Microbial Community Analysis of Food-Spoilage Bacteria in Commercial Custard Creams Using Culture-Dependent and Independent Methods

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ABSTRACT

Custard cream is made from highly nutritive raw materials such as milk and sugar and is easily spoiled by the multiplication of specific microbial contaminants or residents. However, this spoilage microbial community has not been studied. We determined the spoilage microbiota in commercial custard creams using culture-dependent and independent methods. Using the culture-dependent analysis with various agar media, 185 bacterial colonies and 43 eukaryal colonies were isolated from 7 commercial custard cream products. All bacterial isolates were morphologically, physiologically, and genetically identified as bacilli, staphylococci, lactic acid bacteria, and psychrotrophic gram-negative rods. Using culture-independent molecular analysis, the PCR-denaturing gradient gel electrophoresis technique, spoilage of the commercial custard creams was found to be caused by bacilli, staphylococci, lactic acid bacteria, psychrotrophic gram-negative rods, *Anoxybacillus* sp., *Caurobacter* sp., and *Streptococcus* sp. bacteria. The detected spoilage bacteria were the same species as previously detected in spoiled milk products and shown in other reports, suggesting that spoilage bacteria in a raw material easily grow in processed foods made from milk. We determined the spoilage microbial communities in commercial custard creams, and these are the first data concerning spoilage microbiota in nonfermented processed foods using a culture-independent analysis. Our study will be useful for the manufacture and safe preservation of dairy products because the first step toward safe food preservation by food manufacturers is to understand the spoilage microbiota in a target food to select optimal preservatives and to reduce the use of food additives.

Key words: custard cream, spoilage microbiota, 16S rDNA, polymerase chain reaction-denaturing gradient gel electrophoresis

INTRODUCTION

Custard creams used in cream puffs, pastries, and cakes are made from highly nutritive raw materials such as milk, starch, and sugar. Custard creams shipped by suppliers are generally aseptic; however, they encounter bacterial contamination during reprocessing, preservation, transportation, and sales. Growth of the contaminating bacteria not only causes deterioration in taste and flavor but can also cause food poisoning (e.g., *Staphylococcus aureus*). To avoid this, suppliers have to control the bacterial growth by sterilization using heat and food additives. However, many customers do not want artificial additives because these artifacts may negatively affect the flavor and impair health. Therefore, suppliers have recently tried to control bacterial growth using as few additives as possible or using biopreservatives such as bacteriocins (Kawai and Saito, 2002; Cotter et al., 2005).

The milk spoilage microbiota was previously determined using a culture-dependent analysis (Boor and Murphy, 2002) wherein the bacterial species of isolates were identified from their morphological, physiological, and genetic characteristics. Using culture, the predominant spoilage in pasteurized milk was revealed to be caused by thermotolerant bacteria surviving pasteurization (*Micrococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Corynebacterium* spp. and the spores of *Bacillus* and *Clostridium*) and psychrotrophic gram-negative motile rods as postpasteurization contaminants. However, spoilage microbiota of custard creams made primarily from milk has not been studied.

Recently, culture-independent molecular methods have been developed to improve understanding of a total microbiota because the use of selective culture-dependent methods does not determine the entire environmental bacterial diversity (Amann et al., 1995). Total DNA samples extracted from a complex microbial environment are used to identify bacterial species composed of the dominant population using PCR and denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993). The species-specific 16S rDNAs amplified

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from the extracted DNA are separated on the basis of sequence differences using DGGE, and are sequenced to identify the bacterial species. The culture-independent analysis using the PCR-DGGE technique can rapidly detect both cultivable and uncultivable bacteria and has been used for many studies concerning environmental microbial ecology (Torsvik et al., 1998; Ercolini, 2004). The microbial communities of fermented foods such as pozol (Ampe et al., 1999; ben Omar and Ampe, 2000), fermented sausage (Cocolin et al., 2001; Fontana et al., 2005a,b), Sicilian cheese (Randazzo et al., 2002), cassava (Ampe et al., 2001; Miambi et al., 2003), kimchi (Lee et al., 2005), and fermented dairy products (Fasoli et al., 2003; Obodai and Dodd, 2006) have been determined using the PCR-DGGE technique, but few microbiota analyses of putrid foods have been performed using the culture-independent method.

In this study, spoilage bacterial communities of commercial custard creams were determined using the culture-dependent and independent methods with the objective to aid in determining and optimizing the kinds and the amount of food additives to extend the shelf life and preserve this fragile food.

MATERIALS AND METHODS

Sampling

Custard cream samples (A to G) were selected from 7 types of commercial cream puffs, pastries, and cakes containing custard creams produced at our factory (Tsukishima Foods Industry, Tokyo, Japan). They were accelerated to putrefy by holding at 15 or 30°C for 15 or 30 d in a noncontaminative environment until $>10^4$ cfu/g of viable microbial cells were detected using culture on plate count agar (PCA; Eiken Chemical, Tokyo, Japan). Samples A and B were from shelf-stable products and samples C to G were chilled products.

Microbial DNA Extraction from Commercial Custard Creams

The bacterial cells in the putrid custard creams were disrupted to extract the total DNA: 0.8 mL of the 1:10 custard cream dilution in PBS (pH 7.3) was mixed with 1.0 g of glass beads (0.1 mm, Tomy Seiko, Tokyo, Japan) and crushed using a Micro Smash MS-100R (Tomy Seiko) for 5 min at 5,000 rpm. Purification of the total DNA extracted from the disrupted aqueous phase was performed using a QIAamp DNA Blood Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions and the method of Cenci-Goga et al. (2004).

Isolation of Bacterial Strains

Custard creams diluted with PBS were inoculated on PCA, nutrient agar (NA; Oxoid, Hampshire, UK), trypticase soy agar (TSA; Difco, Detroit, MI), and de Man, Rogosa, Sharpe agar (MRS, Difco) plates. The plates were incubated at 25, 30, and 37°C for 24 h under aerobic and anaerobic conditions. After incubation, 4 or 5 colonies were isolated from each plate. The eukaryal colonies were determined using PCR amplification of 26S rRNA genes (26S rDNA) with the primers NL1 and LS2 shown in Table 1 according to the method of Cocolin et al. (2000).

PCR-DGGE of the Isolates

To easily classify the isolated bacterial strains according to their species and to clarify the diversity of bacterial species present in the putrid custard cream, PCR-DGGE for all of the isolates was performed using the V3 region on the 16S rRNA genes (16S rDNA), which is the species-specific sequence and shows a constant mobility in the DGGE gel. In this study, isolates having the same DGGE banding pattern were considered as the same species and one or more representative isolate(s) were used in the next identification steps (sequencing and morphological, physiological, and genetic analyses).

The 16S rDNA V3 region of all isolates were amplified using direct-colony PCR with the bacterial universal primers gc338f including a GC clamp and 518r (Table 1). Amplification was performed in a 20- μ L reaction mixture containing 10 $\times$ PCR buffer (Takara Bio, Shiga, Japan), 200 μ M dNTPs, 0.4 μ M each primer, 0.5 U of *Taq* polymerase (Takara Bio), and bacterial cell template DNA. The cycling program was 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s; and then a final extension of 72°C for 5 min. The PCR products were electrophoresed in 2.0% (wt/vol) agarose gels and the bands were observed using ethidium bromide stain. The amplicons were purified using a commercial kit, MagExtractor (Toyobo, Osaka, Japan) and were separated using DGGE with a Bio-Rad Dcode System (Bio-Rad Laboratories, Hercules, CA) following the procedure described by Muyzer et al. (1993). Denaturing gels consisted of 6.5% (vol/vol) polyacrylamide and 1 $\times$ Tris-acetate EDTA with a gradient ranging from 40 to 60% urea-formamide [100% was defined as 7 M urea and 40% (vol/vol) formamide]. The electrophoresis was performed for 12 h at 100 V in 0.5 $\times$ Tris-acetate EDTA buffer at 65°C. Gels were stained for 60 min using SYBR Gold nucleic acid gel stain solution (Invitrogen, Carlsbad, CA) and photographed under UV transillumination.

Table 1. Polymerase chain reaction primers used in this study

Primer	Sequence (5'–3')	Target	Region	Reference
NL1	GCCATATCAATAAGCGGAGGAAAAG	Eukarya	26S rDNA	Cocolin et al. (2000)
LS2	ATTCCCAAACAACCTCGACTC	Bacteria	16S rDNA	Lane (1991)
27f	AGAGTTTGATCMTGGCTCAG		16S rDNA V3	Kroes et al. (1999)
1525r	AAGGAGGTGATCCAGCC		16S rDNA V3	Lane (1991)
gc338f	CGCCCGCCGCGCGCGGGCGGGGCGGGGCGGGG GCACGGGGGACTCCTACGGGAGGCAGCAG		16S rDNA V6–8	Muyzer et al. (1993)
518r	ATTACCGCGGCTGCTGG	<i>B. thuringiensis</i>	16S rDNA V6–8	Iwamoto et al. (2000)
EUB f933	GCACAAGCGGTGGAGCATGTGG			
EUB f933-GC-clamp	CGCCCGCCGCGCGCGGGCGGGGCGGGGCGGGG- CACGGGGGGGCACAAGCGGTGGAGCATGTGG			
EUB r1387	GCCCGGGAACGTATTACCG			
Cry1a	CTGGATTTACAGGTGGGGATAT		<i>cry1</i>	Bravo et al. (1998)
Cry1b	TGAGTCGCTTCGCATATTGACT			
Cry3a	TTAACCGTTTTTCGCAGAGA		<i>cry3</i>	Ceron et al. (1995)
Cry3b	TCCGCACTTCTATGTGTCCAAG			
Cry4a	CAGGTACCGGTGGAATGAATTATA		<i>cry4</i>	Rosenquist et al. (2005)
Cry4b	GCTCTAGAGACTTCTACTTTAGTA			
Cry8a	ATGAGTCCAAATAATCTAAATG		<i>cry8</i>	Bravo et al. (1998)
Cry8b	TTTGATTAATGAGTTCTTCCACTCG			
Cry11a	TTAGAAGATACGCCAGATCAAGC		<i>cry11</i>	
Cry11b	CATTTGTACTTGAAGTTGTAATCCC			
Cyta	AACCCCTCAATCAACAGCAAGG		<i>cyt</i>	
Cytb	GGTACACAATAGATAACGCCACC			
p-gyrA-f	CAGTCAGGAAATGCGTACGTCCTT	<i>B. subtilis</i>	Partial <i>gyrA</i>	Chun and Bae (2000)
p-gyrA-r	CAAGGTAATGCTCCAGGCATTGCT			

16S rDNA Sequence of the Isolates

The 16S rDNA V1–3 and V6–8 regions from the representative isolates selected by differences in the band position of the PCR-DGGE were amplified using the direct-colony PCR with the primer pairs 27f + 518r and EUB f933 + EUB r1387 (Table 1), respectively. The PCR mixture and the cycling program were the same as above, except for the annealing temperature at 54°C for the V1–3 region and at 62°C for the V6–8 region. After electrophoresis and purification, the amplicons were sequenced using the dideoxy chain termination method with the BigDye Terminators v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequence identities were determined using BLAST comparisons to the GenBank database.

Morphological Analysis

All of the selected isolates were observed using an optical microscope (Olympus BX-40, Olympus, Tokyo, Japan) and a transmission electron microscope (Hitachi H-8100, Tokyo, Japan). Optical microscopy was performed after the isolates were gram-stained (Nissui Pharmaceutical, Tokyo, Japan).

Physiological Analysis

Physiological analyses of the selected isolates were performed using the oxidase producing test (Eiken

Chemical), sulfide indole motility (**SIM**) medium (Eiken Chemical), API 50 CH (bioMérieux, Marcy l'Etoile, France), and API Staph (bioMérieux) following the manufacturer's instructions.

Species and Subspecies Identification

Food spoilage *Bacillus cereus* is phenotypically and genotypically similar to *Bacillus thuringiensis*, where *B. thuringiensis* secretes the insecticidal crystal (**Cry**) protein coded on the *cry* gene. To determine if the isolates secreted the Cry protein using optical microscopy, the *B. cereus*-like isolates were stained using Ziehl-Neelsen carbol-fuchsin stain solution (Merck KGaA, Darmstadt, Germany) according to the method of Harmon (1982). *Bacillus cereus* IFO13494, IFO13690, and JCM2152^T were employed as negative controls, and *B. thuringiensis* NBRC13865 and NBRC13866 were used as positive controls. Possession of the primary *cry* genes (*cry1*, *cry3*, *cry4*, *cry8*, and *cry11*) and *cyt* gene coding for the supplementary cytolytic protein was determined using direct-colony PCR with the primers listed in Table 1 using the methods of Ceron et al. (1995), Bravo et al. (1998), and Rosenquist et al. (2005).

Bacillus subtilis is divided into 2 subspecies, *B. subtilis* ssp. *subtilis* and *B. subtilis* ssp. *spizizenii*, on the basis of cell-wall chemistry and DNA-DNA relatedness especially in partial *gyrA* sequences coding for the DNA gyrase subunit A. The PCR-amplified *gyrA* using primers p-gyrA-f and p-gyrA-r (Table 1) from *B. subtilis*

strains isolated from the putrid custard cream was sequenced according to the method of Chun and Bae (2000) and its sequence was compared with those in the GenBank database.

Leuconostoc mesenteroides is divided into 3 subspecies, *Leu. mesenteroides* ssp. *mesenteroides*, *Leu. mesenteroides* ssp. *cremoris*, and *Leu. mesenteroides* ssp. *dextranicum*. To determine the subspecies we employed tests for acid production from arabinose on the API 50 CH (bioMérieux) and dextran formation in 5 and 10% (wt/vol) sucrose agar with reference to the method of Beukes et al. (2001).

Identification of *Achromobacter denitrificans* was performed using an oxidation fermentation basal medium (Eiken Chemical) adding 1.0% (wt/vol) xylose. The closely related *Achromobacter xylosoxidans* is able to metabolize xylose but *A. denitrificans* does not grow in the xylose medium.

Two similar fluorescein-producing *Pseudomonas* species, *P. fluorescens* and *P. putida*, were differentiated using the test for gelatin liquefaction at 22°C for 60 h according to the method of Blazevec et al. (1973).

Culture-Independent Analysis using PCR-DGGE and Genome Sequencing

In addition to the culture-dependent method described above, spoilage bacterial communities in the commercial custard creams were determined with a culture-independent method using the PCR-DGGE technique. The 16S rDNA sequences of the spoilage bacteria were amplified using the touchdown PCR method with the bacterial universal primers, 27f and 1525r (Table 1). The total DNA extracted from the putrid custard creams were used as the first templates. Then, the V6–8 regions of the 16S rDNA were amplified using the touchdown PCR method with EUB f933-GC-clamp including a GC clamp and EUB r1387 primers (Table 1). The reaction mixture was 10 × PCR buffer (Toyobo), 200 mM dNTPs, 1.0 mM MgSO₄, 0.3 μM each primer, 0.4 U of *KOD plus* DNA polymerase (Toyobo), and 0.8 μL of appropriately diluted template DNA in a final volume of 20 μL. The first PCR program was 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 59 to 49°C (decreasing 2°C per cycle 5 times) for 30 s, 68°C for 2 min, and a final extension at 68°C for 7 min. The second PCR program was 94°C for 2 min; followed by 36 cycles: 94°C for 15 s, 67 to 57°C (decreasing 2°C per cycle 6 times) for 30 s, 68°C for 1 min; and then 68°C for 5 min. After PCR, electrophoresis, and purification of the PCR products, the amplicons were separated using DGGE as described above. The DGGE bands were excised with a sterile scalpel, ground with a sterile glass slide, and eluted in 20 μL of sterile water.

The 16S rDNA V6–8 amplicons from each DGGE band were reamplified and sequenced as described above. The spoilage bacterial communities in the commercial custard creams were determined using the sequence data compared with the GenBank database.

RESULTS

Determining Spoilage Bacterial Isolate Identity using 16S rDNA

Each sample of custard cream (A to G) had >10⁴ cfu/g of viable cells using the colony count on PCA. A total of 228 colonies were isolated on PCA, NA, TSA, and MRS agar plates from the diluted solutions of each sample. Using PCR amplification of the 16S and 26S rDNA showed that 185 colonies among the 228 colonies were bacterial cells and the other 43 colonies were eukaryal cells.

For the 185 colonies identified as bacterial cells, the V3 regions of the 16S rDNA were amplified using the bacterial universal primers gc338f and 518r, and their amplicons were used for DGGE analysis. The DGGE banding patterns after gel staining showed 185 isolates classified into some groups of the same species as shown in Table 2. Then, 1 to 3 strain(s) were selected as representatives from each group (Table 2) and the V1–3 and the V6–8 regions of their 16S rDNA were sequenced. Closest relative species in each group as shown in Table 2 were identified from the sequence identities as determined using BLAST comparisons to the GenBank database. This analysis showed that the spoilage bacterial 16S rDNA sequences were from *Enterococcus faecalis*, *Enterococcus faecium*, *Lactococcus lactis* ssp. *lactis*, and *P. fluorescens* and were identified at the species level; other spoilage bacteria were semi-identified to genus and general group.

Morphological and Physiological Analysis of the Isolates

The 26 bacterial strains (Table 2) selected by PCR-DGGE were observed by optical microscopy using Gram stain and transmission electron microscopy. The stainability and morphology of the 26 strains are shown in Table 3. The observations correlated with the estimation of their bacterial species (semi-) identified from their partial 16S rDNA sequences.

Physiological characteristics and motility of the 26 strains using the oxidase test and the SIM medium test are shown in Table 3. The patterns of carbohydrate metabolism determined by the API kits identified the strains closer to species (Table 3). *Bacillus amyloliquefaciens*, *B. subtilis*, *Lactobacillus paracasei* ssp. *paracasei*, *Leu. mesenteroides*, *Staphylococcus saprophyticus*

Table 2. Species identification of the spoilage bacteria isolated from the putrid custard creams using PCR-denaturing gradient gel electrophoresis and sequencing of the V1–3 and V6–8 regions on their 16S rDNA

Sample	Number	Representative strain(s)	Closest relative (identity >99%)
A	33	AK1014	<i>Staphylococcus pasteurii</i> , <i>Staphylococcus warneri</i>
B	32	AK1048, AK1052	<i>Enterococcus faecalis</i>
	1	AK1040	<i>Achromobacter denitrificans</i> , <i>Achromobacter xylosoxidans</i>
C	39	AK1075	<i>A. denitrificans</i> , <i>A. xylosoxidans</i>
D	19	AK1114, AK1124	<i>Bacillus anthracis</i> , <i>Bacillus cereus</i> , <i>Bacillus thuringiensis</i>
	7	AK1121, AK1129	<i>Staphylococcus cohnii</i> , <i>Staphylococcus saprophyticus</i> , <i>Staphylococcus succinus</i> , <i>Staphylococcus xylosus</i>
	2	AK1113, AK1123	<i>A. denitrificans</i> , <i>A. xylosoxidans</i>
	2	AK1106	<i>Bacillus amyloliquefaciens</i> , <i>Bacillus subtilis</i>
	1	AK1107	<i>B. amyloliquefaciens</i> , <i>B. subtilis</i>
E	13	AK1141, AK1144, AK1145	<i>A. denitrificans</i> , <i>A. xylosoxidans</i>
	8	AK1163	<i>Leuconostoc mesenteroides</i> , <i>Leuconostoc pseudomesenteroides</i>
	7	AK1155	<i>Lactococcus lactis</i> ssp. <i>lactis</i>
	2	AK1165	<i>Lactobacillus paracasei</i> , <i>Lactobacillus casei</i>
	1	AK1157	<i>Enterococcus faecium</i>
F	12	AK1177	<i>A. denitrificans</i> , <i>A. xylosoxidans</i>
	3	AK1171	<i>B. anthracis</i> , <i>B. cereus</i> , <i>B. thuringiensis</i>
G	15	AK1192	<i>A. denitrificans</i> , <i>A. xylosoxidans</i>
	2	AK1194	<i>B. anthracis</i> , <i>B. cereus</i> , <i>B. thuringiensis</i>
	2	AK1195, AK1196	<i>Pseudomonas fluorescens</i>

ssp. *saprophyticus*, and *Staphylococcus warneri* were identified as the primary spoilage bacteria forming communities in the putrid custard creams.

Individual Identification of Isolated Spoilage Bacteria

Bacillus cereus-like isolates (AK1114, AK1124, AK1171, AK1194, and AK1232) were identified as *B. cereus* or *B. thuringiensis* using genetic and physiological analysis. The secretion of the Cry protein and possession of its related genes (*cry* and *cyt* genes) occur only in *B. thuringiensis* strains. We did not find the Cry protein, *cry* genes, or *cyt* gene in the negative control strains or in the 5 target strains. The positive control strains secreted the Cry protein and possessed the *cryI* gene. Therefore, all of the isolated *B. cereus*-like strains were identified as *B. cereus*.

The subspecies of *B. subtilis* AK1107 was determined using *gyrA* gene sequencing. The homology with 99% identity was with *B. subtilis* ssp. *subtilis* strain KCTC3135, whereas the strain had 94% identity with *B. subtilis* ssp. *spizizenii* NRRL B-23049. Hence, strain AK1107 was identified as *B. subtilis* ssp. *subtilis*.

Subspecies of *Leu. mesenteroides* AK1163 were determined by acid production from arabinose and dextran formation from sucrose. Because strain AK1163 produced an acid from arabinose and dextran from sucrose, it was identified as *Leu. mesenteroides* ssp. *mesenteroides*.

Species identification of the 9 strains (Tables 2 and 3) suspected as *Achromobacter* sp. bacteria was performed by determining oxidation of xylose medium.

None of the strains were able to oxidize xylose and they were consequently identified as *A. denitrificans*.

Pseudomonas fluorescens AK1195 and AK1196 genetically identified were confirmed as *P. fluorescens* by the ability to liquefy gelatin.

Using the culture-dependent analysis, the spoilage bacteria were isolated and the spoilage microbiota in the commercial custard creams was determined (Table 4). This showed that spoilage of custard creams was caused by bacilli, enterococci, staphylococci, lactic acid bacteria, psychrotrophic gram-negative motile rods, or eukaryal cells.

Culture-Independent Analysis

In addition to the culture-dependent analysis, spoilage bacterial communities in the commercial custard creams were determined with a culture-independent analysis using the PCR-DGGE technique and DNA sequencing. Sample solutions for the PCR-DGGE containing the 16S rDNA V6–8 region with a GC clamp were amplified from the total DNA in the putrid custard creams. Representative bands (numbered 1 to 21) from the DGGE profiles are shown in Figure 1. The DNA amplicons extracted from each band were sequenced and their homologies with closest relatives in the GenBank database were determined using BLAST comparisons (Table 5). The microbiota found using the culture-independent method were the same species (*B. amyloliquefaciens*, *B. cereus*, *B. subtilis*, *Staph. warneri*, *E. faecalis*, *Lc. lactis*, *Leu. mesenteroides*, and *Pseudomonas* sp.) as those isolated using the culture-dependent analysis; however, *Anoxybacillus* sp., *Caurobacter* sp.,

Figure 1. Polymerase chain reaction-denaturing gradient gel electrophoresis profiles of the V6–8 region of 16S rDNA prepared from putrid custard creams. The sequenced bands (1–21) are identified.

and *Streptococcus* sp. bacteria were not isolated by the culture-dependent method but found using the independent method. *Achromobacter denitrificans* was detected in the culture-dependent analysis and was not found using the independent method. The spoilage microbiota in the commercial custard creams determined using the culture-independent method was similar to that using the culture-dependent method.

DISCUSSION

A highly nutritive food such as custard cream is easily spoiled by multiplication of specific microbial contaminants similar to using high-nutrient selective media. To lower the risk of spoilage microbiota in a target food, the identity of the organisms should be known. However, spoilage communities in nonfermented processed products such as custard cream are not understood using molecular biological techniques, whereas the bacterial communities in fermented dairy products such as cheese (Randazzo et al., 2002) and yogurt (Fasoli et al., 2003) have been comprehensively investigated. In this study, the spoilage microbiota in putrid

Table 3. Morphological and physiological characteristics of the isolated bacteria¹

Species from 16S rDNA	Strain	Gram stain	Morphology	Oxidase	SIM medium			API	
					H ₂ S	Indole	Motility	Identification	(%)
Species from 16S rDNA									
<i>Bacillus amyloliquefaciens</i> -like	AK1106	+	Bacilli	+	—	—	+	<i>B. amyloliquefaciens</i>	98.6
<i>Bacillus cereus</i> -like	AK1114, 1124, 1171, 1194, 1232	+	Bacilli	+	—	—	+	<i>B. cereus</i> / <i>Bacillus thuringiensis</i>	99.8
<i>Bacillus subtilis</i> -like	AK1107	+	Bacilli	+	—	—	+	<i>B. subtilis</i>	83.5
<i>Enterococcus faecalis</i>	AK1048, 1052	+	Cocci	—	—	—	—	Not tested	
<i>Enterococcus faecium</i>	AK1157	+	Cocci	—	—	—	—	Not tested	
<i>Lactobacillus paracasei</i> -like	AK1165	+	Bacilli	—	—	—	—	<i>Lb. paracasei</i> ssp. <i>paracasei</i>	94.1
<i>Lactococcus lactis</i> ssp. <i>lactis</i>	AK1155	+	Cocci	—	—	—	—	<i>Lc. lactis</i> ssp. <i>lactis</i>	99.9
<i>Leuconostoc mesenteroides</i> -like	AK1163	+	Cocci	—	—	—	—	<i>Leu. mesenteroides</i>	96.9
<i>Staphylococcus saprophyticus</i> -like	AK1121, 1129	+	Cocci	—	—	—	—	<i>Staph. saprophyticus</i> ssp. <i>saprophyticus</i>	99.2
<i>Staphylococcus warneri</i> -like	AK1014	+	Cocci	—	—	—	—	<i>Staph. warneri</i>	86.6
<i>Achromobacter denitrificans</i> -like	AK1040, 1075, 1113, 1123, 1141, 1144, 1145, 1177, 1192	—	Bacilli	+	—	—	+	Not tested	
<i>Pseudomonas fluorescens</i>	AK1195, 1196	—	Bacilli	+	—	—	+	Not tested	

Table 4. The spoilage microbiota in the commercial custard creams using the culture-dependent method

Sample	Identified species	Ratio (%)
A	<i>Staphylococcus warneri</i>	33/33 (100)
B	<i>Enterococcus faecalis</i>	32/33 (97.0)
	<i>Achromobacter denitrificans</i>	1/33 (3.0)
C	<i>A. denitrificans</i>	23/39 (59.0)
	Eukarya	16/39 (41.0)
D	<i>Bacillus cereus</i>	19/31 (61.3)
	<i>Staphylococcus saprophyticus</i> ssp. <i>saprophyticus</i>	7/31 (22.6)
	<i>A. denitrificans</i>	2/31 (6.5)
	<i>Bacillus amyloliquefaciens</i>	2/31 (6.5)
	<i>Bacillus subtilis</i> ssp. <i>subtilis</i>	1/31 (3.2)
E	<i>A. denitrificans</i>	13/32 (40.6)
	<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i>	8/32 (25.0)
	<i>Lactococcus lactis</i> ssp. <i>lactis</i>	7/32 (21.9)
	<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i>	2/32 (6.3)
	<i>Enterococcus faecium</i>	1/32 (3.1)
	Eukarya	1/32 (3.1)
F	<i>A. denitrificans</i>	12/23 (52.2)
	Eukarya	8/23 (34.8)
	<i>B. cereus</i>	3/23 (13.0)
G	Eukarya	18/37 (48.6)
	<i>A. denitrificans</i>	15/37 (40.5)
	<i>B. cereus</i>	2/37 (5.4)
	<i>Pseudomonas fluorescens</i>	2/37 (5.4)

custard creams was determined using culture-dependent and independent methods.

Using the culture-dependent analysis, we isolated and identified bacilli (*B. amyloliquefaciens*, *B. cereus*, and *B. subtilis* ssp. *subtilis*), staphylococci (*Staph. saprophyticus* ssp. *saprophyticus* and *Staph. warneri*), lactic acid bacteria (*E. faecalis*, *E. faecium*, *Lb. paracasei* ssp. *paracasei*, *Lc. lactis* ssp. *lactis*, and *Leu. mesenteroides* ssp. *mesenteroides*), and gram-negative bacteria (*A. denitrificans* and *P. fluorescens*) as the principal spoilage bacteria in the custard creams (Table 4). The

dominant bacterial species was the psychrotrophic gram-negative *A. denitrificans* detected in 6 samples (66 colonies) of the 7 samples (228 colonies; Table 4). *Achromobacter denitrificans* and *P. fluorescens* are generally known as heat-sensitive psychrotrophic gram-negative motile rods isolated frequently in rotten milk and eggs (Ray, 2004). It is therefore suspected that they contaminated the custard creams after heat pasteurization and increased under refrigerated storage and during the sales period; these species were barely detected in the shelf-stable samples (A and B) that underwent

Table 5. The spoilage microbiota in the commercial custard creams using the culture-independent method

Sample	Band	Closest relative	Identity (%)
A	1	<i>Staphylococcus pasteurii</i> strain LF-2/ <i>Staph. warneri</i> ATCC27836	99
	2	<i>Staph. pasteurii</i> strain LF-2/ <i>Staph. warneri</i> ATCC27836	99
	3	<i>Staph. pasteurii</i> strain LF-2/ <i>Staph. warneri</i> ATCC27836	99
	4	<i>Staph. pasteurii</i> strain LF-2/ <i>Staph. warneri</i> ATCC27836	99
B	5	<i>Enterococcus faecalis</i> strain GM/ <i>Lactobacillus plantarum</i> strain PFK2	99
	6	<i>E. faecalis</i> strain GM/ <i>Lb. plantarum</i> strain PFK2	99
C	7	Uncultured bacterium clone Thompsons87	89
	8	<i>Bacillus subtilis</i> ssp. <i>subtilis</i> strain 168	74
	9	Uncultured <i>Bacillus</i> hs2 43	96
	10	<i>Anoxybacillus</i> sp. CBS-5	99
D	11	<i>Bacillus atrophaeus</i> strain BCRC 17123/ <i>Bacillus pumilus</i> strain XJU-4/ <i>B. subtilis</i> strain N10	97
	12	<i>B. subtilis</i> strain N10	97
	13	<i>B. subtilis</i> strain h-g/ <i>Bacillus amyloliquefaciens</i> strain BCRC14193	99
E	14	<i>Lactococcus lactis</i> strain P2	100
	15	<i>Leuconostoc mesenteroides</i> isolate F170	85
F	16	<i>Streptococcus</i> sp. 2061A	95
	17	<i>Streptococcus</i> sp. 2061A	89
	18	Uncultured <i>Caulobacter</i> sp. clone Esch5-28	91
G	19	<i>Bacillus cereus</i> strain FRA000041	87
	20	<i>Pseudomonas</i> sp. PRGB06	91
	21	<i>Pseudomonas</i> sp. PRGB06	94

severe heat treatment during food reprocessing. Further, because heat-sensitive eukaryal fungi were also not detected in samples A and B, it is suggested that shelf-stable custard cream may be spoiled only by the heat-resistant gram-positive bacteria. The predominant spoilage bacterial species of the gram-positive species was *B. cereus* isolated in 3 samples (24 colonies) of 7 samples (228 colonies; Table 4). *Bacillus cereus* is well known as a major food spoilage and poisoning organism forming heat-resistant spores and producing toxins that cause emetic and diarrheal syndromes (Schoeni and Wong, 2005). Some strains of *B. cereus* isolated in this study survived heat pasteurization, presumably using spore forms. This suggests that measures such as food preservatives and refrigeration should be considered during custard cream formulation and preservation, so that heat-resistant spores do not propagate.

In culture-independent analysis, PCR-DGGE for the V6–8 region on 16S rDNA was performed (Figure 1); PCR-DGGE for the V3 region was rejected because there were fewer bands for the V3 region (data not shown) than for the V6–8 region (Figure 1). A sequence and homology search of the DGGE banding amplicons in the V6–8 region determined that the spoilage microbiota in the commercial custard creams consisted of bacilli, staphylococci, lactic acid bacteria, *Pseudomonas* sp., *Anoxybacillus* sp., *Caulobacter* sp., and *Streptococcus* sp. bacteria (Table 5). The plural bands in the V6–8 region were identified as the same species, possibly indicating that single-base-change mutants were caused by heteroduplex formation (Jensen and Straus, 1993). The spoilage microbiota detected using the culture-independent method was similar to that in the culture-dependent analysis. However, the dominant species in the culture-dependent analysis (*A. denitrificans*) was not detected using the culture-independent analysis. The reason may be insufficient DNA extraction from the small-sized *A. denitrificans* using the bead extraction method or PCR bias using unsuitable universal primers (Kanagawa, 2003). *Anoxybacillus* sp., *Caulobacter* sp., and *Streptococcus* sp. bacteria were genetically detected only by the culture-independent analysis, in which it is difficult to determine whether they had existed before or were viable after heat pasteurization, because viable and dead cells in the putrid custard creams were detected using this 16S rDNA detection method.

Most of the detected spoilage bacteria in this study were the same as those detected previously in rotten milk (Boor and Murphy, 2002). This result suggests that spoilage bacteria growing in a raw material are easy to grow in a processed food made from the same raw material. As the first step for safe food preservation, food manufacturers should understand the spoil-

age microbiota in a target food to select optimal preservatives and thus reduce the need for food additives. However, in the real-world situation, indistinct and inappropriate preservatives are qualitatively and quantitatively added to processed foods and this is not preferable for manufacturers and consumers. If the spoilage microbiota is known as reported here, antimicrobial measures suitable for each food can be adopted.

The 2 experimental techniques used herein differ in important ways. Using the culture-dependent method, viable cells were isolated and only cultivable cells could be detected, whereas using the culture-independent method, both cultivable and uncultivable species are detected rapidly and easily. However, viable cells cannot be isolated using the culture-independent method and bacterial species cannot be completely identified because the method relies on extracted DNA or RNA sequences as templates for PCR. A more accurate microbiota analysis is possible by combining both methods and compensating for their strengths and weaknesses. Previously, combined analyses have been performed on soil (Ellis et al., 2003) and fermented food microbiota (Rantsiou et al., 2005). However, few studies concerning spoiled foods using the culture-independent method have been performed. Our data suggest that more spoilage microbiota in various food products need to be examined using both the culture-dependent and independent methods to develop future food preservation methods.

REFERENCES

- Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59:143–169.
- Ampe, F., N. ben Omar, C. Moizan, C. Wachter, and J. P. Guyot. 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Appl. Environ. Microbiol.* 65:5464–5473.
- Ampe, F., A. Sirvent, and N. Zakhia. 2001. Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization. *Int. J. Food Microbiol.* 65:45–54.
- ben Omar, N., and F. Ampe. 2000. Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl. Environ. Microbiol.* 66:3664–3673.
- Beukes, E. M., B. H. Bester, and J. F. Mostert. 2001. The microbiology of South African traditional fermented milks. *Int. J. Food Microbiol.* 63:189–197.
- Blazevic, D. J., M. H. Koepche, and J. M. Matsen. 1973. Incidence and identification of *Pseudomonas fluorescens* and *Pseudomonas putida* in the clinical laboratory. *Appl. Microbiol.* 25:107–110.
- Boor, K. J., and S. C. Murphy. 2002. Microbiology of market milks. Pages 91–122 in *Dairy Microbiology Handbook*. 3rd edition. R. K. Robinson, ed. John Wiley and Sons Inc., New York, NY.
- Bravo, A., S. Sarabia, L. Lopez, H. Ontiveros, C. Abarca, A. Ortiz, M. Ortiz, L. Lina, F. J. Villalobos, G. Pena, M. E. Nunez Valdez, M. Soberon, and R. Quintero. 1998. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl. Environ. Microbiol.* 64:4965–4972.

- Cenci-Goga, B. T., S. Crotti, S. Costarelli, C. Rondini, M. Karama, and P. Bennett. 2004. Detection of *tet(M)* gene from raw milk by rapid DNA extraction followed by a two-step PCR with nested primers. *J. Food Prot.* 67:2833–2838.
- Ceron, J., A. Ortiz, R. Quintero, L. Guereca, and A. Bravo. 1995. Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. *Appl. Environ. Microbiol.* 61:3826–3831.
- Chun, J., and K. S. Bae. 2000. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. *Antonie Van Leeuwenhoek* 78:123–127.
- Cocolin, L., L. Bisson, and D. A. Mills. 2000. Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* 189:81–87.
- Cocolin, L., M. Manzano, C. Cantoni, and G. Comi. 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Appl. Environ. Microbiol.* 67:5113–5121.
- Cotter, P. D., C. Hill, and R. P. Ross. 2005. Bacteriocins: Developing innate immunity for food. *Nat. Rev. Microbiol.* 3:777–788.
- Ellis, R. J., P. Morgan, A. J. Weightman, and J. C. Fry. 2003. Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl. Environ. Microbiol.* 69:3223–3230.
- Ercolini, D. 2004. PCR-DGGE fingerprinting: Novel strategies for detection of microbes in food. *J. Microbiol. Methods* 56:297–314.
- Fasoli, S., M. Marzotto, L. Rizzotti, F. Rossi, F. Dellaglio, and S. Torriani. 2003. Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *Int. J. Food Microbiol.* 82:59–70.
- Fontana, C., P. S. Cocconcetti, and G. Vignolo. 2005a. Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. *Int. J. Food Microbiol.* 103:131–142.
- Fontana, C., G. Vignolo, and P. S. Cocconcetti. 2005b. PCR-DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. *J. Microbiol. Methods* 63:254–263.
- Harmon, S. M. 1982. New method for differentiating members of the *Bacillus cereus* group: Collaborative study. *J. Assoc. Off. Anal. Chem.* 65:1134–1139.
- Iwamoto, T., K. Tani, K. Nakamura, Y. Suzuki, M. Kitagawa, M. Eguchi, and M. Nasu. 2000. Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. *FEMS Microbiol. Ecol.* 32:129–141.
- Jensen, M. A., and N. Straus. 1993. Effect of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of bacterial ribosomal DNA spacer regions. *PCR Methods Appl.* 3:186–194.
- Kanagawa, T. 2003. Bias and artifacts in multitemplate polymerase chain reaction (PCR). *J. Biosci. Bioeng.* 96:317–323.
- Kawai, Y., and T. Saito. 2002. Bacteriocins of lactic acid bacteria. Pages 99–112 in *The Biol.-defensive Function of Dairy Foods*. K. Shimazaki and H. Otani, ed. Research Signpost, Trivandrum, India.
- Kroes, I., P. W. Lepp, and D. A. Relman. 1999. Bacterial diversity within the human subgingival crevice. *Proc. Natl. Acad. Sci. USA* 96:14547–14552.
- Lane, D. J. 1991. 16S/23S rRNA sequencing. Pages 115–175 in *Nucleic Acid Techniques in Bacterial Systematics*. E. R. Stackebrandt and M. Goodfellow, ed. John Wiley and Sons Inc., New York, NY.
- Lee, J. S., G. Y. Heo, J. W. Lee, Y. J. Oh, J. A. Park, Y. H. Park, Y. R. Pyun, and J. S. Ahn. 2005. Analysis of kimchi microflora using denaturing gradient gel electrophoresis. *Int. J. Food Microbiol.* 102:143–150.
- Miambi, E., J. P. Guyot, and F. Ampe. 2003. Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods. *Int. J. Food Microbiol.* 82:111–120.
- Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695–700.
- Obodai, M., and C. E. Dodd. 2006. Characterization of dominant microbiota of a Ghanaian fermented milk product, nyarmie, by culture- and nonculture-based methods. *J. Appl. Microbiol.* 100:1355–1363.
- Randazzo, C. L., S. Torriani, A. D. Akkermans, W. M. de Vos, and E. E. Vaughan. 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Appl. Environ. Microbiol.* 68:1882–1892.
- Rantsiou, K., R. Urso, L. Iacumin, C. Cantoni, P. Cattaneo, G. Comi, and L. Cocolin. 2005. Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. *Appl. Environ. Microbiol.* 71:1977–1986.
- Ray, B. 2004. Spoilage of specific food groups. Pages 269–288 in *Fundamental Food Microbiology*. 3rd edition. B. Ray, ed. CRC Press, Boca Raton, FL.
- Rosenquist, H., L. Smidt, S. R. Andersen, G. B. Jensen, and A. Wilcks. 2005. Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiol. Lett.* 250:129–136.
- Schoeni, J. L., and A. C. Wong. 2005. *Bacillus cereus* food poisoning and its toxins. *J. Food Prot.* 68:636–648.
- Torsvik, V., F. L. Daae, R. A. Sandaa, and L. Ovreas. 1998. Novel techniques for analyzing microbial diversity in natural and perturbed environments. *J. Biotechnol.* 17:53–62.