



HAL
open science

PCR Detection of Thermophilic Spore-Forming Bacteria Involved in Canned Food Spoilage

Sylviane Prevost, Stéphane André, Fabienne Remize

► **To cite this version:**

Sylviane Prevost, Stéphane André, Fabienne Remize. PCR Detection of Thermophilic Spore-Forming Bacteria Involved in Canned Food Spoilage. *Current Microbiology*, 2010, 61 (6), pp.525–533. 10.1007/s00284-010-9648-8 . hal-01201607

HAL Id: hal-01201607

<https://hal.univ-reunion.fr/hal-01201607>

Submitted on 17 Apr 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

PCR Detection of Thermophilic Spore-Forming Bacteria Involved in Canned Food Spoilage

S. Prevost, S. Andre, F. Remize

Abstract Thermophilic bacteria that form highly heat-resistant spores constitute an important group of spoilage bacteria of low-acid canned food. A PCR assay was developed in order to rapidly trace these bacteria. Three PCR primer pairs were designed from rRNA gene sequences. These primers were evaluated for the specificity and the sensitivity of detection. Two primer pairs allowed detection at the species level of *Geobacillus stearothermophilus* and *Moorella thermoacetica/thermoautrophica*. The other pair allowed group-specific detection of anaerobic thermophilic bacteria of the genera *Thermoanaerobacterium*, *Thermoanaerobacter*, *Caldanerobium* and *Caldanaerobacter*. After a single enrichment step, these PCR assays allowed the detection of 28 thermophiles from 34 cans of spoiled low-acid food. In addition, 13 ingredients were screened for the presence of these bacteria. This PCR assay serves as a detection method for strains able to spoil low-acid canned food treated at 55°C. It will lead to better reactivity in the canning industry. Raw materials and ingredients might be qualified not only for quantitative spore contamination, but also for qualitative contamination by highly heat-resistant spores.

Introduction

The thermal process applied during canning results in products, which remain stable for long periods at ambient temperature. The minimum levels of heat treatment were

determined on safety concerns to prevent the development of *Clostridium botulinum* [10]. However, this thermal treatment is not always sufficient to inactivate all spore-forming bacteria, especially those that are highly heat-resistant and non-pathogenic [13].

Spore-forming thermophilic bacteria are recognized as the main cause of spoilage of canned food [3, 8, 18]. The typical species of thermophilic bacteria found in canned products are *Thermoanaerobacterium thermosaccharolyticum* and *Moorella thermoacetica* for obligate anaerobes, plus *Geobacillus stearothermophilus* for facultative anaerobes [2, 5, 9]. *G. stearothermophilus* is the typical species responsible of “flat sour” spoilage, due to the production of acids but not gas from saccharides [7]. Thermophilic anaerobes usually produce abundant gas, which result in swelling of cans. *M. thermoacetica* produces acetate as main fermentation product, and as such is termed acetogenic and used for biotechnological applications [7, 14]. These bacteria share the characteristic of being able to form highly heat-resistant spores [2, 4, 11]. The decimal reduction times is well established for *G. stearothermophilus* to a few min at 121°C [20, 26]. The spores of *M. thermoacetica* strains, which survive autoclaving, have decimal reduction times between 23 and 111 min at 121°C depending on sporulation conditions [4].

Thermal process setting requires taking into account spore heat resistance and population level, together with their ability to grow under ambient storage conditions [13, 17, 19].

Therefore, not only the number of heat-resistant spores but also the nature of bacteria is an important parameter regarding the potentially high heat resistance of endospores. In addition, identification of the bacteria, which cause canned food spoilage, can implement corrective and preventive action, as the thermal resistance of the spores

S. Prevost · S. Andre · F. Remize (✉)
CTCPA Centre Technique de la Conservation des Produits
Agricoles, Site Agroparc, ZA de l'aéroport, BP 21 203,
84911 AVIGNON cedex 9, France
e-mail: fremize@ctcpa.org

and the conditions of growth and sporulation are known. These elements are then used in the processing lines, e.g. to control the thermal treatment applied, or to reduce the level of spore contamination at different steps of the process before heat treatment, through enhancement of hygiene procedures.

Nowadays, identification is performed by 16S rRNA gene sequencing, a method that requires multiple culture steps to achieve isolation and therefore is time-consuming and sometimes unsuccessful if the bacteria fail to grow. The improvement of industrial reactivity requires the design of molecular methods for fast detection of contaminants. In case of spoiled canned food, molecular tools need to detect thermophilic spore-forming bacteria. Specific primers that amplify 16S rRNA gene and 16S–23S rRNA gene internal transcribed spacer (ITS) have been widely used as a target, and have given promising results [16, 21, 27]. In this study, three PCR primer pairs were developed from publicly available sequences to detect the most frequent spoilage thermophilic spore formers: *M. thermoacetica*, *G. stearothermophilus*, *Thermoanaerobacterium* spp. The specificity and the sensitivity of the primer sets were evaluated on DNA extracted from thermophilic bacteria obtained from collections and isolated from canned food samples. In addition, the potential of the PCR assay was assessed with non-stable canned food and ingredients.

Materials and Methods

Bacterial Strains and Culture Conditions

The strains used for specificity assays are shown in Tables 1 and 2. Types and reference strains were obtained from various culture collections and maintained in the laboratory. Other strains were collected and isolated from non-stable canned food and were identified by 16S rRNA gene sequencing.

Aerobic bacteria were grown in brain–heart infusion (BHI) broth or in BCP glucose agar (dextrose tryptone agar), whereas anaerobic bacteria were grown in Rosenow Cysteine broth complemented with brain fragments and marble chips or in meat-liver glucose agar complemented with 2% yeast extract.

Thermophilic strains were incubated at 55°C. *Geobacillus* and *Thermoanaerobacterium* cultures were incubated for 2 days, while *Moorella* cultures were incubated for 5 days.

Other bacteria were incubated at 37°C for 48 h.

PCR Primers Design

Target sequences were selected from the National Centre for Biotechnology Information database. The sequences of

the 16S rRNA gene and ITS 16S–23S regions were aligned in order to search for specific primer sites. A pair of oligonucleotides for each bacterial group was selected and synthesized by Eurogentec. The sequences and positions of oligonucleotides are presented in Table 3.

DNA Preparation and PCR Optimization

DNA was prepared from picked colonies or from 1 ml liquid culture with the InstaGene™ (Bio-Rad, Marnes-la-Coquette, France) matrix following manufacturer's instructions.

All amplification steps were performed in 25 µl tubes with an Applied Biosystem GeneAmp PCR system 9700. The reactions were performed with 5 µl of DNA matrix, 0.2 mM dNTPs, 1× *Taq* reaction buffer, 2 units of *Taq* polymerase AmpliTaq (Applied Biosystem, Courtaboeuf, France), 0.3 µM of each primer and 1.5–3 mM MgCl₂. The PCR was cycled once at 94°C for 5 min, 40 repetitions at 94°C for 1 min, 65°C as indicated for 1 min, 72°C for 1 min, and once at 72°C for 10 min. On completion of cycling, amplicons were directly analysed by electrophoresis in TAE 1% agarose gel.

The specificity was optimized by adjusting annealing temperature and magnesium chloride concentration.

16S rRNA gene amplification was performed with primers FD1 and RD1 [29]. Sequencing was carried out by Eurofins MWG Operon (Ebersberg, Germany).

Sensitivity Assays

The sensitivity was determined for each of the three PCR primer pairs with DNA from different strains. The level and purity of the purified DNA were evaluated by measuring OD 260 and 280 nm and confirmed by amplification with universal primers. The level of DNA was adjusted to 1 µg for 5 µl and serially diluted in triplicate in ultrapure water. Each DNA extract corresponding to one dilution level was tested for PCR amplification in duplicate, so that at least six PCR tests were obtained for each concentration. The lowest amount of DNA with a positive amplification in most tests was chosen as the sensitivity limit.

Examination of Canned Food

Non-stable canned products were detected after an incubation of 7 days at 55°C according to NF V08-408 [1]. Samples of 25 or 50 g of non-stable products were diluted twofold in peptone water and homogenized with a stomacher for 1 min. The stomacher bag filtrate was used to inoculate broth, which was then incubated at 55°C for 2–5 days. Identification was performed from positive

Table 1 List of strains used for *G. stearothermophilus* detection specificity tests

| Species | Bacterial strains | No of strains | Source | PCR Gbs ^a |
|--|--|---------------|---------------|----------------------|
| <i>G. stearothermophilus</i> | DSM 1550, 2804 001, 2804 017, 2804 019, 2804 030, 2804 033, 2804 037, 2804 038, 2804 039, 2804 052, 2804 053, 2804 054, 2804 055, 2804 056, 2804 057, 2804 116, 2804 129, 2804 144, 2804 145 | 19 | DSMZ CTCPA | + |
| <i>Geobacillus kaustophilus</i> | DSM 7263 | 1 | DSMZ | – |
| <i>Geobacillus subterraneus</i> | DSM 13 552 | 1 | DSMZ | – |
| <i>Geobacillus thermocatenulatus</i> | DSM 730 | 1 | DSMZ | – |
| <i>Geobacillus thermodenitrificans</i> | DSM 465 | 1 | DSMZ | – |
| <i>Geobacillus thermoglucosidasius</i> | DSM 2 542 | 1 | DSMZ | – |
| <i>Geobacillus thermoleovorans</i> | DSM 5 366 | 1 | DSMZ | – |
| <i>Geobacillus uzenensis</i> | DSM 13 551 | 1 | DSMZ | – |
| <i>Geobacillus caldxylosilyticus</i> | 2801 003 | 1 | CTCPA | – |
| <i>Geobacillus</i> sp | 2810 004 | 1 | CTCPA | – |
| <i>Bacillus amyloliquefaciens</i> | 3101 006 | 1 | CTCPA | – |
| <i>Bacillus cereus</i> | ATCC 14579 | 1 | ATCC | – |
| <i>Bacillus circulans</i> | 3104 002 | 1 | CTCPA | – |
| <i>Bacillus coagulans</i> | 3105 003, 3105 034 | 2 | CTCPA | – |
| <i>Bacillus licheniformis</i> | 3107 028 | 1 | CTCPA | – |
| <i>Bacillus smithii</i> | 3108 002, 3108 004, 3108 017 | 3 | CTCPA | – |
| <i>Bacillus subtilis</i> | 3111 032, 3111 033, 3111 035, 3111 038 | 4 | CTCPA | – |
| <i>Paenibacillus graminis</i> | 2910 001 | 1 | CTCPA | – |
| <i>Paenibacillus macerans</i> | 2903 006, 2903 009, 2903 012, 2903 014 | 4 | CTCPA | – |
| <i>Paenibacillus turicensis</i> | 2907 001 | 1 | CTCPA | – |
| <i>Lactobacillus sakei</i> | 3009 005 | 1 | CTCPA | – |
| <i>Enterococcus faecalis</i> | ATCC 29 212 | 1 | ATCC | – |
| <i>Escherichia coli</i> | DSM 30 083 | 1 | DSMZ | – |
| <i>Listeria monocytogenes</i> | ATCC 19 115 | 1 | ATCC | – |
| <i>M. thermoacetica</i> | DSM 521 | 1 | DSMZ | – |
| <i>M. thermoautotrophica</i> | DSM 1974 | 1 | DSMZ | – |
| <i>M. thermoacetica/thermoautotrophica</i> | 1901 056, 1901 057, 1901 058, 1901 059, 1901 060 | 5 | CTCPA | – |
| <i>Thermoanaerobacterium thermosaccharolyticum</i> | 2506 006, 3224 001 | 2 | CTCPA | – |
| <i>Clostridium novyi</i> | 3210 014, 3210 017, 3213 011 | 3 | CTCPA | – |
| <i>Clostridium sporogenes</i> | DSM 1734, 3213 018, 3213 020, 3222 004 | 4 | DSMZ CTCPA | – |
| <i>Clostridium thermopalmarium/thermobutyricum</i> | 3219 001, 3226 006 | 2 | CTCPA | – |

^a To detect *G. stearothermophilus*

culture broth tubes, preferentially anaerobic tubes if available. It was further confirmed by 16S rRNA gene sequencing of DNA extracted from isolated colonies obtained from the same broth tube by plating onto the adequate medium.

Examination of Ingredients

Samples of 25 g of ingredients were diluted 10-fold in peptone water and homogenized with a stomacher for

Table 2 List of strains used for *Moorella* and *Thermoanaerobacterium* detection specificity tests

| Species | Bacterial strains | No. of strains | Source | PCR Moo ^a | PCR Thm ^b |
|--|--|----------------|--------|----------------------|----------------------|
| <i>M. thermoacetica</i> | DSM 521 | 1 | DSMZ | + | – |
| <i>M. thermoautotrophica</i> | DSM 1974 | 1 | DSMZ | + | – |
| <i>M. thermoacetica/thermoautotrophica</i> | 1901 043, 1901 056, 1901 057, 1901 058, 1901 059, 1902 051 | 6 | CTCPA | + | – |
| <i>M. thermoacetica/thermoautotrophica</i> | 1901 003, 1901 066, 1901 067, 1901 071, 1901 044 | 5 | CTCPA | + | nd |
| <i>M. thermoacetica/thermoautotrophica</i> | 1901 060, 1902 006 | 2 | CTCPA | + | + |
| <i>Moorella glycerini</i> | DSM 11 254 | 1 | DSMZ | – | nd |
| <i>Moorella mulderi</i> | DSM 14 980 | 1 | DSMZ | – | nd |
| <i>Thermoanaerobacter mathranii/thermocopriae</i> | 2105 001, 3220 003, 2503 001 | 3 | CTCPA | – | + |
| <i>Thermoanaerobacterium aciditolerans/aotearoense</i> | 2502 001, 2503 020, 2503 018, 2503 008, 2501 001 | 5 | CTCPA | – | + |
| <i>T. aciditolerans</i> | DSM 16 487 | 1 | DSMZ | + | + |
| <i>T. aciditolerans/aotearoense</i> | 2503 014, 2503 019 | 2 | CTCPA | + | + |
| <i>Thermoanaerobacterium saccharolyticum</i> | 2505 001, 2506 007, 2503 012 | 3 | CTCPA | – | + |
| <i>Thermoanaerobacterium</i> sp | 2503 009 | 1 | CTCPA | – | + |
| <i>Thermoanaerobacterium thermosaccharolyticum</i> | DSM 571 | 1 | DSMZ | – | + |
| <i>T. thermosaccharolyticum</i> | 2506 006, 3224 001, 2503 010 | 3 | CTCPA | – | + |
| <i>T. thermosaccharolyticum</i> | 2503 006, 2503 015, 2506 011 | 3 | CTCPA | + | + |
| <i>Thermoanaerobacterium zeeae</i> | 2507 001 | 1 | CTCPA | – | + |
| <i>Thermoanaerobacterium</i> sp | 2506 012 | 1 | CTCPA | – | – |
| <i>Thermoanaerobacterium saccharolyticum</i> | DSM 7060 | 1 | DSMZ | – | – |
| <i>Caldanaerobium fjensis</i> | 2503 013 | 1 | CTCPA | – | + |
| <i>Clostridium bifermentans</i> | DSM 630 | 1 | DSMZ | – | – |
| <i>Clostridium cochlearium</i> | 3206 002 | 1 | CTCPA | – | – |
| <i>Clostridium novyi</i> | 3210 014, 3210 017, 3210 011 | 3 | CTCPA | – | – |
| <i>Clostridium sporogenes</i> | 3213 018, 3213 020, 3222 004, DSM 1734 | 4 | DSMZ | – | – |
| <i>Clostridium thermobutyricum/thermopalmarium</i> | 3219 001, 3226 006 | 2 | CTCPA | – | – |
| <i>Clostridium thermobutyricum/thermopalmarium</i> | 3226 004, 3226 005 | 2 | CTCPA | nd | – |
| <i>Clostridium thermopalmarium</i> | DSM 2544 | 1 | DSMZ | nd | – |
| <i>Bacillus amyloliquefaciens</i> | 3110 006 | 1 | CTCPA | – | – |
| <i>Bacillus coagulans</i> | 3105 034 | 1 | CTCPA | – | – |
| <i>Bacillus coagulans</i> | 3105 003 | 1 | CTCPA | – | nd |
| <i>Bacillus coagulans</i> | DSM1 | 1 | DSMZ | nd | – |
| <i>Bacillus licheniformis</i> | 3107 028 | 1 | CTCPA | – | – |
| <i>Bacillus licheniformis</i> | 3107 001, 3107 014 | 2 | CTCPA | – | nd |
| <i>Bacillus smithii</i> | 3108 017 | 1 | CTCPA | – | – |
| <i>Bacillus subtilis</i> | 3111 032, 3111 033, 3111 038 | 3 | CTCPA | – | – |
| <i>G. stearothermophilus</i> | 2804 144, 2804 145 | 2 | CTCPA | – | – |
| <i>Paenibacillus macerans</i> | 2903 006, 2903 014 | 2 | CTCPA | – | – |
| <i>Lactobacillus sakei</i> | 3009 005 | 1 | CTCPA | – | – |

^a To detect *M. thermoacetica*; ^b To detect the *Thermoanaerobacterium* group; nd not determined

1 min. The obtained stomacher bag filtrate was treated for 10 min at 100°C to eliminate vegetative cells and then used for serial dilutions in peptone salt broth. For CFU

determination, the dilutions were poured into two media. BCP glucose agar was used to determine aerobic thermophilic counts and was incubated for 2 days at 55°C. Meat-

Table 3 Primers used in this study

| Reaction | Region | Name | Sequence |
|--------------------------|------------------|--------|----------------------------|
| PCR <i>Moorella</i> F | rRNA 16S | FD1 | AGAGTTTGATCCTGGCTCAG |
| PCR <i>Moorella</i> R | rRNA 16S | R3 | AAAGGCTATTCGCCTTTAAGAC |
| PCR <i>Thm</i> F | rRNA 16S | 772F2 | TGGCGAAAGCGGCTCTCTGG |
| PCR <i>Thm</i> R | rRNA 16S | 1239R | CCCCACCTTCCTCCGTG |
| PCR <i>Geobacillus</i> F | rRNA ITS 16S–23S | Fits2 | GGGGAAGCGCCGCGTTCGG |
| PCR <i>Geobacillus</i> R | rRNA ITS 16S–23S | Rits2 | GTGCAAGCACCCCTTGCAGGCGAAGA |
| Universal PCR F | rRNA 16S | FD1 | AGAGTTTGATCCTGGCTCAG |
| Universal PCR R | rRNA 16S | RD1 | GGMTACCTTGTACGAYTTC |
| Sequencing R | rRNA 16S | S6-16S | GTATTACCGCGCTGCTG |

liver glucose agar was used to determine anaerobic thermophilic counts and was incubated for 5 days at 55°C. For PCR assay, up to 10 colonies if available were randomly picked prior to DNA isolation.

Results

PCR Specificity for *G. stearothermophilus*

A PCR assay was developed from available sequence data to detect the aerobic thermophile *G. stearothermophilus*. A specific primer pair was designed in the rRNA internal transcribed sequence (ITS) 16S–23S (Table 3). The assay optimization resulted in the choice of an annealing temperature of 65°C with 1.5 mM MgCl₂.

The specific amplification of a 302-bp fragment was obtained for 19 *G. stearothermophilus* isolates, whereas no amplicon was obtained from nine strains belonging to other *Geobacillus* species (Table 1). Forty-one other non-*Geobacillus* strains were not detected by the assay. Among them, the most closely related species were the thermophilic species *Bacillus coagulans*, *Bacillus licheniformis* and *Bacillus smithii* belonging to *Bacillaceae* family.

PCR Specificity for the Detection of *M. thermoacetical thermoautotrophica* and *Thermoanaerobacterium* Group

The PCR assay developed to detect *M. thermoacetical thermoautotrophica* detection triggered the 16S rRNA gene region (Table 3). From a genetic point of view, *M. thermoacetica* and *M. thermoautotrophica* present differences in 16S rRNA gene sequences of less than 1% [5]. The forward primer FD1 was described by Weisburg et al. [29] for use as a universal 16S rRNA coding region amplification primer. A reverse primer R3 was designed by sequence alignments. The use of these primers allowed the

amplification of a single 488-bp fragment at an annealing temperature of 65°C with 3 mM MgCl₂.

PCR specificity was assayed against 15 *M. thermoacetica/thermoautotrophica* isolates, 1 *Moorella glycerini* and 1 *Moorella mulderi* (Table 2). All *M. thermoacetica/thermoautotrophica* strains were detected, while other species of the same genus were not. Fifty-one other isolates were used for exclusivity assays. Among them, anaerobic thermophilic bacteria accounted for 33 of the isolates. Unexpectedly, three *Thermoanaerobacterium aciditolerans/aotearoense* and three *T. thermosaccharolyticum* strains generated positive signals. Sequencing of the 16S rRNA coding region of these strains did not show higher similarity with the R3 primer than did DNA from other *Thermoanaerobacterium* strains like DSM 7060, which did not generate positive signals with the *M. thermoacetical thermoautotrophica* detection assay.

Primers were designed in the 16S rRNA gene region by alignment from all *Thermoanaerobacterium* sequences available (Table 3). A maximal score was obtained for DNA regions from the following genera: *Thermoanaerobacter*, *Thermoanaerobacterium*, *Caldanaerobium* and *Caldanaerobacter*. These primers were also checked against other spore-forming bacteria sequences. PCR optimization was performed with cycle conditions identical to the PCR runs for *M. thermoacetical thermoautotrophica* and *G. stearothermophilus*. It showed that 1.5 mM MgCl₂ was optimal regarding specificity. Twenty-two isolates of the industrial and collection strains of *Thermoanaerobacterium* spp, three isolates of *Thermoanaerobacter thermocopriae/mathranii* and one isolate of *Caldanaerobium fijensis* were assayed. Two of them, DSM 7060 and 2506 012, were not detected by this assay (Table 2), but were amplified by universal PCR. Sequence analysis from the former did not reveal any mismatch. Ten isolates of the closely related species *M. thermoacetica* were tested and two of these led to positive detection. As expected, the 29 isolates from other species, including anaerobic

thermophiles such as *Clostridium novyi* and *Clostridium thermopalmarium*, did not lead to positive detection.

The PCR test to detect *M. thermoacetica/thermoautotrophica* isolates was able to detect the species. The other PCR assay detected *Thermoanaerobacterium* and its closest relatives, and was named the *Thermoanaerobacterium* group detection assay. For two *M. thermoacetica/thermoautotrophica* and six *Thermoanaerobacterium* sp strains, a positive signal was obtained with the two PCR assays that trigger anaerobic bacteria rRNA gene. In these cases, complementary experiments such as sequencing of 16S rRNA coding region are necessary to confirm the identification.

Sensitivity of PCR Detection

Sensitivity assays were performed with the three PCR assays herein described as well as with the universal PCR assay (Fig. 1). The most sensitive PCR was the one aiming to detect *G. stearothermophilus*. The sensitivity level of this assay was evaluated at 0.5 pg. The least sensitive assay was that for *M. thermoacetica* detection, with a level of 500 pg. The detection limit for the *Thermoanaerobacterium* group PCR assay was estimated at 50 pg. It was identical to the latter for universal PCR assay.

Detection of Thermophilic Bacteria in Non-Stable Canned Products

In order to test whether the PCR assays were appropriate for the investigation of non-stable canned food, we tested 34 products, which had failed the stability test performed at 55°C (Table 4). After a single enrichment step and quick DNA extraction, the samples were tested with the three PCR assays.

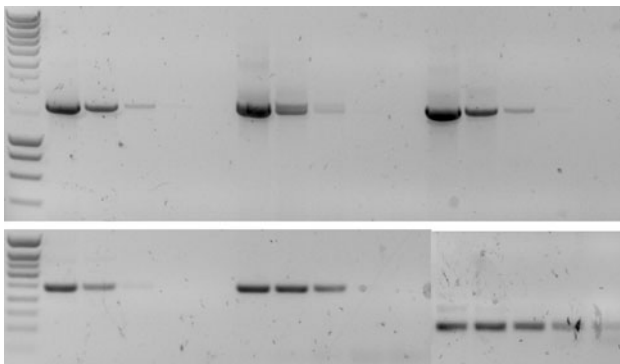


Fig. 1 Sensitivity of the PCR assays. *Upper part*: 16S rRNA gene amplification; *lower part*: specific detection PCR. *From left to right*: ladder, five lanes *M. thermoacetica* DNA as template, five lanes *T. thermoasaccharolyticum* DNA as template, five lanes *G. stearothermophilus* DNA as template. The five lanes correspond to 5 ng, 50 pg, 5 pg and 0.5 pg of template DNA

Geobacillus stearothermophilus was detected in seven non-stable samples. Fourteen samples tested positive for *M. thermoacetica/thermoautotrophica* and seven were positive for *Thermoanaerobacterium* group bacteria. Seven samples generated negative PCR tests even though the cultures were visually positive. These samples were used to isolate and identify bacteria by 16S rRNA gene sequencing. Identification showed the presence of two strains of *B. licheniformis*, one *B. smithii*, one *C. novyi*, one *C. thermopalmarium* and one *Paenibacillus graminis*.

Detection of Thermophilic Bacteria in Ingredients

Ingredients may be the entrance point for highly heat-resistant bacteria in food [19]. In particular, milk powders, which are produced by a heating process, are known to present thermophilic bacteria spore contamination occasionally [6, 22]. Thermophilic bacteria spores were counted in 13 ingredients such as spices, milk powder and aromas. DNA was extracted from randomly picked colonies and used in PCR assays. *G. stearothermophilus* or *Thermoanaerobacterium* group bacteria were detected by the assay in several samples (Table 5). No correlation was observed between colony counts and positive PCR tests. This was expected because heat-resistant spores present a remarkable diversity in milk powders and spices [19, 22].

Discussion

Moorella thermoacetica, *G. stearothermophilus* and *Thermoanaerobacterium* spp are among the most frequent contaminants causing low-acid canned food spoilage [2, 5, 9]. These thermophilic bacilli are of hygienic concern to the manufacturers and processors of low-acid canned food, and the ability to monitor these contaminants before or after thermal processing would have economic benefits. Remarkably, the first reports on the presence of thermophilic bacteria causing flat souring or swelling of canned food date from about 80 years [28], but rapid microbiology tools were rarely reported in spite of their recognized value [9, 15]. This study describes the development of a PCR assay to detect thermophilic bacteria forming highly heat-resistant spores that may pass through retort process of low-acid canned food.

An extensive specificity study is reported with both collection strains and strains collected from industrial environments. The possibility that strains from the same taxonomical group but isolated from different sources result in a negative result with a detection PCR is well documented for *Bacillus sporothermodurans* [12, 27]. A high specificity level was established for our PCR assay designed at the species level for *G. stearothermophilus*. A

Table 4 Detection of thermophilic bacteria from 55°C non-stable canned food

| Sample | Product | PCR positive detection and/or rRNA gene sequencing identification ^a |
|--------|------------------------------|--|
| CT196 | Canned fish-based course | <i>G. stearothermophilus</i> ^a |
| CT197 | Canned meat | <i>G. stearothermophilus</i> ^a |
| CT198 | Canned fish-based course | <i>G. stearothermophilus</i> ^a |
| CT199 | Meat dumpling | <i>G. stearothermophilus</i> ^a |
| CT248 | Canned meat | <i>G. stearothermophilus</i> ^a |
| CT260 | Sweet corn | <i>G. stearothermophilus</i> |
| CT263 | Sweet corn | <i>G. stearothermophilus</i> |
| D8137 | Fish dumpling | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| D8161 | Pre-cooked meal with meat | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| D8162 | Cooked vegetables | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| CT090 | Pre-cooked meal with meat | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| CT027 | Pre-cooked meal with chicken | <i>M. thermoacetica/thermoautotrophica</i> |
| CT071 | Green peas and carrots | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| CT073 | Green peas | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| CT089 | Green peas | <i>M. thermoacetica/thermoautotrophica</i> |
| CT141 | Mixed vegetables | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| CT201 | Peas | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| CT217 | Peas | <i>M. thermoacetica/thermoautotrophica</i> |
| CT256 | Peas | <i>M. thermoacetica/thermoautotrophica</i> |
| CT274 | Pre-cooked meal with meat | <i>M. thermoacetica/thermoautotrophica</i> |
| CT195 | Pre-cooked meal with meat | <i>M. thermoacetica/thermoautotrophica</i> ^a |
| CT086 | Peas | <i>Thermoanaerobacterium</i> group ^a |
| CT109 | Peas | <i>Thermoanaerobacterium</i> group ^a |
| CT124 | Peas | <i>Thermoanaerobacterium</i> group |
| CT125 | Pre-cooked meal with meat | <i>Thermoanaerobacterium</i> group ^a |
| CT149 | French beans | <i>Thermoanaerobacterium</i> group ^a |
| CT070 | Peas | <i>Thermoanaerobacterium</i> group ^a |
| CT072 | Paté | <i>Thermoanaerobacterium</i> group ^a |
| CT135 | Soup | <i>Bacillus licheniformis</i> ^a |
| CT087 | Paté | <i>Bacillus licheniformis</i> ^a |
| CT213 | Paté | <i>Bacillus smithii</i> ^a |
| CT228 | Pre-cooked meal with meat | <i>Clostridium novyi</i> ^a |
| CT169 | Paté | <i>Clostridium thermopalmarium</i> ^a |
| CT017 | Pre-cooked meal with fish | <i>Paenibacillus graminis</i> ^a |

^a Corresponds to strains, which were isolated and further identified by 16S rRNA gene sequencing

species-specificity level was obtained for the *M. thermoacetica/thermoautotrophica* detection assay, whereas the PCR assay for the *Thermoanaerobacterium* group detected all tested *Thermoanaerobacterium* species, but also the closest relatives belonging to *Thermoanaerobacter* and *Caldanaerobium* genera. These two PCR assays, which trigger thermophilic obligate anaerobes, presented some overlaps regarding the detected isolates.

All three PCR assays were able to detect low levels of DNA. However, high sensitivity is not an absolute requirement since these assays were developed with an enrichment step from canned food or from colonies picked from ingredients.

This assay showed its relevance for the identification of bacteria responsible for low-acid canned spoilage at 55°C. It allowed the cause to be detected rapidly and without isolation of bacteria in more than 80% of examined cases.

In addition, this assay was useful for ingredient qualification. Indeed, thermophilic spore counts, without species consideration, are an important issue for risk assessment [23–25]. It takes into account the large diversity of spore formers in food environment. However, also the detection of highly heat-resistant species is important. Adjustment of food retort settings takes into account historic data collection about spore contamination levels of ingredients and raw materials. In order to optimize sterilization processes,

Table 5 Spore enumeration and PCR assays of ingredients and raw materials

| Product | Thermophilic anaerobic spores counts (cfu/g) | Thermophilic aerobic spores counts (cfu/g) | PCR positive detection |
|---------------------------|--|--|--|
| Cocoa and dry milk | 1,700 | 220 | <i>G. stearothermophilus</i> |
| Milk powder | 20 | <10 | <i>G. stearothermophilus</i> |
| Milk powder | 1,200 | 400 | <i>G. stearothermophilus</i> |
| Spices | 100 | nd | <i>G. stearothermophilus</i> |
| Cocoa and dry milk | 1,800 | 220 | <i>G. stearothermophilus</i> |
| Milk powder | 1,000 | 50 | <i>G. stearothermophilus</i> |
| Pre-cooked meal with meat | <10 | 40 | <i>G. stearothermophilus</i> |
| Milk powder | 1,000 | 50 | <i>G. stearothermophilus</i> and <i>Thermoanaerobacterium</i> group |
| Milk powder | nd | 400 | None |
| Dry garlic | nd | 1,000 | None |
| Spices | nd | 40 | None |
| Pre-cooked meal with meat | <10 | 320 | None |

the heat resistance of contaminating spores must be considered. The tools developed here enable ingredients to be qualified. Similarly, PCR methods were developed for *B. sporothermodurans* and were further used to explore possible contamination routes [27].

Acknowledgements This work received financial support from FranceAgriMer and French canners.

References

- Anonymous (1997) Contrôle de la stabilité des produits appertisés et assimilés—Méthode de routine, in Microbiologie des aliments. AFNOR (Association Française de Normalisation), Paris
- Ashton D (1981) Thermophilic microorganisms involved in food spoilage: thermophilic anaerobes not producing hydrogen sulfide. *J Food Protect* 44(2):146–148
- Ashton D, Bernard D (1992) Thermophilic anaerobic spore-formers. In: Vanderzantz C, Splittstoesser DF (eds) Compendium of methods for the microbiological examination of foods, 3rd edn. American Public Health Association, Washington, DC, pp 309–316
- Byrer DE, Rainey FA, Wiegel J (2000) Novel strains of *Moorella thermoacetica* form unusually heat-resistant spores. *Arch Microbiol* 174(5):334–339
- Carlier JP, Bedora-Faure M (2006) Phenotypic and genotypic characterization of some *Moorella* sp. strains isolated from canned foods. *Syst Appl Microbiol* 29(7):581–588
- Cooper RM, McKillip JL (2006) Enterotoxigenic *Bacillus* spp. DNA fingerprint revealed in naturally contaminated nonfat dry milk powder using rep-PCR. *J Basic Microbiol* 46(5):358–364
- De Vos P, Garrity GM, Jones D et al (eds) (2009) *Bergey's manual of systematic bacteriology*, vol 3. The Firmicutes, 2nd edn. Springer, Dordrecht, p 1450
- Denny CB, Corlett DAJ (1992) Canned foods—tests for cause of spoilage. In: Vanderzantz C, Splittstoesser DF (eds) Compendium of methods for the microbiological examination of foods, 3rd edn. American Public Health Association, Washington, DC, pp 1051–1092
- Dotzauer C, Ehrmann M, Vogel R (2002) Occurrence and detection of *Thermoanaerobacterium* and *Thermoanaerobacter* in canned food. *Food Technol Biotechnol* 40(1):21–26
- Esty JR, Meyer KF (1922) The heat resistance of the spores of *B. botulinus* and allied anaerobes XI. *J Infect Dis* 31:650–663
- Feeherry FE, Munsey DT, Rowley DB (1987) Thermal inactivation and injury of *Bacillus stearothermophilus* spores. *Appl Environ Microbiol* 53(2):365–370
- Herman LM, Vaerewijck MJ, Moermans RJ et al (1997) Identification and detection of *Bacillus sporothermodurans* spores in 1, 10, and 100 milliliters of raw milk by PCR. *Appl Environ Microbiol* 63(8):3139–3143
- Hornstra LM, Ter Beek A, Smelt JP et al (2009) On the origin of heterogeneity in (preservation) resistance of *Bacillus* spores: input for a 'systems' analysis approach of bacterial spore outgrowth. *Int J Food Microbiol* 134(1–2):9–15
- Karnholz A, Kusel K, Gossner A et al (2002) Tolerance and metabolic response of acetogenic bacteria toward oxygen. *Appl Environ Microbiol* 68(2):1005–1009
- Kotzekidou P (1996) A microtitre tray procedure for a simplified identification of *Bacillus* spp. in spoiled canned foods. *Food Microbiol* 13:35–40
- Liu D (2008) Preparation of *Listeria monocytogenes* specimens for molecular detection and identification. *Int J Food Microbiol* 122(3):229–242
- Mafart P (2000) Taking injuries of surviving bacteria into account for optimising heat treatments. *Int J Food Microbiol* 55:175–179
- Olson KE, Sorrells KM (1992) Thermophilic flat sour spore-formers. In: Vanderzantz C, Splittstoesser DF (eds) Compendium of methods for the microbiological examination of foods, 3rd edn. American Public Health Association, Washington, DC, pp 299–308
- Oomes SJ, van Zuijlen AC, Hehenkamp JO et al (2007) The characterisation of *Bacillus* spores occurring in the manufacturing of (low acid) canned products. *Int J Food Microbiol* 120(1–2): 85–94
- Penna TC, Machoshvili IA, Taqueda ME et al (2000) The effect of media composition on the thermal resistance of *Bacillus stearothermophilus*. *PDA J Pharm Sci Technol* 54(5):398–412
- Robert H, Gabriel V, Fontagne-Faucher C (2009) Biodiversity of lactic acid bacteria in French wheat sourdough as determined by molecular characterization using species-specific PCR. *Int J Food Microbiol* 135(1):53–59

22. Ruckert A, Ronimus RS, Morgan HW (2004) A RAPD-based survey of thermophilic bacilli in milk powders from different countries. *Int J Food Microbiol* 96(3):263–272
23. Rueckert A, Ronimus RS, Morgan HW (2005) Development of a rapid detection and enumeration method for thermophilic bacilli in milk powders. *J Microbiol Methods* 60(2):155–167
24. Rueckert A, Ronimus RS, Morgan HW (2005) Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus*. *J Appl Microbiol* 99(5):1246–1255
25. Rueckert A, Ronimus RS, Morgan HW (2006) Development of a real-time PCR assay targeting the sporulation gene, *spo0A*, for the enumeration of thermophilic bacilli in milk powder. *Food Microbiol* 23(3):220–230
26. Sasaki K, Shintani H, Itoh J et al (2000) Effect of calcium in assay medium on D value of *Bacillus stearothermophilus* ATCC 7953 spores. *Appl Environ Microbiol* 66(12):5509–5513
27. Scheldeman P, Herman L, Goris J et al (2002) Polymerase chain reaction identification of *Bacillus sporothermodurans* from dairy sources. *J Appl Microbiol* 92(5):983–991
28. Shaw M (1928) Thermophilic bacteria in canned foods. *J Infect Dis* 43(5):461–474
29. Weisburg WG, Barns SM, Pelletier DA et al (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173(2):697–703