Note

Development of a Quantitative PCR Assay for Thermophilic Spore-Forming *Geobacillus stearothermophilus* in Canned Food

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The thermophilic spore forming bacteria *Geobacillus stearothermophilus* is recognized as a major cause of spoilage in canned food. A quantitative real-time PCR assay was developed to specifically detect and quantify the species *G. stearothermophilus* in samples from canned food. The selected primer pairs amplified a 163-bp fragment of the 16S rRNA gene in a specific PCR assay with a detection limit of 12.5 fg of pure culture DNA, corresponding to DNA extracted from approximately 0.7 CFU/mL of *G. stearothermophilus*. Analysis showed that the bacterial species *G. stearothermophilus* was not detected in any canned food sample. Our approach presented here will be useful for tracking or quantifying species *G. stearothermophilus* in canned food and ingredients.

Key words : *Geobacillus stearothermophilus* / Flat sour spoilage / Real-time PCR / Spore-forming thermophilic bacteria.

The spoilage control and production safety in the canning industries are based on the simple principles: a minimal heat-treatment for low-acid foods ($F_0 = 3$ min, the “*botulinum* cook”) and an additional heat-treatment to guarantee microbiological stability. Microbial spoilage of canned food is often caused by thermophilic and highly heat-resistant spore-forming bacteria such as *Geobacillus stearothermophilus* in the food industry (Logan and De Vos, 2009; Burgess et al., 2010; Prevost et al., 2010). The canned food F_0 value observed in industry is strongly related to the heat resistance of the strains isolated. *G. stearothermophilus* has been regularly isolated from products treated at moderate or high heat levels (F_0 between 5 and 20 min and F_0 above 20 min) (Andre et al, 2013). It is speculated that spores in these products may come from the soil or may result from sporulation during the storage conditions and treatment of ingredients. In cooked food, a variety of ingredients are involved and may carry microbial contaminants (Membre and van Zuijlen, 2011). Despite a long history of description of these

bacteria, the origin of spores in canned food is poorly understood.

Developments in molecular technique enable faster and more sensitive analysis than classical microbiological procedures. Real-time PCR based identification and quantification is a suitable alternative because it is a comparatively easy, rapid and simple method (Postollec et al., 2011) and it can be completed within several hours. Real-time PCR techniques have been developed with a wide variety of microorganisms, with emphasis on the main food-borne pathogens responsible for substantial medical and economic problems. In the last two decades, culture-independent techniques have greatly improved our understanding of the composition, activities, and dynamics of microbial communities. Nowadays, PCR-based methods, in particular quantitative PCR, are predominantly used to detect, identify and quantify both pathogens and beneficial microbial species, such as fermenting microbes or probiotics (Le Drian et al., 2010, Malorny et al., 2008). ISO standards have also been established, and provide guidelines to quantitatively detect food-borne pathogens by PCR (ISO 22174: 2005, ISO/TS 20836: 2005, ISO 20837: 2006, ISO 20838:2006).

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PCR is a faster, more sensitive, and more specific method than traditional culture-based techniques, and enable detection of sub-dominant populations, even in the absence of a selective enrichment medium and in the presence of other dominant populations. Furthermore, combined with reverse transcription (RT), quantitative PCR can also estimate transcript amounts, therefore providing information on microbial gene activity (Postollec et al., 2011). In this study, we developed a real-time PCR assay for identification and quantification of *G. stearrowthermophilus*, and applied the direct detection of this species to the examination of samples from commercial canned food.

A total of 35 strains from 30 bacterial species,

including four *G. stearrowthermophilus* strains, and other close and distant genera, listed in Table 1, were used for primer specificity studies. *Geobacillus* group strains were incubated at 55–60 °C in Nutrient Broth (EIKEN CHEMICAL Co., LTD. Tokyo, Japan). Both aerobic and anaerobic other thermophilic strains were incubated at 55–60 °C according to the reference of growth information.

Genomic DNA was extracted using an Ultra clean DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions with minor modifications for the extraction of genomic DNA including spore DNA (Rose et al., 2011). Briefly, microbial cell pellets were collected from 1 ml of undiluted cell

TABLE 1. Microbial strains used in this study

Species	Strains and sources* ¹
<i>Geobacillus stearrowthermophilus</i>	NBRC 12550 ^T (ATCC 12980)
<i>Geobacillus stearrowthermophilus</i>	NBRC 12983 (ATCC 12976)
<i>Geobacillus stearrowthermophilus</i>	NBRC 13737 (ATCC 7953)
<i>Geobacillus stearrowthermophilus</i>	NBRC 100862 (DSM 1550)
<i>Geobacillus caldoolosilycus</i>	NBRC 107762 ^T (ATCC 700356)
<i>Geobacillus jurassicus</i>	NBRC 107829 ^T (DSM 15726)
<i>Geobacillus kaustophilus</i>	NBRC 102445 ^T (ATCC 8005)
<i>Geobacillus thermoglucosidasius</i>	NBRC 107763 ^T (ATCC 43742)
<i>Geobacillus toebii</i>	NBRC 107807 ^T (DSM 14590)
<i>Geobacillus zhalhae</i>	NBRC 101842 ^T (DSM 18318)
<i>Bacillus subtilis</i>	NBRC 13719 ^T (ATCC 6051)
<i>Bacillus coagulans</i>	ATCC 80078
<i>Bacillus licheniformis</i>	NBRC 12200 ^T (ATCC 14580)
<i>Paenibacillus polymyxa</i>	NBRC 15309 ^T (ATCC 842)
<i>Staphylococcus aureus</i>	NBRC 100910 ^T (ATCC 12600)
<i>Escherichia coli</i>	NBRC 102203 ^T (ATCC 11775)
<i>Clostridium acetobutylicum</i>	NBRC 13948 ^T (ATCC 824)
<i>Clostridium clariflavum</i>	NBRC 101661 ^T (DSM 19732)
<i>Clostridium kluyveri</i>	NBRC 12016 ^T (DSM 555)
<i>Clostridium thermocellum</i>	NBRC 103400 ^T (ATCC 27405)
<i>Moorella thermoacetica</i>	JCM 9319 ^T (ATCC 35608)
<i>Moorella thermoacetica</i>	JCM 9320 ^T (ATCC 39073)
<i>Moorella thermoacetica</i>	24-1 (our collection)
<i>Moorella glycerini</i>	DSM 11254 ^T
<i>Moorella humiferrea</i>	DSM 23265 ^T
<i>Moorella mulderi</i>	DSM 14980 ^T
<i>Moorella stamsii</i>	DSM 26217 ^T
<i>Moorella thermoautotrophica</i>	DSM 1974 ^T
<i>Moorella perchloratireducens</i>	ATCC BAA-1531
<i>Ammonifex</i> sp.	NBRC 100904
<i>Caldanaerobacter sybterraneus</i> subsp. <i>tengcongensis</i>	NBRC 100824 ^T
<i>Carboxydotherrmus pertinax</i>	NBRC 107576 ^T (DSM 23698)
<i>Tepidanaerobacter syntrophicus</i>	NBRC 100060 ^T (DSM 15584)
<i>Thermaneromonas toyohensis</i>	NBRC 101528 ^T (DSM 14490)
<i>Thermanaerobacter cellulolyticus</i>	NBRC 14436

*¹ NBRC, NITE Biological Resource Center (Kisarazu, Chiba, Japan); T, type strain; references in parentheses indicate the corresponding reference number in an alternative collection, ATCC, American Type Culture Collection (Manassas, VA, USA), DSM, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

suspension and then resuspended in 300 μ L of Microbead solution (without beads) and 50 μ L of MD1 solution. Suspensions were heated at 95 $^{\circ}$ C for 20 min and then centrifuged at $10,000 \times g$ for 2 min. Three hundred microliters of the supernatant were added to 100 μ L of MD2 solution, and then tubes were inverted several times, incubated at 4 $^{\circ}$ C for 10 min, and centrifuged at $10,000 \times g$ for 2 min. Three hundred microliters of the supernatant were then added to 900 μ L of MD3 solution. Two 600 μ L aliquots of supernatant/MD3 solution were sequentially added to the spin column and centrifuged at $10,000 \times g$ for 30 s. Each flow-through was discarded. Three hundred microliters of solution MD4 were then added to the spin column, centrifuged as above, and the flow-through was discarded. One hundred microliters of solution MD5 were added to the spin column, which was then centrifuged at $10,000 \times g$ for 1 min and the flow-through was retained for PCR and real-time PCR assays. For all other reference strains, pure cultures were grown, and bacterial DNA was extracted using an Ultra clean DNA isolation kit following the manufacturer's instructions (MO BIO). Finally, genomic DNA concentrations were determined using a spectrophotometer (μ Quant, BioTek Instruments, Inc., USA) and diluted to the appropriate concentration prior to use.

The 16S rRNA gene of *G. stearothermophilus* NBRC 12550^T was chosen as the target for amplification. Overall, sequences from 120 bacterial species including *Geobacillus* species, species belonging to a different genus from *Bacillaceae* such as *Bacillus*, *Listeria*, *Paenibacillus*, *Sporolactobacillus*, others belonging to *Clostridium*, and non-related taxa were obtained from the GenBank nucleotide database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/genbank/>), and from the National Institute of Technology and Evaluation Biological Resource Center (<http://www.nbrc.nite.go.jp/e/>). Alignment of the 16S rRNA gene sequence from *G. stearothermophilus* and other related and nonrelated taxa was performed with ClustalX (Thompson et al., 1997). Based on the alignment, four primer pairs were selected using Primer3Plus (<http://www.bioinformatics.nl/>

FIG. 1. (a) Amplification curve profiles. (b) Melting curve profiles. (c) Gel images of electrophoresis of 10-fold serial diluted standard curves of *Geobacillus stearothermophilus* NBRC 12550^T from real-time PCR.

Supporting information Fig.1. Image of primer positions in the nucleotide sequence of the 16S rRNA gene of *Geobacillus stearothermophilus* strain NBRC 12550^T

TABLE 2. DNA sequence of primers used in this study

Primer name	Primer sequence (5'-3') ^{*1} Forward	T _m (°C)	Primer name	Primer sequence (5'-3') ^{*1} Reverse	T _m (°C)
Gv 1F	GATTGGGGCTTGCCCTGA	66.1	Gv3R	GCAAGTGACAGCCCAAAGG	65.8
			Gv5R	GGCTTTCACATCAGACTTAAGAGACC	66
			Gv6R	TCAGGTGCAGGCCAGAGA	66.2
			Gv8R	ACCGAATGCTGGCAACTAGAG	65.3

^{*1} Primer design was carried out using Primer 3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>).

cgi-bin/primer3plus/primer3plus.cgi; Untergasser et al., 2007). Primer sequences are listed in Table 2. Primer positions in the *G. stearothersophilus* 16S rRNA gene region are illustrated in Supporting Information Fig. 1. The overall theoretic specificities of the newly designed primers were checked using BLAST search.

Quantitative PCR was performed using SYBR Green chemistry. The reactions were carried out in a 25 μ L volume containing 12.5 μ L of 2 $\times$ SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio, Shiga, Japan), 0.2 μ M of each primer, 1 μ L of microbial genomic DNA template, and 5 to 10 μ L of DNA samples from canned food samples, in a Thermal Cycler Dice Real Time System TP800 (Takara Bio). The optimum cycling parameters were: 95°C for 30 s, followed by 36 cycles of 95°C for 10 s, 62°C for 30 s, and 72°C for 1 min. A melting curve was generated after the last amplification cycle using a temperature range of 64–95°C, and a temperature transition rate of 0.5°C. The cycle threshold (C_t) and the melting temperature of amplification products were calculated automatically. The amplicon obtained from real-time PCR analysis was confirmed as a single DNA band that corresponded to the expected product size. Negative controls and all non-*Geobacillus* strains showed no peaks in the melting profiles. The absence of nonspecific products and primer dimers was confirmed by agarose gel electrophoresis.

Among the designed four primer sets, Gv1F (5'-GATTGGGGCTTGCCTTGA-3') and Gv3R (5'-GCAAGTGACAGCCCAAAGG-3') were successfully used for species-specific detection of *G. stearothersophilus* by real-time PCR assay. Using 50 pg of template DNA from non-*G. stearothersophilus* strain (Table 1), no C_t reading was obtained within the 36 amplification cycles for any of the isolates (data not shown). The amplicon obtained from real-time PCR analysis was confirmed as a single band that corresponded to the expected product size (Fig. 2). Negative controls and all non-*G. stearothersophilus* strains also showed no peaks in the melting profiles. The reproducible distinct melting points (T_m) was 87.3 °C (Fig. 2). The other primer pairs Gv1F/Gv5R and Gv1F/Gv6R demonstrated poorer results than Gv1F/Gv3R in terms of productivity. Consequently, the primer pair Gv1F/Gv3R was used for the specific detection and quantification of this species in this study.

The sensitivity and efficiency of the real-time PCR assays was determined using purified genomic DNA from the representative *G. stearothersophilus* NBRC 12550^T strain. Genomic DNA was extracted in triplicate from undiluted cell suspension samples (OD_{600} = 0.2–0.3) and the DNA concentration quantified. As a result, three individual suspensions of *G. stearothersophilus* with optical densities of 0.254, 0.229, and

0.234 at 600 nm were prepared, and the corresponding cell counts were respectively determined to be 3.8×10^7 CFU/mL, 2.6×10^7 CFU/mL, and 7.1×10^7 CFU/mL.

FIG. 2. (A) Standard curve (●) obtained by plotting log CFU/reaction against the C_t obtained from real-time PCR analysis of serially diluted solutions of DNA extracted from *G. stearothersophilus* NBRC 12550^T. The linear regression equation was $y = -1.48 (\pm 0.1) \ln(x) + 33.356 (\pm 0.302)$ with a coefficient of determination of $R^2 = 0.9954-0.9989$. PCR efficiencies were 96.1% to 127.9%. Additionally shown are spiking experiment curves used to evaluate the efficiency of this method for detection of *G. stearothersophilus* from samples containing non-target species. (B) Spiking experiment curves from canned whole corn. The linear regression equation was $y = -1.309 (\pm 0.057) \ln(x) + 34.176 (\pm 0.505)$ with a coefficient of determination of $R^2 = 0.9863-0.9917$. (C) Spiking experiment curves from boiled azuki-beans. The linear regression equation was $y = -0.893 (\pm 0.011) \ln(x) + 28.85 (\pm 0.049)$ with a coefficient of determination of $R^2 = 0.9879-0.9923$. The symbols (▲), (◆), and (■) indicated spiked 1, spiked 2, and spiked 3, respectively.

based on the plate counting of colonies. The genomic DNA concentrations of these individual samples were 14.5 ng/ μ L, 9.5 ng/ μ L, and 12.5 ng/ μ L, respectively. Consequently, a 10-fold serial dilution series with concentrations ranging from 12.5 ng to 1.25 fg was prepared using sterile distilled water. Standard curves using *G. stearothermophilus* were produced by plotting the C_t values from three replicate PCR assays against the bacterial count (Fig. 2A).

For the extraction of DNA from commercial canned food for detecting the species of *G. stearothermophilus*, sample preparation was conducted as follows. Five kinds of samples that included two different brands of canned whole corn, boiled-beans (azuki bean, *Vigna angularis*), boiled mushrooms, and boiled-shellfish (Manila clam, *Ruditapes philippinarum*) were used. In the first step, individual canned samples were treated by sonication for 5 min. Then, microbial pellets were collected from the submerged liquid samples (approximately 25 to 40 ml) by centrifugation at $7,800 \times g$ for 40 min at 4 °C, and the pellets were washed three times with phosphate buffered saline (PBS, pH 7.4). Then, microbial DNA was extracted according to the manufacturer's instructions, with minor modifications mentioned above.

Conventional PCR analysis was also carried out using Emerald Amp PCR Master Mix (Takara Bio) in an S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Thermal cycling conditions were as follows: 95°C for 4 min, 30 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 1.5 min, followed by 72°C for 7 min. Amplification products were separated on 1.5% (w/v) agarose gels and stained with ethidium bromide. For all of the primer pairs, the minimum detection sensitivity was 125 fg, which is equivalent to approximately 7.1 CFU/mL of *G. stearothermophilus*. Meanwhile, primer pairs Gv1F-Gv5R, and Gv1F-Gv8R amplified a secondary weak product in addition to the single amplicon of the expected size. Therefore, Gv1F-Gv3R and Gv1F-Gv6R were suitable primer sets for the conventional PCR assay.

In the spiking experiments, the quantitative PCR assay was tested for its ability to detect *G. stearothermophilus* in canned food containing a diversity of non-pathogenic microorganisms. The microbial pellets were prepared from liquid samples of canned whole corn and boiled-beans (azuki beans, *Vigna angularis*), respectively. 1 ml of *G. stearothermophilus* microbial cell suspension ($OD_{600} = 0.291$) was serially diluted and submerged into *Geobacillus*-free microbial pellets derived from canned food samples (whole corn and boiled azuki-beans). These pellets were prepared to confirm the negative results in the real-time PCR assay with a 40-cycle amplification. Microbial DNA was then

extracted from the pellets as described above. Then, genomic DNA extracted from the spiked samples was subjected to real-time PCR, and the C_t values were plotted (Fig. 2B and 2C). The results for whole corn samples were $y = -1.309 (\pm 0.057) \ln(x) + 34.18 (\pm 0.505)$ and $R^2 = 0.9863$ to 0.9917 , and for boiled azuki-beans were $y = -0.893 (\pm 0.011) \ln(x) + 28.85 (\pm 0.049)$, and $R^2 = 0.9879$ to 0.9923 .

The results revealed C_t values ranging from 10^1 to 10^6 CFU/mL, and each curve was highly linear. The statistical analyses revealed no significance differences ($P > 0.05$) among the slopes, intercepts, and R^2 values between the spiked results from whole corn and the reference *G. stearothermophilus* strain (Fig. 2B). Meanwhile, there were significant differences ($P < 0.05$) among slopes, intercepts between the spiked results from boiled-beans and the reference *G. stearothermophilus* strain (Fig. 2C). We concluded that amplification efficiency and target gene detection were not affected by the presence of non-target microbial DNA, except for when target DNA was only present in low concentrations. Furthermore, this method may be affected by the genomic DNA extraction efficiency when only a small amount of target DNA was present together with non-target DNA, and different types of food samples were assayed.

To identify *G. stearothermophilus* from canned food, samples were prepared as described above, and subjected to the real-time PCR assay by using the primer pair Gv1F-Gv3R. The C_t values obtained from the assay were used to calculate the CFU/ml of *G. stearothermophilus* in each sample on the standard curve (Fig. 2A). As for the detection limit by using the primer pair Gv1F-Gv3R, the assay theoretically allowed the estimation of numbers of microorganisms to 0.65 CFU/mL. Quantitative real-time PCR results showed negative results for *G. stearothermophilus* in all samples. Meanwhile, a conventional PCR assay and a direct incubation produced negative results for all samples. It was suggested these organisms 1) may not be major inhabitants of phyllosphere environments of vegetable tissues and/or marine foods, or 2) may be inactive due to treatments during the processing of canned products.

In addition, in a one-step incubation, the pellets from canned food submerged samples were incubated at 55 °C for 10 days, and one hundred microliter samples were spread onto nutrient agar plates and incubated at 55 °C for 3 days. The colony formation was observed (at least $>10^3$ CFU/ml) for boiled-beans. Two colonies were picked up randomly and subjected to genomic DNA extraction and sequence determination for the identification of microorganisms. Based on the phylogenetic analysis of 16S rRNA sequences, these strains

were found to be identical to that of *G. stearothermophilus* at 97 % and 95 %, respectively. It was suggested these spores may have been germinated through incubation at 55 °C. The discrepancy in the results between real-time PCR and incubation analyses from boiled-bean samples must to be explored in further analyses. Taken together, it suggests that measures such as additional heat treatment and the risk assessment of the storage environments might be needed to ensure the adequate handling of canned food.

Andre et al. (2013) investigated the bacterial species responsible for non-stability after prolonged incubation of low acid canned food at 55 °C by examining 455 samples collected from 122 French canneries over 10 years. These results showed three genera were responsible for more than 80 % of all non-stability cases: mostly *Moorella* (36 %) and *Geobacillus* (35 %), and less frequently *Thermoanaerobacterium* (10 %). Among thermophilic bacteria, *G. stearothermophilus* is recognized as a major cause of spoilage in canned foods and is frequently detected after a 7-day incubation at 55°C (Andre et al., 2013). Recently, Prevost et al. (2010) reported that thermophilic bacteria including *G. stearothermophilus*, *M. thermoacetica*/*M. thermoautotrophica*, and *Thermoanaerobacterium* group were detected in canned food spoilage via cultivation methods. Thermophilic bacteria spores were counted in 13 ingredients such as spices, milk powder and aromas (Prevost et al., 2010). In the past, several studies pointed out that thermophilic bacteria spore contamination occasionally occurred, particular, in milk powder produced by a heating process (Cooper et al., 2006, Ruckert et al., 2004). The ingredients may be the entrance point for highly heat resistant bacteria in food (Oomes et al., 2007). Thus, spore contamination in canned food may be related to the inclusion of various kinds of ingredients susceptible to microbial contamination. In addition, spore contamination probably comes from the soil or may result from sporulation during food processing. Hence, control of the large diversity of spore-forming microorganisms in the food environment and adjustment of food retort settings should be based on data collection about spore contamination levels in ingredients and raw materials. In order to optimize sterilization processes, the heat resistance of contaminating spores must be considered.

In this study, a highly specific real-time PCR assay was established to detect and quantify *G. stearothermophilus* in canned food. This method is highly reliable, rapid and can be directly performed within several hours. The current approach could be useful for tracking and monitoring bacterial contaminants in various kinds of food and/or food ingredients. Screening for the spoilage-causing microorganisms in raw mate-

rials, ingredients and samples from processing lines could help identify points or sites of contamination in canned food industry for improve safety management and hygiene control.

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