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Development of Polymerase Chain Reaction and Multiplex Polymerase Chain Reaction for Simple Identification of Thermoanaerobic Spore-forming Bacteria

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Thermoanaerobic spore-forming bacteria such as *Thermoanaerobacter*, *Moorella*, *Thermoanaerobacterium*, and *Caldanaerobius* produce spores with extremely high heat resistance. They are known to spoil various sealed, sterile drinks; in particular, low-acid drinks distributed at high temperatures, such as canned coffee containing milk. These bacteria are difficult to culture and identify on the basis of traditional biochemical characteristics. We developed novel primers for single and multiplex PCR methods for simple identification of these bacteria at the genus level. Bacteria were correctly identified approximately 2 h after DNA extraction among 86 strains of 35 species of Gram-positive and -negative bacteria including various spore-forming bacilli. Furthermore, new Loop-Mediated Isothermal Amplification (LAMP) primers were designed to develop a specific detection method for *Thermoanaerobacter mathranii* and *Thermoanaerobacter thermocopriae*, highly problematic microbes in the food industry due to their extremely high resistance to heat and various antibacterial agents. Our LAMP method using the novel primers was able to easily detect these microbes. Our present methods effectively improve upon the complicated procedures employed in the quality control of raw materials and products in the food industry.

Keywords: *Moorella*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Caldanaerobius*, low-acid drink

Introduction

It is extremely difficult to control microbes in low-acid soft drinks and food stored or sold at high temperatures, such as coffee or tea containing milk. A variety of control methods are required to remedy this situation. Bacteria responsible for spoilage of these foods are thermophilic anaerobes that form spores with extremely high heat resistance, such as *Moorella*, *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Caldanaerobius* (Ashton *et al.*, 1981; Yamamoto *et al.*, 1991). Generally, these microbes are isolated from hot springs or soils (Larsen *et al.*, 1997) and are non-

pathogenic. In foods, however, many of these microbes exist in powdered raw materials manufactured by spray-dry processing or high-temperature extraction or reduction processing (Akutsu *et al.*, 2008; Pollach *et al.*, 2002; Sakurai *et al.*, 2000). Bacteria contaminate sealed products through the raw materials and are able to survive under typical sterile conditions because of their high heat resistance (Tanaka *et al.*, 1998; Enda *et al.*, 1989; Byrer *et al.*, 2000). As a result, they survive in the product, continuing to grow during storage in vending machines exposed to high temperatures. The microbes produce acid and gas in sealed foods such as canned

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coffee. Hence, various countermeasures have been applied to prevent bacterial spoilage of soft drinks, for example, prevention of contamination by inspection of materials, control of microbes during processing, and supplementation with food additives with bacteriostatic activity (Enda *et al.*, 1992). The number of spoilage cases caused by these microbes has decreased; however, they continue to occur occasionally in various low-acid drinks and foods (Carlier *et al.*, 2006; Prevost *et al.*, 2010; Andre *et al.*, 2013) distributed at high temperatures. Therefore, the rapid and accurate detection and identification of thermoanaerobic spore-forming bacteria in products and raw materials is essential to prevent food spoilage. In particular, a method for the rapid detection of *Thermoanaerobacter mathranii* or *Thermoanaerobacter thermocopriae* is vital for quality control because these species have high resistance to heat and bacteriostatic agents. In the present study, we developed polymerase chain reaction (PCR) and multiplex PCR methods for the rapid identification of thermoanaerobic spore-forming bacteria using primers designed to distinguish closely related microbes such as *Thermoanaerobacter*, *Thermoanaerobacterium*, *Moorella*, and *Caldanaerobius*. We also designed primers for the Loop-Mediated Isothermal Amplification (LAMP) method to identify *T. mathranii* and *T. thermocopriae*, which are the most important spoilage microbes of soft drinks.

Materials and Methods

Strains and culture Genus *Bacillus*, *Paenibacillus*, *Caldanaerobius*, *Moorella*, *Thermoanaerobacter*, and *Thermoanaerobacterium* used in this study were provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Japan Collection of Microorganisms (JCM), Institute of Fermentation, Osaka (IFO), NITE Biological Resource Center (NBRC), Belgian Coordinated Collection of Microorganisms (BCCM), and the Japan Canners Association (JCA) (Table 3). These bacterial strains were detected or isolated from low-acid drinks or canned foods distributed under high temperatures. Other bacterial strains were isolated from various low-acid drinks or their raw materials and were identified by 16S rDNA gene analysis. The bacteria were inoculated to modified TGC (mTGC) medium (Nissui Pharmaceuticals, Tokyo, Japan) or mTGC medium with 2.0% agar, and incubated for 3–7 days at their respective optimal temperatures: 60°C for *Thermoanaerobacterium*, *Thermoanaerobacter* and *Caldanaerobius*, and 55°C for *Moorella*. Aneropack - Kenki (Mitsubishi Gas Chemical, Tokyo, Japan) was used for anaerobic culture. Other bacteria were incubated at 37°C.

DNA preparation method Bacterial cells grown on mTGC medium or mTGC agar were harvested by centrifugation at 14,000 rpm for 1 min, followed by washing twice with water. A 100-μL aliquot of Prepman Ultra Reagent (Life Technologies, Carlsbad, CA, USA) was added to the washed pellet and resuspended thoroughly by pipetting. The suspension was heated at 100°C for 10 min and then cooled to room temperature. After

centrifugation at 14,000 rpm for 1 min, 50 μL of the supernatant was recovered and diluted three-fold with sterile water; the resulting solution was used as the DNA solution.

Primer design 16S rDNA sequence data were used for designing primers to specifically detect thermoanaerobic bacteria, since DNA sequence data for this gene is abundant. The 16S rDNA sequences of each bacterial strain were compared.

Species-specific PCR Illustra RTG (Ready-to-Go) PCR Beads (GE Healthcare 27-9559-01; Piscataway, NJ, USA) were used for the PCR. Template DNA (1 μL), 10 pmol forward primer, 10 pmol reverse primer and RTG PCR Beads were mixed in a reaction tube and water was added for a final volume of 25 μL. The PCR conditions were as follows: 95°C for 2 min, 30 cycles of 95°C for 30 s; 62°C for 30 s; and 72°C for 30 s, and 72°C for 2 min as a final extension. A C1000 Touch Thermal Cycler (BIORAD, Hercules, CA, USA) or iCycler (BIORAD) were used for the PCR. PCR products were confirmed by 2% agarose gel electrophoresis using SEA KEM GTG AGAROSE (FMC Bio Products, Philadelphia, PA, USA) and stained with ethidium bromide.

Determination of PCR detection limits Template DNA was prepared from 0.1 pg/μL to 10 ng/μL from *Moorella thermoacetica* DSM521, *T. mathranii* DSM11426, and *Caldanaerobius polysaccharolyticum* DSM13641. PCR was performed with primers MooF2 and MooR2 for *M. thermoacetica*, TbmcomF and TbrR2 for *T. mathranii*, CpszF1 and CpszR2 for *C. polysaccharolyticum* and the PCR products were detected according to the method described above.

Multiplex PCR Multiplex PCR was used to simplify the test method distinguishing thermoanaerobic bacteria. As shown below, six types of primers were added to the reaction mixture, and multiplex PCR was performed. Illustra RTG PCR Beads were used; the reaction mixture contained 1 μL template DNA solution and each primer (10 pmol each of CpszF1, CpszR2, TbmotherF, TbrR2, MooF2, and MooR2) and sterile water was added up to 25 μL. PCR was performed under the same conditions as used for strain-specific PCR: 95°C for 2 min, 30 cycles of 95°C for 30 s; 62°C for 30 s; and 72°C for 30 s, and 72°C for 2 min as a final extension. A C1000 Touch thermal cycler or iCycler were used for PCR. The amplicons were confirmed by 2% agarose gel electrophoresis using SEA KEM GTG AGAROSE (FMC Bio products) and stained with ethidium bromide.

LAMP method Online primer design support software PrimerExplorer (i) was used to design LAMP primers specific to *T. mathranii* based on their 16S rDNA sequences. A Loopamp DNA Amplification Kit (Eiken Chemical Co., Tokyo, Japan) was used for the LAMP method experiment. The reaction mixture contained 2 μL of template DNA solution and *T. mathranii* specific Tbr1 LAMP primer set (40 pmol each of Tbr1FIP and Tbr1BIP, 5 pmol each of Tbr1F3 and Tbr1B3), 1 μL Bst DNA polymerase, and 12.5 μL 2x Reaction mixture; sterile water was added to a final volume of 25 μL. The LAMP reaction was conducted at 60°C for 90

min using the iCycler (BIORAD). The amplicons were confirmed by turbidity or Fluorescent Detection Reagent (Eiken Chemical Co.).

Determination of LAMP method detection limit *T. mathranii* DSM11426 was cultured with mTGC broth at 60°C for 2 days. The cultured broth was serially diluted from 10⁰ cfu/mL to 10⁵ cfu/mL. One mL of each cultured broth was harvested by centrifugation at 14,000 rpm for 1 min, followed by washing twice with water. A 10-μL aliquot of Prepman Ultra Reagent was added to the washed pellet and resuspended thoroughly by pipetting. The suspension was heated at 100°C for 10 min and then cooled to room temperature. After centrifugation at 14,000 rpm for 1 min, 5 μL of the supernatant was recovered, and 2 μL of the solution was used as the DNA template. The LAMP assay was performed with the Tbr1 primer set and the detection limit was determined.

Detection and simple identification of *Thermoanaerobic* bacteria in raw materials In order to test whether the genomic assays were useful for investigation of the raw materials of canned coffee drinks, we used 10 g of each raw material. For determination of bacterial counts, 10-g samples were diluted in 50 mL water and heated at 100°C for 10 min. The sample solution was mixed with 200 mL of 5/4 fold mTGC medium including 2% agar. The entire mixture was poured into petri dishes and then incubated anaerobically for 5 days at 60°C. Detected colonies were identified by 16S rDNA sequences. For PCR and LAMP assays, 50 mL of the heat treated sample solution was mixed with 200 mL of 5/4 fold mTGC broth and then incubated at 60°C for 5 days. Aliquots (1 μL) of the cultured media were used as the templates.

Results

Primer design The primers CpszF1 and CpszR2, specific to *C. polysaccharolyticum* and *Caldanaerobius zaeae*, as well as the primers specific to *Moorella*, MooF2 and MooR2, were designed based on their 16S rDNA sequence data. TbmcomF and TbrR2 primers specific to both *Thermoanaerobacter* and *Thermoanaerobacterium* were designed. TbmotherF was designed as a primer to detect *Thermoanaerobacterium*. Primers specific to *Thermoanaerobacter* could not be designed based on their 16S rDNA sequences (Table 1). To design specific primers, all 16S rDNA sequences of the genera *Bacillus*, *Clostridium*, and *Geobacillus* in the databases were referenced.

Specificity of PCR primers To detect *Moorella*, MooF2 and MooR2 primers were used. PCR products of approximately 470 bp were amplified with template DNAs from *M. thermoacetica* and *Moorella thermoautotrophica* (Fig. 1, A, lanes 6 and 7). No PCR products were amplified with DNAs from other anaerobic spore-forming bacteria of the genera *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Caldanaerobius*.

For *Caldanaerobius*, CpszF1 and CpszR2 were used to amplify the approximately 210 bp PCR product. The PCR product was amplified with template DNA from *C. polysaccharolyticum* among the

Table 1. Primers for the identification of thermophilic anaerobic spore-forming bacteria

Primer	Sequence
CpszF1	CACGTGAGCAACCTGCCTTT
CpszR2	AGTCCCAGTGTGGCCGTA
TbmcomF	GATAACACCTCGAAAGGGGT
TbrR2	TAGTTAGCCGGGGCTTTTCGT
MooF2	ATGCAAGTCGAGCGGTCTTT
MooR2	CCGGGGCTTCCTCCTCA
Tbmother	GCGTGGACAATCTACCCTGT

Table 2. LAMP primers for the specific detection of *T. mathranii*

Primer	Sequence
Tbr1B3	GCGGGCCCATCCTTAAGC
Tbr1BIP	GTAATACTGGATAAGCTCCTTATCCTTTCTCCCTATAGGATG
Tbr1F3	GGAGAGTTTGATCCTGGCTC
Tbr1FIP	GCCACCCAACCTACGTTTGTAGTAGGACGAACGCTGGCG

Fig. 1. PCR products amplified with each primer and the genomic DNA prepared from thermoanaerobic bacteria. MooF2/MooR2 (A), CpszF1/CpszR2 (B), TbmcomF/ TbrR2 (C), TbmotherF/ TbrR2 (D). Lanes: M, 100 bp DNA ladder; 1, *T. mathranii* NBS-041; 2, *T. mathranii* DSM11426; 3, *T. thermohydrosulfuricus* ATCC 35045; 4, *C. polysaccharolyticum* DSM13641; 5, *T. thermosaccharolyticum* ATCC 7956; 6, *M. thermoacetica* DSM521; 7, *M. thermoautotrophica* DSM1794; 8, *G. stearothermophilus* JCM2501

bacteria tested. (Fig. 1, B, lane 4). To detect *Thermoanaerobacter* and *Thermoanaerobacterium*, TbmcomF and TbrR2 primers were used to amplify the approximately 352 bp PCR products. Amplicons were generated with DNAs from *T. mathranii*, *Thermoanaerobacter thermohydrosulfuricus*, and *Thermoanaerobacterium thermosaccharolyticum*. No PCR products were observed for the other microbes (Fig. 1, C, lanes 1 - 3, and 5). To detect *Thermoanaerobacterium*, Tbmother and TbrR2 primers were designed. An approximately 375 bp PCR product was amplified using the primer set with the template DNA from *T. thermosaccharolyticum*. No PCR products were amplified with DNA from the other microbes (Fig. 1, D, lane 5). Table 3 shows the specificity test results of all the primer sets on bacterial strains used in this study. No PCR products were amplified with DNAs from any of the *Bacillus* and *Clostridium*

Table 3. List of strains and specificity of PCR and LAMP methods using novel primers for various bacteria.

Species	Bacterial strain	Source	Primer sets					LAMP TbrI set
			MooF2 /MooR2	CpsZF1 /CpsZR2	TbmomF/ TbrR2	TbmomF/ TbrR2	Multiplex	
<i>Moorella thermoacetica</i>	DSM 521	DSMZ	-	-	-	-	-	-
	JCA 5802, 5805, 5806, 5807, 5808, 5809, 5803, 5810	JCA	-	-	-	-	-	-
	NBS 003, 075, 076, 077, 078	Asahi soft drinks	-	-	-	-	-	-
<i>Moorella thermoautotrophica</i>	DSM 1974	DSMZ	-	-	-	-	-	-
<i>Thermoanaerobacter thermohydrosulfuricus</i>	ATCC 35045	ATCC	-	-	-	+	-	-
<i>Thermoanaerobacter mathranii</i>	DSM 11426	DSMZ	-	-	-	-	-	-
	JCA 5910, 5913, 5915	JCA	-	-	-	-	-	-
	NBS 017, 018, 019, 020, 021, 022, 023, 024, 025, 026, 030, 031, 032, 035, 036, 037, 038, 039, 041, 045, 046, 081, 082	Asahi soft drinks	-	-	-	-	-	-
<i>Thermoanaerobacter thermocapriac</i>	JCM 7501, ATCC 33223	JCM	-	-	-	-	-	-
<i>Thermoanaerobacter italicus</i>	DSM 9252	DSMZ	-	-	-	-	-	-
<i>Thermoanaerobacter sulfurophilus</i>	DSM 11584	DSMZ	-	-	-	-	-	-
<i>Thermoanaerobacter brockii</i> subsp. <i>lactiethylicus</i>	DSM 9801	DSMZ	-	-	-	-	-	-
<i>Thermoanaerobacterium thermosaccharolyticum</i>	ATCC 7956	ATCC	-	-	-	-	-	-
	JCA 5604, 5617, 5623, 5625, 5626, 5631	JCA	-	-	-	-	-	-
<i>Thermoanaerobacterium thermosulfurigenes</i>	DSM 2229	DSMZ	-	-	+	+	-	-
<i>Thermoanaerobacterium</i> spp.	JCA 5923, 5633, 5640, 5643	JCA	-	-	-	-	-	-
<i>Caldanaerobius zoeae</i>	DSM 13642	DSMZ	-	-	-	-	-	-
<i>Caldanaerobius polysaccharolyticum</i>	DSM 13641	DSMZ	-	-	-	-	-	-
	JCA 5920, 5930	JCA	-	-	-	-	-	-
<i>Geobacillus stearothermophilus</i>	JCM 2501(T.S.)	JCM	-	-	-	-	-	-
<i>Bacillus cereus</i>	JCM 2152(T.S.)	JCM	-	-	-	-	-	-
<i>Bacillus circulans</i>	JCM 2504(T.S.)	JCM	-	-	-	-	-	-
<i>Bacillus licheniformis</i>	IFO 12200(T.S.)	IFO	-	-	-	-	-	-
<i>Bacillus pumilus</i>	IFO 12092(T.S.)	IFO	-	-	-	-	-	-
<i>Bacillus sphaericus</i>	JCM 2502(T.S.)	JCM	-	-	-	-	-	-
<i>Bacillus thuringiensis</i>	ATCC 10792(T.S.)	ATCC	-	-	-	-	-	-
<i>Bacillus subtilis</i>	JCM 1465(T.S.)	JCM	-	-	-	-	-	-
<i>Bacillus megaterium</i>	JCM 2506(T.S.)	JCM	-	-	-	-	-	-
<i>Bacillus coagulans</i>	JCM 2257(T.S.)	JCM	-	-	-	-	-	-
<i>Bacillus pumilus</i>	LMG 18418	BCCM	-	-	-	-	-	-
<i>Brevibacillus agri</i>	NBRC 15538(T.S.)	NBRC	-	-	-	-	-	-
<i>Brevibacillus brevis</i>	NBRC 15304(T.S.)	NBRC	-	-	-	-	-	-
<i>Brevibacillus formosus</i>	NBRC 15716(T.S.)	NBRC	-	-	-	-	-	-
<i>Brevibacillus laterosporus</i>	NBRC 15654(T.S.)	NBRC	-	-	-	-	-	-
<i>Pantoea polymyxa</i>	JCM 2507(T.S.)	JCM	-	-	-	-	-	-
<i>Pantoea polymyxa</i>	JCM 2500(T.S.)	JCM	-	-	-	-	-	-
<i>Clostridium fermentans</i>	JCM 1390(T.S.)	JCM	-	-	-	-	-	-
<i>Clostridium histricum</i>	JCM 1391(T.S.)	JCM	-	-	-	-	-	-
<i>Clostridium pasteurianum</i>	JCA 5511	JCA	-	-	-	-	-	-
<i>Enterobacter cloacae</i>	JCM 1232	JCM	-	-	-	-	-	-
<i>Escherichia coli</i>	ABS 078	Asahi soft drinks	-	-	-	-	-	-

DSMZ:Deutsche Sammlung von Mikroorganismen und Zellkulturen, JCM:Japan Collection of Microorganisms, NBRC:NITE Biological Resource Center, BCCM:Belgian Co-ordinated Collection of Microorganisms, JCA:Japan Camers Association, IFO: Institute of Fermentation, Osaka

Fig. 2. PCR detection limit using each primer set. Lanes: M, 100 bp DNA ladder, 1-6: *M. thermoacetica* DSM521 DNA with MooF2/MooR2 primers; Lane 7-12, *T. mathranii* DSM11426 DNA with TbmomF/TbrR2 primers; Lanes 13-18, *C. polysaccharolyticum* DSM13641 DNA with CpsZF1/CpsZR2 primers; (Lanes 1, 7 and 13, 0.1 pg; Lane 2, 8 and 14, 1 pg; Lanes 3, 9 and 15, 10 pg; Lanes 4, 10 and 16, 100 pg; Lane 5, 11 and 17, 1 ng; Lane 6, 12 and 18, 10 ng/ μ L)

strains examined in this study. No PCR products were observed for *Geobacillus stearothermophilus*, a strain detected frequently with other thermophilic anaerobes from low-acid soft drinks and food stored or sold at high temperatures. The results suggested that each of the primer sets was specific to the target thermoanaerobic spore-forming bacteria.

Detection limit PCR was performed with each primer set with 0.1 pg, 1 pg, 10 pg, 100 pg, 1 ng, and 10 ng of DNA from each bacteria. The detection limit of the PCR assay was 1 pg/ μ L template DNA from each bacteria (Fig. 2).

Multiplex PCR Although we have constructed a single PCR method specific to each of the genera, four different PCRs were required to distinguish thermoanaerobic spore-forming bacteria using the current method. Multiplex PCR was used to simplify the test method to distinguish the thermoanaerobic bacteria. Therefore, multiplex PCR was developed by mixing six types of primers in one PCR mixture. PCR products of 474 bp, 352 bp, 352 bp, and

Fig. 3. Amplicons produced by multiplex PCR using a mixture of all the primer sets. Multiplex PCR was performed in the presence of all the primer sets (MooF2/MooR2, CpszF1/CpszR2 and TbmotherF/TbrR2) in a single tube, including each of the DNA templates prepared from the thermoanaerobes. Lanes: M, 100 bp DNA ladder; 1, *M. thermoacetica* DSM521; 2, *M. thermoautotrophica* DSM1794; 3, *T. thermohydrosulfuricus* ATCC 35045; 4, *T. mathranii* DSM11426; 5, *T. thermocopriae* JCM7501; 6, *T. italicus* DSM9252; 7, *T. sulfurophilus* DSM11584; 8, *T. brockii* subsp. *lactiethylicus* DSM9801; 9, *T. thermosaccharolyticum* ATCC 7956; 10, *T. thermosulfurigenes* DSM2229; 11, *C. polysaccharolyticum* DSM13641; 12, *C. zeae* DSM13642

210 bp were amplified with template DNAs respectively from the genera *Moorella*, *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Caldanaerobius* (Fig. 3). Table 3 shows the specificity test results of multiplex PCR for bacterial strains used in this study. No PCR products were amplified with DNAs from any of the *Bacillus* and *Clostridium* strains examined in this study.

Specificity of the LAMP method for *T. mathranii* For specific detection of *T. mathranii*, 4 primers for the LAMP method were designed and tested under the various conditions (Table 2). All 27 strains of *T. mathranii* were positive using the LAMP method. Among 86 strains, which included 35 species of spore-forming bacteria, only 2 strains of *T. thermocopriae* were positive, except the *T. mathranii* strains (Table 3).

Detection limit of LAMP assay LAMP assay was performed with the Tbr1 LAMP primer set with template DNA from 10^1 to 10^5 cells. The detection limit of the LAMP assay was 10^2 cfu/mL (Fig. 4, tube No. 2).

Detection and simple identification of Thermoanaerobic bacteria in raw materials Thermophilic anaerobic bacterial spores in 12 samples of sugar and 5 samples of milk powder were counted and identified. *Thermoanaerobacter tencongensis*, *T. mathranii*, and *T. thermosaccharolyticum* were detected in three samples. These bacteria were detected by PCR and LAMP assays (Table 4). No correlation was observed between colony counts and genomic assays.

Discussion

Thermoanaerobic spore-forming bacteria are frequently detected in spoiled processed foods. These microbes have species-

Fig. 4. Detection limit of LAMP assay for *T. mathranii* DSM11426, confirmed by turbidity (A) or fluorescence (B). 1, 10^1 cfu/mL; 2, 10^2 cfu/mL; 3, 10^3 cfu/mL; 4, 10^3 cfu/mL; 5, 10^4 cfu/mL

specific characteristics. The D values at 121°C of spores of *M. thermoacetica* and *M. thermoautotrophica*, highly heat-resistant bacteria, are reported to be approximately 5-80 min or more depending on the strain. *T. mathranii* and *T. thermocopriae* are some of the most important spoilage bacteria of low-acid soft drinks because of their resistance to bacteriostatic emulsifiers. They are difficult to culture systematically because their optimal culture conditions, such as culture temperature, aeration condition and auxotrophy, differ greatly. Furthermore, these bacteria perish easily after reaching maximal growth, making it difficult to perform characteristic tests or passage and to obtain detailed information for characterization. Thus, on-site identification of these thermoanaerobic bacteria cannot be performed easily.

16S rDNA gene sequence analysis is often used to identify these bacterial strains. However, the analysis cannot be performed easily at food companies during material storage or process management because expensive machinery and complicated procedures are required. Here, we developed a simple method to distinguish thermoanaerobic bacteria with very similar characteristics such as *Moorella*, *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Caldanaerobius*. Although studies on the specific detection of these species have been reported previously (Yamamoto *et al.*, 2001; Prevost *et al.*, 2010), these methods were not sufficiently rapid or simple for use in soft drink manufacturing.

Our methods are superior to conventional methods for the identification of thermoanaerobic bacterial strains that are typically difficult to distinguish. Indeed, they can be identified relatively easily with inexpensive machinery approximately 2 h after DNA extraction; in contrast to the use of advanced techniques such as DNA sequencing. We also successfully developed a simple test system based on the LAMP method for detecting *T. mathranii* and *T. thermocopriae*, which are thought to have the highest resistance to heat and emulsifiers. Our method requires no equipment except for a water bath.

For the practical application of our methods to detect and identify problematic thermoanaerobic spore-forming bacteria during the quality control of raw materials and products, further detailed studies using field isolates of thermoanaerobes are required. Our present methods effectively improved the

Table 4. Anaerobic spore enumeration and genomic assays of raw materials of canned coffee.

Product	Anaerobic spore count		Isolation and Identification	PCR method	LAMP
	No	(CFU/10g)			
Sugar	1	6	<i>Thermoanaerobacter tencongensis</i>	<i>Thermoanaerobacterium</i> or/and <i>Thermoanaerobacter</i>	-
	2	0	-	-	-
	3	7	<i>G. stearothermophilus</i>	-	-
	4	1	<i>G. kaustophilus</i>	-	-
	5	2	<i>Bacillus smithii</i>	-	-
	6	0		-	-
	7	0		-	-
	8	41	<i>T. thermosaccharolitycum</i>	<i>Thermoanaerobacterium</i> or/and <i>Thermoanaerobacter</i>	-
	9	238	<i>T. thermosaccharolitycum</i> , <i>T. mathranii</i>	<i>Thermoanaerobacterium</i> or/and <i>Thermoanaerobacter</i>	+
	10	151	<i>G. kaustophilus</i>	-	-
	11	2	<i>G. stearothermophilus</i>	-	-
	12	2	<i>B. coagulans</i>	-	-
Milk powder	1	460	<i>G. kaustophilus</i>	-	-
	2	660	<i>G. kaustophilus</i>	-	-
	3	185	<i>G. stearothermophilus</i>	-	-
	4	365	<i>G. stearothermophilus</i>	-	-
	5	430	<i>G. kaustophilus</i>	-	-

complicated procedures currently used in the quality control of raw materials and products.

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