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Simple Identification of Some *Bacillus* Species and Related Genera in Food by RAPD Analysis Combined with Morphological Observation

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Template DNA from *Brevibacillus brevis*, *Geobacillus stearothermophilus*, *Paenibacillus macerans*, *P. polymyxa* and 10 species of *Bacillus* was used with the commercially available RAPD Analysis Primers to generate DNA fragments by PCR. Different RAPD band patterns were generated from the DNA of 14 different *Bacillus* species and related genera with Primer 1 or 4. The result suggested that the single use of Primer 1 or 4 is effective for the simple identification of these strains of *Bacillus* and related genera by the RAPD analysis. By the RAPD analyses of the template DNA prepared from bacterial colonies formed on nutrient agar plates after cultivation at 35°C for 24 to 48 h, *B. brevis*, *B. circulans*, *B. firmus*, *B. licheniformis*, *P. macerans*, *P. polymyxa*, *B. pumilus*, *B. sphaericus*, *B. subtilis* and *B. thuringiensis* were identified by the combination of the present RAPD analysis using Primer 4 and morphological observations of colonies, vegetative cells and spores. For identification of *B. coagulans* and *G. stearothermophilus*, the RAPD analyses of the template DNA prepared from colonies formed on standard plate agar after cultivation at 55°C for 24 to 48 h might be effective though further improvements of the assay are required for identification of these *Bacillus* species. For simple identification of *B. cereus* and *B. megaterium* by the PCR-based method, the use of other RAPD primers or a specific PCR primer set should be developed in addition to morphological observations of colonies, vegetative cells and spores.

Key words : *Bacillus*/RAPD/Identification.

INTRODUCTION

The *Bacillus* species and related genera are of great concern in the food industries since they produce heat resistant spores. Some species such as *B. anthracis* and *B. cereus* are pathogenic to humans and animals (Keim et al., 1997). *B. licheniformis*, *B.*

subtilis, and *B. pumilus* comprise the *subtilis* group. The relevance of the *subtilis* group as food poisoning organisms has been recognized recently (Turnbull, 1997). Other species such as *B. coagulans* and *Geobacillus stearothermophilus* are of major concern in food production, where they can cause problems in production lines because they are resistant to thermal treatments (Blake and Weimer, 1997). Furthermore, *G. stearothermophilus* causes the flat, sour type spoilage of canned milk coffee. *B. subtilis* and *B.*

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licheniformis are also frequently isolated from various canned foods. In order to optimize the condition of thermal sterilization of processed foods, it is necessary to clarify the target *Bacillus* species. Recently, the *Bacillus* species have been grouped into five distinct clusters according to the phylogenetic heterogeneity of the *Bacillus* genus based on 16S rRNA gene sequences (Ash et al., 1991). Such phylogenetic studies have led to the creation of new *Bacillus*-derived genera: *Alicyclobacillus* (Wisotzkey et al., 1992), *Paenibacillus* (Ash et al., 1993), *Virgibacillus* (Heyndrickx et al., 1998), *Gracilibacillus* and *Salibacillus* (Wainø et al., 1999) and *Geobacillus* (Nazina et al., 2001).

In the traditional approaches to identify a strain of the genus *Bacillus*, several complicated physiological tests are required. To reduce the labor and time required for their identification, a kit for identification of *Bacillus* (Berkley et al., 1984) and a random amplified polymorphic DNA (RAPD) fingerprinting assay (Ronimus et al., 1997) have been reported. Ronimus et al. (1997) have reported that the primer with DNA sequence 5'-GGACGACAAG-3' was the most suitable for the identification of the *Bacillus* species by RAPD assay among 20 primers tested. The first report of the RAPD procedure by Williams et al. (1990) showed how it could be used to identify molecular markers linked to genes of specific interest and discussed the possibility of using this technique to identify genotypes or plant varieties. The RAPD analysis has been applied to the identification and detection of bacteria such as *Listeria* (Mazurier and Werners, 1992), *Lactobacillus* (Du Plessis and Dicks, 1995) and *Salmonella* (Miyamoto et al., 1998). Some primers for the RAPD analysis of pathogenic *E. coli* are commercially available (Poulsen and Jensen, 1991).

In this study we tested six different commercially available oligonucleotide primers for the simple identification of some *Bacillus* species and related genera by RAPD analysis. The *Bacillus* species and related genera isolated from food samples and identified by using the commercially available biochemical screening kit were analyzed by the present RAPD analysis using the selected primer combined with morphological observation of colonies, vegetative cells and spores.

MATERIALS AND METHODS

Strains

Brevibacillus brevis IFO 3331, *Bacillus coagulans* IFO 12583^T, *Geobacillus stearothermophilus* IFO 12550^T, *B. thuringiensis* IFO 3951, *B. thuringiensis* IFO 13865 and *B. thuringiensis* IFO 13866 were

obtained from the Institute for Fermentation, Osaka (Osaka, Japan). *B. cereus* JCM 2152^T, *B. circulans* JCM 2504^T, *B. firmus* JCM 2512^T, *B. licheniformis* JCM 2505^T, *B. megaterium* JCM 2506^T, *B. pumilus* JCM 2508^T, *B. sphaericus* JCM 2502^T, *B. subtilis* JCM 1465^T, *Paenibacillus macerans* JCM 2500^T and *P. polymyxa* JCM 2507^T were purchased from the Japan Collection of Microorganisms (Wako, Saitama, Japan). *Bacillus circulans* NBRC 13627, *B. coagulans* NBRC 3557, *B. coagulans* NBRC 3886, *B. coagulans* NBRC 12714, *B. firmus* NBRC 3330, *B. megaterium* NBRC 12068, *B. megaterium* NBRC 13498, *B. sphaericus* NBRC 3525, *B. sphaericus* NBRC 3527, *P. macerans* NBRC 3490, *P. macerans* NBRC 15041, *P. polymyxa* NBRC 3020 and *P. polymyxa* NBRC 13003 were purchased from NITE Biological Resource Center, Biotechnology Center, National Institute of Technology and Evaluation, Chiba, Japan. *B. thuringiensis* B and *B. thuringiensis* sotto were the stock culture strains of our laboratory.

For RAPD analyses of strains of *Bacillus* and the related genera, which were isolated from chicken soup stock and identified by using the API 50 CHB system (Bio Mérieux S.A., France), the following strains were used: 9 *B. brevis* strains; 4 *B. cereus* strains; 4 *B. licheniformis* strains; 6 *B. pumilus* strains; 1 *B. sphaericus* strain; 35 *G. stearothermophilus* strains; and 2 *B. subtilis* strains.

Culture conditions

Unless otherwise noted, the bacteria were cultured overnight at 37°C in Tryptic Soy Broth (TSB) (Difco Co., Ltd., Detroit, Mich, USA) or on Nutrient Agar (Difco Co., Ltd., Detroit, Mich, USA). *B. coagulans* and *G. stearothermophilus* were cultured at 50° and 60°C, respectively, for preparation of genomic DNA.

Primers

The RAPD Analysis Primers (Amersham Biosciences UK Limited, Amersham, UK) used for RAPD analysis are listed in Table 1.

TABLE 1. The nucleotide sequences of RAPD Analysis Primers

Primers	Nucleotide sequences
1	5'-GGTGCGGGAA-3'
2	5'-GTTTCGCTCC-3'
3	5'-GTAGACCCGT-3'
4	5'-AAGAGCCCGT-3'
5	5'-AACGCGCAAC-3'
6	5'-CCCGTCAGCA-3'

Preparation of genomic DNA

Genomic DNA was extracted from bacterial cells by using DNeasy Tissue kit (Quiagen K. K., Tokyo, Japan). The DNA was dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (TE buffer).

PCR conditions

Polymerase chain reaction (PCR) was performed in a PCR Express (Hybaid Limited, Ashford, Middlesex, UK). For RAPD analysis using the RAPD Analysis Primers, PCR reaction was carried out in a 50 μ l-mixture containing 10mM Tris-HCl (pH8.8), 50mM KCl, 1.5mM MgCl₂, 200 μ M of each of dATP, dTTP, dCTP and dGTP, 40pmole of the primer, 20ng of template DNA, and 1.5U of Super Taq DNA polymerase (Sawady technology Inc., Tokyo, Japan). The reaction solution was overlaid with an equal volume of mineral oil and 45 cycles of amplification were done with an amplification cycle constituted of template DNA denaturation at 95°C for 30 sec, primer-template annealing at 40°C for 30 sec and primer extension at 65°C for 45 sec.

Detection of amplified DNA

An aliquot (10 μ l) of the PCR reaction mixture was analyzed by gel electrophoresis in a 2% agarose gel and 40 mM Tris-acetate buffer (pH 3.0) containing 1mM EDTA at 3V/cm for 45 min. The gel was stained in ethidium bromide solution (10 μ g/ml) and visualized with an UV transilluminator. The λ DNA-*Hind* III digest and 100bp DNA ladder (New England Biolabs, USA) were used for estimation of the size of PCR products.

Morphological observations

After cultivation of isolates and type strains of each *Bacillus* species and the related genera on Nutrient agar (Difco Co., Ltd., Detroit, Mich, USA) at a suitable temperature for 24 to 48 h, colonies were observed through a Nikon Stereoscopic microscope SMZ-2T (Nikon, Japan). These colonies were picked up and suspended in water and the vegetative cells and spores were observed through a Nikon Optiphot phase contrast microscope at 1000 $\times$ magnification.

RESULTS

DNA band patterns generated by using RAPD Analysis Primers

Template DNA from *B. brevis*, *P. macerans*, *P. polymyxa*, *G. stearothermophilus* and 10 species of *Bacillus* were used with the RAPD Analysis Primers to generate DNA fragments by PCR. Among the 14 species tested, 11 strains were type strains. Figure 1

FIG. 1. The DNA band patterns generated with RAPD primers and genomic DNA from some *Bacillus* species and related genera. The commercially available RAPD Analysis Primers 1 to 6 were used for the RAPD analysis and the RAPD band patterns were shown in (A) to (F), respectively. Lanes: m, λ DNA-*Hind* III digest; M, 100 bp DNA ladder; 1, *B. brevis* IFO 3331; 2, *B. cereus* JCM 2152^T; 3, *B. circulans* JCM 2504^T; 4, *B. coagulans* IFO 12583^T; 5, *B. firmus* JCM 2512^T; 6, *B. licheniformis* JCM 2505^T; 7, *P. macerans* JCM 2500^T; 8, *B. megaterium* JCM 2506^T; 9, *P. polymyxa* JCM 2507^T; 10, *B. pumilus* JCM 2508^T; 11, *B. sphaericus* JCM 2502^T; 12, *G. stearothermophilus* IFO 12550^T; 13, *B. subtilis* JCM 1465^T; 14, *B. thuringiensis* B.

shows RAPD band patterns obtained for these strains after 45 cycles of amplification. The RAPD analysis using Primer 2 generated only 1 band with genomic DNA from *B. sphaericus* and *B. subtilis*. These strains also showed the same RAPD band pattern with Primer 3. By the use of Primer 3, the RAPD band patterns were also same in *B. coagulans* and *B. pumilus*. Similar RAPD band patterns were generated from the DNA of *B. licheniformis* and *G. stearothermophilus* with the same primer. With Primer 5, the same RAPD band pattern was generated from *B. coagulans* and *B. sphaericus*. The RAPD band patterns generated with Primer 6 were similar between *B. circulans* and *B. macerans*. Different RAPD band patterns were generated from the DNAs of 14 different *Bacillus* species and related genera with Primer 1 and 4. The results suggested that the single use of Primer 1 or 4 may be effective for the simple identification of some *Bacillus* strains and related genera frequently detected in foods by RAPD analysis. In this study, Primer 4 was used for RAPD analyses of *Bacillus* and related genera isolated from food samples.

RAPD band patterns generated with Primer 4 from the DNA of some strains including isolates from food.

Genomic DNA was prepared from some strains of *Bacillus* and related genera including isolates from foods. Figures 2, 3 and 4 show RAPD band patterns generated from the genomic DNA of some strains with Primer 4.

The 9 strains of *B. brevis* isolated from food generated a predominant band at 450 bp, the same band which was generated with *B. brevis* IFO 3331 (Fig. 2A). *B. brevis* isolates also produced bands at 1200 and 1500 bp, which were not produced in *B. brevis* IFO 3331. It seems that these *B. brevis* strains generate the same RAPD band at 450 bp with Primer 4. The 4 *B. cereus* isolates produced the same band pattern by the RAPD analysis with Primer 4 (Fig. 2B). The patterns were slightly different from that of *B. cereus* type strain. *B. circulans* NBRC 13627 generated the same RAPD band pattern with that of the *B. circulans* type strain, JCM 2504^T (Fig. 2C). *B. coagulans* NBRC 3557 and 12714 generated slightly different RAPD band patterns from that of the type strain JCM 12583^T with Primer 4 (Fig. 2D). The RAPD band patterns of NBRC 3886 and the 3 strains isolated from foods were quite different from that of the type strain. *B. firmus* NBRC 3330 generated the same RAPD band pattern with that of *B. firmus* type strain, JCM 2512^T (Fig. 2E). Three *B. licheniformis* isolates produced the same RAPD band patterns (Fig. 3A, Lane 2, 3, and 4). Another *B. licheniformis*

FIG. 2. The DNA band patterns generated with Primer 4 and genomic DNA from some strains of *Bacillus* and related genera. Lanes: m, λ DNA-Hind III digest; M, 100 bp DNA ladder; (A) 1, *B. brevis* IFO 3331; (A) 2 to 10, *B. brevis* isolated from food; (B) 1, *B. cereus* JCM 2152^T; (B) 2 to 5, *B. cereus* isolated from food; (C) 1, *B. circulans* JCM 2504^T; (C) 2, *B. circulans* NBRC 13627; (D) 1, *B. coagulans* IFO 12583^T; (D) 2, *B. coagulans* NBRC 3557; (D) 3, *B. coagulans* NBRC 12714, (D) 4, *B. coagulans* NBRC 3886; (D) 5 to 7, *B. coagulans* isolated from food; (E) 1, *B. firmus* JCM 2512^T; (E) 2, *B. firmus* NBRC 3330.

isolate (Fig. 3A, Lane 5) showed a similar band pattern with that of the type strain JCM 2505^T. A RAPD band at about 550 bp was generated from all the *B. licheniformis* strains tested. *P. macerans* NBRC 3490 produced the same band pattern with that of the type strain JCM 2500^T (Fig. 3B). *P. macerans* NBRC 15041 generated bands at both 1700 and 300 bp, which were also generated from the genomic DNA of the type strain and NBRC 3490. The RAPD band patterns generated from 3 different *B. megaterium* strains were different from one another (Fig. 3C). Although all 3 *B. polymyxa* strains produced RAPD bands at both 500 and 420 bp, *B. polymyxa* NBRC 3020 and 13003 did not generate additional RAPD bands that were generated in the type strain JCM 2507^T (Fig. 3D). All 7 *B. pumilus* strains tested

FIG. 3. The DNA band patterns generated with Primer 4 and genomic DNA from some strains of *Bacillus* and related genera. Lanes: m, λ DNA-*Hind* III digest; M, 100 bp DNA ladder; (A) 1, *B. licheniformis* JCM 2505^T; (A) 2 to 5, *B. licheniformis* isolated from food; (B) 1, *P. macerans* JCM 2500^T; (B) 2, *P. macerans* NBRC 3490; (B) 3, *P. macerans* NBRC 15041; (C) 1, *B. megaterium* JCM 2506^T; (C) 2, *B. megaterium* NBRC 12068; *B. megaterium* NBRC 13498; (D) 1, *P. polymyxa* JCM 2507^T; (D) 2, *P. polymyxa* NBRC 3020; (D) 3, *P. polymyxa* NBRC 13003; (E) 1, *B. pumilus* JCM 2508^T; (E) 2 to 7, *B. pumilus* isolated from food; (F) 1, *B. sphaericus* JCM 2502^T; (F) 2, *B. sphaericus* NBRC 3525; (F) 3, *B. sphaericus* NBRC 3527; (F) 4, *B. sphaericus* isolated from food.

generated RAPD bands at both 700 and 850 bp (Fig. 3E). The 2 *B. pumilus* isolates (Fig. 3E, Lane 2 and 3) generated several additional bands, which were different from those of the type strain and other isolates. *B. sphaericus* NBRC 3525 generated the same RAPD band pattern with that of the type strain JCM 2502^T though that of strain NBRC 3527 was slightly different from those of the type strain and NBRC 3525 (Fig. 3F). A RAPD band at 850 was clearly detected in the type strain, NBRC 3525 and the isolate 31, but was faint in NBRC 3527. *G. stearothermophilus* IFO 12550^T, the type strain, generated a thick band at 750 bp, faint ones at 1500, 1100 and 850 (Fig. 1D, Lane 12). *G. stearothermophilus* isolates showed

FIG. 4. The DNA band patterns generated with Primer 4 and genomic DNA from some strains of *Bacillus* and related genera. Lanes: m, λ DNA-*Hind* III digest; M, 100 bp DNA ladder; (A) 1, *G. stearothermophilus* IFO 12550^T; (A) 2 to 35, *G. stearothermophilus* isolated from food; (B) 1, *B. subtilis* JCM 1465^T; (B) 2 and 3, *B. subtilis* isolated from food; (C) 1, *B. thuringiensis* B; (C) 2, *B. thuringiensis* sotto; (C) 3, *B. thuringiensis* IFO 3951; (C) 4, *B. thuringiensis* IFO 13865; (C) 5, *B. thuringiensis* IFO 13866.

various RAPD band patterns (Fig. 4A). These bands were estimated to be 2500, 1800, 1500, 1000, 850, 750 and/or 450 bp. These strains were classified into 7 groups according to their RAPD band patterns. *B. subtilis* JCM 1465, the type strain, generated a thick RAPD band at 700 bp. The same band was also generated in the 2 *B. subtilis* isolates (Fig. 4B). A thick RAPD band and a faint RAPD band were detected at 850 and 750 bp, respectively, in both *B. thuringiensis* sotto and *B. thuringiensis* B, but at 750 and 850 bp, respectively, in the strains obtained from IFO (Fig. 4C). Although several additional bands were generated from the 3 IFO strains, it seems that *B. thuringiensis* strains commonly generate DNA bands at 850 and 750 bp by RAPD analysis with Primer 4.

Simple method to identify some *Bacillus* species and related genera by RAPD analysis combined with morphological observation

For simple identification of some *Bacillus* species isolated from food samples by the conventional aerobic plating assay of the sample using Nutrient Agar after heat treatment at 80°C for 10 min, the RAPD analysis using Primer 4 combined with morphological observations of colonies, vegetative cells and spores might be effective. Figure 5 shows the proposed

FIG. 5. The proposed simple method to identify some *Bacillus* species and related genera in food.

* *B. cereus* could be identified by using selective media.

***B. megatrium* could be identified with the formation of unstained granules in vegetative cells.

method to identify some *Bacillus* species and related genera in food. After cultivation at 35 °C, *B. subtilis* and *B. licheniformis* can be distinguished from other species by their characteristic appearance of colonies: *B. subtilis* forms irregular, surface-dull and wrinkled colonies; *B. licheniformis* forms rough, surface-dull mounds and lobes consisting of the slime of

accumulated colonies. *B. sphaericus* can be distinguished from other species since it forms spherical spores. *B. cereus*, *B. megaterium* and *B. thuringiensis* are distinguished from other species by the morphological observations of colonies, vegetative cells and spores: their colonies are dull and have a frosted glass appearance, their rod shaped cells tend to

occur in chains, and ellipsoidal spores are formed at the central of the cells. RAPD analyses of the template DNA prepared from bacterial colonies formed on the Nutrient Agar after cultivation at 35°C for 24 to 48 h, *B. brevis*, *B. circulans*, *B. firmus*, *B. licheniformis*, *P. macerans*, *P. polymyxa*, *B. pumilus*, *B. sphaericus*, *B. subtilis* and *B. thuringiensis* might be identified by combination of the present RAPD analysis and morphological observation. *B. cereus* and *B. megatrium* could be identified respectively by using selective media and with the formation of unstained granules in vegetative cells. For simple identification of *G. stearothermophilus*, the RAPD analyses of the template DNA prepared from colonies formed on a Nutrient Agar after cultivation at 55 °C for 48 h might be effective.

DISCUSSION

In this paper, we have shown the usefulness of RAPD analysis using commercially available RAPD analysis primers combined with morphological observations of colonies, vegetative cells and spores for the rapid and simple identification of some *Bacillus* species and related genera. The genus *Bacillus* includes a variety of phenotypically heterogeneous species exhibiting physiological and metabolic diversity and DNA base composition. In the traditional approaches to identify a strain of the genus *Bacillus*, complicated physiological tests are required. Several approaches have been proposed to classify *Bacillus* species, such as those emphasizing morphological and biochemical properties (Smith et al., 1952), spore shape and swelling of the sporangium (Gordon et al., 1973), API test (Berkeley et al., 1984). Joung and Côté (2002) have evaluated ribosomal RNA gene restriction patterns for the classification of *Bacillus* species and related genera, and indicated that the patterns can be used to reconstruct the phylogeny of the *Bacillus* species and derived genera. However, their method was complicated and time-consuming for the purpose of rapidly identifying these species, since their method included 4 h-enzyme digestion, 20 h-agarose gel electrophoresis for separation of the digests, Southern hybridization and detection by a radioactive probe.

To reduce the labor and time required for identification, a kit for the identification of *Bacillus* (Berkley et al., 1984), a RAPD fingerprinting assay (Ronimus et al., 1997) have been reported. Ronimus et al. (1997) have reported that the primer 5'-GGACGACAAG-3' was the most suitable for the identification of *Bacillus* species among 20 primers tested. They also showed RAPD band patterns generated from the genomic

DNA of isolates from a biofilm sample growing at 72 °C were closely matched with the pattern generated from *G. stearothermophilus*. However, they did not show RAPD band patterns of the other *Bacillus* species isolated from factories and environment. Soejima et al. (2003) have compared RAPD band patterns of isolates of *B. licheniformis* and *B. cereus* using the same RAPD Analysis primers as those used in this study. They reported that the RAPD Analysis Primers 1, 3 and 4 were effective for RAPD analysis of the *B. licheniformis* strains isolated from soybean milk test products. However, they did not refer to the usefulness of these primers for identification of other *Bacillus* species by RAPD analysis. In our study, different RAPD band patterns were generated from the DNA of 14 different *Bacillus* species and related genera with Primer 1 or 4. Although further studies on the other *Bacillus* species and related genera not tested in this study would be required, our results suggest that the single use of Primer 4 is effective for the simple identification of some *Bacillus* species and related genera frequently isolated from foods by RAPD analysis.

It has been reported that there are still several difficulties in the identification and characterization of new isolates of thermophilic endospore-forming bacteria of the genus *Bacillus* and related genera. This is due to the great genotypic and phenotypic variability that characterizes strains belonging to the species of *G. stearothermophilus* and *B. coagulans*, the traditional thermophilic aerobic spore-forming bacilli. In this study, RAPD band patterns of some *B. coagulans* strains were similar to that of the type strain and those of the other strains isolated from food were different. In the case of *G. stearothermophilus* strains isolated from food, their RAPD band patterns were classified into 7 groups (Fig. 4A). For identification of *B. coagulans* and *G. stearothermophilus*, the RAPD analyses of the template DNA prepared from colonies formed on a Nutrient Agar after cultivation at 55 °C for 48 h might be effective, though further investigations on the other thermophilic endospore-forming bacteria, especially, newly classified *Geobacillus* species such as *G. thermocatenulans* and *G. thermoleovorans* (Nazina et al., 2001), are required. Among the thermophilic bacteria placed in the classical group of *B. stearothermophilus* according to their biochemical properties, strains phylogenetically quite distant from the typical *G. stearothermophilus* group should be considered to be other species as mentioned by Nazina et al. (2001).

B. cereus and *B. thuringiensis* were indistinguishable by pulsed-field gel electrophoresis and multilocus enzyme electrophoresis (Damgaard, 1995).

Although differentiation of *B. cereus* and *B. thuringiensis* was also not possible by using oligonucleotide probes directed against the spacer regions between the 16S and 23S rRNA genes (Yamada et al., 1999), the use of selective media such as NGKG agar and MYP agar are effective for the simple identification of *B. cereus*. *B. cereus* are potential food poisoning bacteria that can produce enterotoxins and an emetic toxin causing diarrhea and vomiting, respectively, in humans (Granum and Lund, 1997). Some strains produce toxins and others do not. In our study, *B. cereus* isolated from food showed different RAPD band patterns with that of the *B. cereus* type strain. Rapid identification of pathogenic *B. cereus* among *Bacillus* strains isolated from food is very difficult. It seems that for simple identification of pathogenic *B. cereus* by the PCR related methods, the use of a new PCR primer set specific to pathogenic *B. cereus* is required. To increase the accuracy of the rapid and simple identification of *Bacillus* species and related genera by RAPD analysis using Primer 4 combined with morphological observations of colonies, vegetative cells and spores, RAPD band patterns generated by other primers should be examined for some *Bacillus* species.

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