCC 23350; 15, ATCC 23845; M, 1 kb size ladder (MBI Fermentas, Glen Burnie, MD, USA). **B**. 1 and 15, *B. subtilis* 168; 2−14, *B. cereus* strains: 2, ATCC 21771; 3, ATCC 25621; 4, ATCC 10701; 5, ATCC 11778; 6, ATCC 13061; 7, ATCC 10987; 8, ATCC 10876; 9, ATCC 9634; 10, ATCC 21768; 11, ATCC 21366; 12, ATCC 21772; 13, ATCC 11240; 14, ATCC 12480; M, 1 kb size ladder (MBI Fermentas). A 2% agarose gel was used and the 0.91 kb bands were marked.

were analyzed by agarose gel [2% (w/v)] electrophoresis. RAPD−PCR patterns of 13 *B. cereus* and 6 other *Bacillus* reference strains were examined using S30, a 10-mer, which generated distinctive bands reproducibly. When the profiles of *B. cereus* strains were compared with those of other *Bacillus* species (Fig. 1A, lanes 10−15), all *B. cereus* strains showed a 0.91 kb band in addition to a 0.5 kb band. All 13 *B. cereus* strains produced the same 0.91 kb band (Fig. 1B) and it was possible to distinguish the 0.91 kb band from the 0.88 kb band of *B. subtilis*.

We previously showed that all *B. subtilis* strains produced 0.5 and 0.88 kb bands when S30 was used as a single primer [8]. The 0.5 kb band corresponded to an internal part of the *ytcP* gene encoding a hypothetical ABC-type transporter and it was also observed in most *B. amyloliquefaciens* and *B. licheniformis* strains [8]. The 0.5 kb band from *B. cereus* ATCC 21772 (Fig. 1A, lane 8) was gel extracted and sequenced. The sequence was basically identical to that from *B. subtilis*, with a few base substitutions (results not shown). The sequence of the 0.5 kb band from *B. subtilis* is shown in Fig. 2 together with the sequence of the 0.91 kb fragment from *B. cereus* ATCC 12480. BLAST of the nucleotide sequence of the 0.91 kb fragment did not produce any matches. BLAST for the translated amino acid sequence (BLASTp) indicated that the fragment might be a part of a gene encoding a transposase of family 11. The



A

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GTGATCGCAGCATCCTTTGCCACAGTAGAAGAAGTCGTGTCGAAAAAATTTATTTTAATA  60
CCGACCACTTTTTCGCTAGATGCTTATCGCTACATTTTTTCAACAGATATTATTTATAAG  120
AGTTTGCTTGTTTCTGTGTTTGTGACAGTGATAGGCACTGCGGTCAGCATGTTTCTTTCG  180
TCACTGATTGCTTACGGGTTATCCCGCCGTGATTTAATCGGCCGGCAGCCGCTCATGTTT  240
CTCGTCGTATTTACGATGCTGTTTAGCGGCGGCATGATTCCGACTTTCCTTGTGGTCAAA  300
TCGCTTGGATTGCTCGATTCTTACTGGGCGCTTATTTTGCCGACAGCCATTAATGCCTTT  360
AACCTGATCATTCTGAAAAACTTCTTTCAAAATATCCCGTCAAGCCTGGAAGAGTCCGCG  420
AAAATTGACGGGTGCAATGATCTGGGCATATTCTTTAAAATTGTGCTGCCGCTGTCTCTT  480
CCTGCGATCGC                                                   491
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B

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TGTGATCGCAGCCGATTTAATCGACGACGGCGAAGGTTGTCAGGGCTTATCATTCGAGTT  60
CAGGATCATGTATCTCATAGGCTTGAAAACCTAAGCCAGATAATGGTTATAGACTCTATT  120
CCAGTTCCAGTTGTAAAGATGGCAAGGGAGAAGTCCCATAAATCTTTTAAGGAATCTTTT  180
GAGGCGGCACCAGCCAAAGGATACAGTGCAGTCAACAAAAGTTGGTTTATCGGATACAAA  240
CTCCACGTTGTCATATATGATAACGGTATTGTCCAACAAAGCGGAATCACGAAAGGCAAT  300
GTCCATGATATCAACTTCTTGAAACAGGTAGAAAATCTACCAGCAGATAAACAATTACTG  360
GGTGATCGGGCATATATATCAAAGACCTTGCAAATGGATTTATTCGACCGTTACCAGGTG  420
AACCTGAAGGTTCCATTTAGAAATAATCAGCATGATTACAGAAAGCATCCCCAAAAATAT  480
AAATCCAAACGCCAAATGGTAGAAACCTTCTTTGCTCAAATGTGTGACCAAATGAATTTG  540
AGGAGAAACTATGCCAAATCTTATCAGGGACTGGTAACCAGATTAACCTCAAAACTCTCT  600
TCCATGGCCATTTTACACTGGATCAACTATCTTAATGGCAGAAAAATCGCTCAAATCAAA  660
CATGCTCTCAGTTTCTAATAGCACAACACGTTATTTTTTAACATTACTAAAGCCCTTCCT  720
ATGAGCCATCCATTTATTTTTTGGGCAACATTCCTCGCCCTTGCCTCGTTGGTTTATTTC  780
TGCGACAAAAAATATGGCATGCTGAGGGATGCCAGTACAGCCACGCCCCGTCCTTACAG  840
CTGGTCGAGGGTACAACTGGCCTGGTGGACGGTTATCGTATTCTCCTCTTATATTACCATC  900
ATCCTGCGATCAC                                                 914
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Fig. 2. Nucleotide sequences of PCR fragments.

A. Sequence of a 0.5 kb fragment conserved in bacilli (EU669668). **B**. Sequence of a 0.91 kb fragment conserved among *B. cereus* reference strains (GU230872).

highest identity score (37%) was observed for transposase from *Flavobacteria bacterium* BAL38 (EAZ95025.1).

Detection of *B. cereus* from spiked *cheonggukjang* was attempted. For this purpose, *cheonggukjang* was prepared

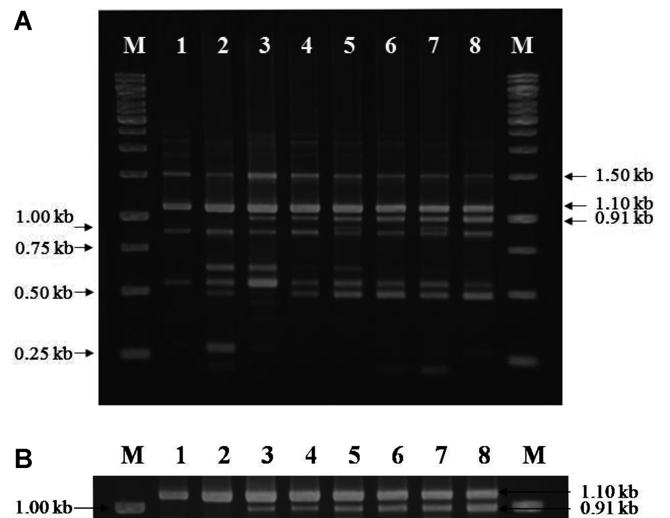

Fig. 3. RAPD−PCR patterns of *cheonggukjang* spiked with *B. cereus*.

A. 1, control (non-spiked *cheonggukjang*); 2−8, *cheonggukjang* samples spiked with *B. cereus* cells (cells/g *cheongukjang*): 2, 10^0 ; 3, 10^1; 4, 10^2; 5, 10^3; 6, 10^4; 7, 10^5; 8, 10^6 M, 1 kb size ladder (MBI Fermentas). **B**. Enlarged picture showing the 0.91 kb fragment.

and *B. cereus* cells were added. Soybean (Tae-Kwang variety, 2007 crop year, Gyeongbuk, Korea) was soaked in water at room temperature for 10 h, and then autoclaved at 121°C for 20 min. Then, it was inoculated with *B. amyloliquefaciens* CH86-1 culture [2% (v/w)], grown in LB overnight. Inoculated soybean was incubated for 48 h at 37°C. After fermentation, *cheonggukjang* was spiked with *B. cereus* ATCC 31429 cells (10^0 to 10^6 CFU/g *cheonggukjang*). Then 20 g of *cheonggukjang* was mixed with 80 ml of 0.1% peptone water and the mixture was homogenized using a stomacher (Seward, Worthing, UK). The total DNA was prepared as described above. RAPD–PCR was done and the results are shown in Fig. 3. It was previously shown that *B. amyloliquefaciens* strains produced two unique bands of 1.1 and 1.5 kb in addition to the common 0.5 kb fragment by RAPD–PCR using the S30 primer [8]. These bands were observed from *cheonggukjang* samples. In addition, the 0.91 kb band specific for *B. cereus* was observed from *cheonggukjang* spiked with *B. cereus* cells of more than 10 cells/g (Fig. 3, lane 3).

RAPD–PCR is a useful method for the rapid identification of microorganisms isolated from foods by producing genus, species, or strain-specific profiles [14]. All *B. cereus* strains tested in this work generated two bands, 0.91 and 0.5 kb. The 0.5 kb band, an internal part of *ytcP*, is a useful marker for bacilli, whereas the 0.91 kb band seems useful as a *B. cereus* species-specific marker. Preliminary results indicated that RAPD–PCR using S30 could be used for the differentiation of *B. cereus* from other species belonging to the *B. cereus* group such as *B. mycoides* and *B. thuringiensis* (results not shown). Thorough studies on these species in sufficient numbers are necessary in the future. The RAPD–PCR using S30 successfully detected *B. cereus* cells from spiked *cheonggukjang* if *B. cereus* cells were present in at least 10^2 cells/g. Since more than 10^5 cells/g food is required to cause either type of *B. cereus* food poisoning [2], the method is sensitive enough to detect *B. cereus* cells present in low numbers in contaminated foods. One disadvantage of the method is that the 0.91 kb *B. cereus* band is sometimes difficult to be differentiated from the 0.88 kb *B. subtilis* band owing to their similar sizes. Adjustments of the electrophoresis conditions such as agarose content of a gel should be helpful to see the size difference, and inclusion of reference strains would also be helpful. The method seems useful for the differentiation of *B. cereus* strains from other closely related bacilli, especially when used together with other identification methods.

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