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A B S T R A C T

Geobacillus stearothermophilus is the main thermophilic spore former involved in flat sour spoilage of canned foods. Three typing methods were tested and applied to differentiate strains at intra-species level: *panC* sequence analysis, REP-PCR and M13-PCR. *panC* gene was highly conserved within the studied strains, suggesting a low intra-specific diversity. This was supported by REP-PCR primary assays and M13-PCR results. M13-PCR profile analysis succeeded in differentiating six closely related groups (at 79% threshold similarity) among 127 strains from a range of spoiled canned food products and from different canneries. Phenotypic traits were investigated among 20 selected strains representing groups and origins. Ranges of growth under different temperatures (from 40 °C to 70 °C), pH (from 5.0 to 6.5), NaCl concentrations (from 1 to 5%) and sporulation conditions poorly differed between strains, but wet heat resistance of spores showed a 20-fold variation between strains. Furthermore, in this study, strains that belonged to the same M13-PCR genetic group did not share phenotypic characteristics or common origin. The work emphasizes a low diversity within the *G. stearothermophilus* species but data from this study may contribute to a better control of *G. stearothermophilus* spoilage in canned food.

1. Introduction

Geobacillus stearothermophilus is a thermophilic spore-forming bacterium with optimal growth between 55 °C and 65 °C (Nazina et al., 2001). *G. stearothermophilus* properties interest several industrial sectors, as a source of enzymes with high temperature stability, producer of antiviral compounds, biological indicator for sterility control, and as a major cause of food spoilage (Cheng et al., 2009; Guizelini et al., 2012; Rivero et al., 2012). *G. stearothermophilus* is responsible for "flat-sour" spoilage of low-acid canned food stored at high temperature (above 40 °C) (Ashton and Bernard, 1992). "Flat sour" results from saccharide fermentation into organic acids without gas production (Kalogridou-Vassiliadou, 1992). *G. stearothermophilus* spores can withstand canned food heat treatment and can further germinate and grow in products. *G. stearothermophilus* is responsible for *ca*. 35% of canned food spoilage during incubation at 55 °C (Ashton and Bernard, 1992; André et al., 2013). It has been detected in many raw and processed foods (canned vegetables, ready-to-eat meals containing meat, fruit preparations, dehydrated ingredients, gelatin extracts…) (Postollec et al., 2012) and in processing facilities. For instance up to 50% milk powders may contain the bacterium, which may represent more than 10% of thermophilic isolates (Rückert et al., 2004) and *G. stearothermophilus* spores were detected in 8.6% and 2.1% of raw carrots and green bean samples, respectively (Sevenier et al., 2012).

High prevalence in food has supported the characterization of *G. stearothermophilus* isolates for survival and growth in canned food, without clear evidence of the variability within the species (Donk, 1920; Gordon and Smith, 1949; Smith and Gordon, 1957; Gibson and Gordon, 1974; Kalogridou-Vassiliadou, 1992; White et al., 1993; Nazina et al., 2001; Coorevits et al., 2012). Genomic studies are now increasingly carried out on thermophilic bacteria to determine the extent of variability and diversity within bacterial groups or species. Several strategies and methods can be used. For

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instance these include rep-PCR profiling and 16S rRNA coding region sequencing applied on thermophilic bacteria from hot springs (Adiguzel et al., 2009), RAPD-PCR or MLVA applied to differentiate thermophilic bacilli in milk powder and tracking of contamination in dairy processing plants (Ronimus et al., 2003; Rückert et al., 2004). Sequencing methods that target highly conserved housekeeping genes such as *recN*, *rpoB* and *recA* have been applied to the *Geobacillus* genus (Zeigler, 2005; Meintanis et al., 2008; Weng et al., 2009; Seale et al., 2012). The diversity of the pathogenic and spore-forming bacterium *Bacillus cereus* was also investigated with success using a M13 sequence-based polymerase chain reaction (M13-PCR) (Guinebretiere and Nguyen-The, 2003), *panC* sequencing and AFLP (Guinebretiere et al., 2008). Analysis of 16S rRNA gene sequence was not discriminatory enough for *G. stearothermophilus* (Dinsdale et al., 2011). In this case, *panC* gene phylogeny could be used as an alternative to 16S rRNA gene phylogeny, as with *B. cereus* (Guinebretiere et al., 2008). Sequence (or partial sequence) of the coliphage M13 is repeated on many genomes and was exploited as primer in DNA sequence based PCR (M13-PCR) (Henderson et al., 1994; Guinebretiere and Nguyen-The, 2003). This resulted in a more rapid DNA fingerprinting technique that discriminates bacterial isolates at the intra-specific level, and potentially up to the strain level. As for RAPD and REP-PCR, M13-PCR is less cumbersome and time-consuming than other typing methods such as genotyping, ribotyping, RFLP, PCR-RFLP and AFLP, and is adapted to studies on a large number of strains (*i.e.* >100). In addition, M13-PCR involves a longer primer and higher hybridization temperatures than RAPD and thus results in more reproducible results.

The aims of this work were (i) to compare the ability of three typing methods (M13-PCR, REP-PCR and *panC* sequencing) to differentiate *G. stearothermophilus* genetic groups, (ii) to evaluate the genetic diversity among a large panel of *G. stearothermophilus* strains from spoiled canned food, and (iii) to explore their phenotype in relation to ability to grow at different temperatures, pH and salt concentrations, ability to sporulate, and spore resistance to heat treatment.

2. Materials and methods

2.1. Strains and culture conditions

A total of 127 *G. stearothermophilus* strains were tested (Supplementary Table 1). Among those, 116 strains were recovered between 2001 and 2010 from spoiled canned food after long-term storage at 55 °C (André et al., 2013). Two isolates (#110 and #112) were isolated from vegetable debris sampled in a cannery blancher. Three isolates (#82, #85 and #86) were isolated from the covering brine of canned vegetable, and one isolate, #63, was isolated from a cannery conveyor surface. They were identified as *G. stearothermophilus* using either a specifically developed PCR tool (SporeTraQ™) (Prevost et al., 2010) that targets the DNA region corresponding to rRNA internal transcribed sequence 16S-23S, or a partial sequencing of 16S rRNA coding region. Five other *G. stearothermophilus* strains were obtained from the DSMZ collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). DSM 22 and DSM 297 were isolated from spoiled canned food. The origin of DSM 5934, DSM 2313 and DSM 6790 strains is not specified. Strains were stored at −80 °C in a cryogenic preservative solution with beads (AES Chemunex, Bruz, France).

A bead of a stock culture was dropped into brain heart infusion (BHI) before incubation for 48 h at 55 °C. A 500 µl volume of this pre-culture was inoculated into BHI and incubated overnight at 55 °C. The resulting culture was used for subsequent inoculation of growth and sporulation media.

2.2. Genetic groups and diversity

2.2.1. DNA extraction

A 100 µl volume of an overnight culture in BHI was spread on dextrose tryptone agar (DTA) and incubated for 16 h at 55 °C before DNA extraction. DNA preparation was obtained as described previously (Guinebretiere and Nguyen-The, 2003) with minor modifications. Two loops of cells were suspended in 550 µl extraction buffer (1.7% sodium dodecyl sulfate, 200 mM Tris-HCl (pH 8), 20 mM EDTA, 200 mM NaCl). The suspension was incubated at 55 °C for 30 min with 25 µl of proteinase K (10 µg μl^{-1}) (Sigma–Aldrich, Saint-Quentin-Fallavier, France). DNA was extracted with one volume of phenol. After centrifuging, the aqueous phase containing DNA was mixed with one volume of phenol, recovered after centrifuging, and then mixed with one volume of chloroform. The aqueous phase was precipitated with cold absolute ethanol. After centrifuging, the pellet was washed with cold 70% ethanol, air dried at room temperature, dissolved in 50 µl sterile Milli-Q water, and stored at −20 °C. DNA was quantified by absorbance at 260 nm (NanoQuant Plate™ Infinite® M200, Tecan Austria). The DNA was extracted from three independent cultures of randomly selected 24 strains to evaluate the reproducibility of genomic analyses.

2.2.2. M13-PCR

The method used followed the PCR protocol as previously described with minor modifications (Guinebretiere and Nguyen-The, 2003). PCR mix (25 µl) contained 50 ng of DNA template, 4.8 mM primer PM13: 5′-GAGGGTGGCGGCTCT-3′ (Eurogentec S.A., Seraing, Belgium), 1.25 mM dNTPs mix (Eurogentec), 6 mM $MgCl_2$ (Sigma–Aldrich), 10% (vol/vol) dimethyl sulfoxide (Sigma–Aldrich), and 1.5 U of Diamond *Taq*® DNA polymerase (Eurogentec) in supplier buffer. PCR amplifications were performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA). PCR cycling parameters included a denaturation step at 94 °C for 3 min followed by 35 cycles each consisting of 94 °C for 1 min, 40 °C for 1 min and 68 °C for 8 min; and a final extension step at 68 °C for 16 min. A negative control containing Milli-Q water instead of DNA and a positive control with the DNA of *B. cereus* strain ATCC 14579 producing a known banding pattern were included in each PCR experiment. All of the DNA preparations were analyzed by M13-PCR twice independently to evaluate the reproducibility of the typing results.

2.2.3. REP-PCR analysis

A set of 24 strains underwent REP-PCR genomic fingerprinting as previously described (Versalovic et al., 1994). Amplification was performed as described above, except that 2.4 µM of each primer REP 1R-I (5′ III IGC ICG ICG ICA TCI GGC 3′) and REP 2-I (5′ ICG ICT TAT CIG GCC TAC 3′) (Eurogentec) was used, and PCR elongation was performed at 65 °C.

2.2.4. panC sequencing

The oligonucleotides used for the PCR amplification of *panC* encoding pantoate-β-alanine ligase were: PCF3, 5′-TCGGDTTYGTNCCGACGATGG-3′ and PCR1, 5′-AAABCGVACGGCVACVGCGA-3′. They were designed from sequence alignment of *panC* genes from several *Geobacillus* sp. (accession numbers: CP001794, CP002442, CP002050, CP001638, CP002293, NC_006510, and CP003125). Amplification reaction was performed in a final volume of 25 µl containing DNA template (50 ng), dNTPs mix (Eurogentec) (0.2 mM), $MgCl_2$ (2 mM), primers (0.4 µM each), and 0.5 U of Diamond *Taq*® DNA polymerase (Eurogentec) in supplier buffer. The cycling program was 94 °C for 4 min; 35 cycles of 94 °C for 40 s, 64 °C for 40 s and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR product was purified with the High Pure PCR Product Purification Kit according to the manufacturer's instructions

(Roche, France). The partial sequencing of the *panC* gene (648 pb) was done with the PCF3 primer by Eurofins MWG Operon (Courtaboeuf, France) according to the classical Sanger method. The 20 *panC* sequences were registered in GenBank under accession numbers KF177233 to KF177252. Sequence alignment was carried out with Clustal Omega at EBI-EMBL (Goujon et al., 2010; Sievers et al., 2011). Percentages of identity were calculated between these sequences and other thermophilic spore-forming bacteria *panC* sequences (accession numbers: NC_006274, NC_004722, NC_003909, NC_006510 and CP000232) available in the GenBank database.

2.2.5. Electrophoresis and data analysis

PCR products from M13-PCR and REP-PCR were analyzed by electrophoresis on 1.5% agarose gels with the molecular mass DNA marker Smart Ladder (Eurogentec) using 0.5 × TAE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8). Gels were stained with ethidium bromide and visualized and photographed on a UV gel imager (Molecular Imager® Gel Doc™ XR System 170-8170, BioRad, France). Band position, similarity coefficient and dendrogram were determined using the BioGene 99.04 software (Vilber Lourmat, Marne-la-Vallée, France). A dendrogram was constructed from the similarity banding pattern of M13-PCR amplification using the unweighted pair group method with arithmetic mean (UPGMA). *B. cereus* ATCC 14579 was chosen as positive control to evaluate the variability generated by the analyses (PCR-electrophoresis-image analysis). Amplification product from the positive control was deposited on each gel, and results were compared to define the confidence interval.

2.3. Growth studies

2.3.1. Growth at different temperatures and pH

Growth at a range of temperature and pH was tested on dextrose tryptone agar (DTA) and in DT broth (DTB) selected for its color stability at high temperature that favors reading. Inoculums were made of an overnight culture in BHI broth diluted 100-fold in peptone salt water to obtain suspensions at 10^5 CFU ml^{-1}. In a first step one a droplet (10 µl) of each bacterial suspension was deposited on DTA. Plates were wrapped in aluminum foil to avoid desiccation and incubated for 14 days. Growth was tested at each of the 24 combinations of incubation temperature (45 °C, 50 °C, 55 °C or 70 °C) and pH (6.5, 6.0, 5.5, 5.0, 4.8 and 4.5). Growth at 40 °C was tested only on DTA pH 6.5. The pH adjustment was performed in sterilized molten DTA by addition of filter-sterile 2 N HCl (Fischer Scientific SAS, Illkirch, France). Strains were deemed positive for growth when the diameter of the colony formed from the droplet inoculum at the agar surface was greater than 1 mm. Colony development was examined daily after 48 h of incubation, until 14 days.

In a second step strains that were negative in at least one combination of pH and temperature were tested in (DTB) to differentiate between absence of growth and weak growth. A 500 µl volume of an overnight culture was inoculated in DTB sterilized at 121.1 °C for 15 min and incubated at 45 °C, 50 °C, 55 °C and 70 °C, adjusted to the required pH with filter-sterile 2 N HCl for a maximum of 14 days. Growth was controlled every 24 h by serial decimal dilution of the culture onto DTA and colony enumeration after 48 h incubation at 55 °C. Strains were deemed positive when they showed at least one 1 log cfu.ml^{-1} increase in DTB after 14 days of incubation. Growth at 37 °C, 39 °C, 40 °C, 72 °C and 75 °C at pH 6.5 was tested in DTB during incubation for 14 days using the same enumeration procedure and was replicated three times with independently prepared inoculums. Incubation temperatures at 37 °C, 39 °C, 40 °C, 72 °C and 75 °C were obtained in a thermostated water-bath and temperature was monitored using a digital thermometer (EcoScan Temp5, Fisher, Illkirch, France).

2.3.2. Growth at different sodium chloride concentrations

DTA was supplemented with sodium chloride to obtain final concentrations in the range 1–5% (wt/vol). The previously described drop method was used to inoculate the medium. Water activity (a_w) DTA was measured for each NaCl concentration (AquaLab, Biotrace International SAS). Incubation was performed at 55 °C for a maximum of 14 days. Strains were deemed positive for growth, as colony diameter at the agar surface was greater than 1 mm.

2.4. Spore production and evaluation of spore heat resistance

2.4.1. Spore production and percentage of sporulation

For spore production, a 2 ml volume of an overnight culture in BHI was spread onto the sporulation medium (SM) on a 145 mm agar plate. The sporulation medium was made of 10 g l^{-1} meat extract, 2 g l^{-1}yeast extract, 0.04 g l^{-1} $MnSO_4.H_2O$ and 15 g l^{-1} agar. Temperatures tested for sporulation were 40, 45, 55 and 70 °C. The spores were also examined under a phase-contrast microscope under a ×1000 magnification to estimate the % of sporulation as the % of phase bright and free spores within the sporulating population after 21 days of incubation at selected temperature and pH. Approximately 30–50 cells were counted in 10 observation fields for each different strain. Spores were harvested when the culture contained more than 50% of free spores. Harvested spores were washed with 5 ml of sterile distilled water, pelleted at 1400 × *g* for 20 min at 4 °C, and suspended again in 5 ml of sterile distilled water. This procedure was replicated four times. Residual vegetative cells and germinated spores were killed by heating suspensions at 100 °C for 10 min. Spore suspensions were stored for 15 days at 4 °C. These spore suspensions were heated at 100 °C for 10 min for counting. One batch of spores was prepared for each strain.

2.4.2. Spore heat resistance and curve fitting with mathematical models

Heat resistance at 120 °C was determined as previously described (André et al., 2013). The number of surviving spores in capillary tubes was evaluated after 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6 and 8 min. Serial decimal dilutions of the content of capillary tubes in 0.2 M phosphate buffer were spread onto bromocresol purple (BCP), and then covered with layer of molten BCP at 50 °C. Plates were incubated at 55 °C for 2 days before colony counts. The time for a 5-$\log_{10}$ reduction (t_{5D}) was used to compare the heat resistance of isolates, to take into account the different forms of survival curves (linear, concave or convex) and to account for a large number of log-reduction. Graphical log plots of surviving population *vs* heating time (log CFU $ml^{-1} = f(t)$) were fitted according to three equation models: log-linear, biphasic log-linear and Weibull.

The log-linear model is based on the following equation:

$$\log N = \log N_0 - \frac{t}{D}$$

where N_0 and N are the initial and final number of cells (CFU ml^{-1}) after a treatment time t (min), respectively, and D is the decimal reduction time (min).

The biphasic log-linear model was applied with the following equation:

$$\log N = \begin{cases} \log N_0 & \text{if} \quad t \leq \text{lag} \\ \log N_0 - \frac{t}{D} & \text{if} \quad t > \text{lag} \end{cases}$$

where N_0 and N are the initial and final number of cells (CFU ml^{-1}) after a treatment time t (min), respectively, D is the decimal reduction time (min), and lag the duration of the latency phase.

The Weibull model (Couvert et al., 2005) was applied with the equation:

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p,$$

where N_0 and N are the initial and final number of cells (CFU ml^{-1}) after a treatment time t (min), respectively, δ is the first reduction decimal time (min) that leads to a 10-fold reduction in surviving population, and p is the fitting parameter. The equation that generates the lowest residual sum of squares (RSS) was used to calculate the time for a 5-log$_{10}$ reduction (t_{5D}).

2.5. Statistical analysis

Qualitative data of the tested variables were analyzed by multiple correspondence analysis (MCA) with the software XLSTAT (AddinsoftTM, Paris, France). The variables tested were either (i) food category/product for isolation, food company, geographical location and year of isolation, or (ii) lowest temperature, highest temperature, lowest pH, highest pH and highest NaCl concentration at which a growth was observed. Observations were isolate numbers and M13-PCR group numbers.

3. Results and discussion

3.1. Genetic diversity of G. stearothermophilus strains examined with M13-PCR, REP-PCR and panC sequencing

An ideal genotyping method should be applicable to all isolates, capable of differentiating unrelated isolates, reproducible both within and between laboratories, rapid, cost-effective, and easy to perform. In this study, M13-PCR, REP-PCR and *panC* sequencing were applied within the species *G. stearothermophilus*. All three techniques had previously been used successfully for species typing in the genus *Bacillus* (Guinebretiere et al., 2001; Guinebretiere and Nguyen-The, 2003; Guinebretiere et al., 2008).

An internal region of *panC* gene was sequenced for 20 selected strains. A 82.7% sequence identity was obtained with *Geobacillus kaustophilus* HTA426 *panC* gene, validating identity of sequences obtained with that method. When compared with the reference *G. stearothermophilus* DSM 22, sequence identity of obtained sequences ranged from 99.4% to 100%. These results indicate that *panC* gene is probably highly conserved within the *G. stearothermophilus* species. We thus expect a low diversity in this species. For this reason *panC* sequence could not be used as a phylogenetic tool for *G. stearothermophilus* strains isolated from canned food spoilage. The low intra-specific diversity in *G. stearothermophilus* species is also underlined by numerous bibliographic data : the *rpoB* housekeeping gene exhibited more than 74% of identity within *Geobacillus* spp. (Meintanis et al., 2008); *recA* and *rpoB* gene sequence comparison discriminates only among *Geobacillus* species (Weng et al., 2009); *recN* is a powerful tool to differentiate species of *Geobacillus* but failed to differentiate strains at intraspecific level (Zeigler, 2003). Also, the use of the sequence 16S-23S rRNA internal transcribed spacers (ITS) have shown no phylogenetic relationships between the different species of *Geobacillus* (Xu and Côté, 2003; Kuisienė et al., 2008), which decrease the interest of use it. All these bibliographic data together with *panC* sequence analysis from this study converge to a low intra-specific diversity in *G. stearothermophilus*.

A series of naturally-occurring repetitive DNA sequences are dispersed in multiple copies throughout bacterial genomes (Gilson et al., 1984; Stern et al., 1984; Hulton et al., 1991; Lupski and Weinstock, 1992; Koeuth et al., 1995). Although the functions of these interspersed repetitive DNA elements remain unknown, their presence is useful for DNA fingerprinting of bacteria. REP-PCR was successfully used by Meintanis et al. (2008) to discriminate *Geobacillus* and *Bacillus* species type strains. In this study, the REP-PCR method was performed on 24 isolates that differed by their isolation origin. A maximum of three to five bands only was observed on the banding patterns, and most of strains exhibited similar electrophoretic profiles, which was not sufficient to efficiently discriminate the isolates (data not shown). In contrast to M13-PCR, the REP-PCR technique resulted in a low number of signals on the banding patterns Hence REP-PCR was not kept to characterize more specifically *G. stearothermophilus* isolates.

Another method that uses a DNA sequence repeated within genomes is M13-PCR. An example of fingerprints generated using M13-PCR on DNA extracted from 20 isolates out of the 127 *G. stearothermophilus* isolates from this study is shown in Fig. 1. The number of detected DNA bands varied up to 20 and band size ranged between 100 and 5000 bp with a confidence interval of 9%. A M13-PCR profile was obtained for each of the 127 strains. A dendrogram was constructed to compare *G. stearothermophilus* isolates (Fig. 2). A confidence interval of 9% was obtained from positive controls from three independent DNA preparations, each

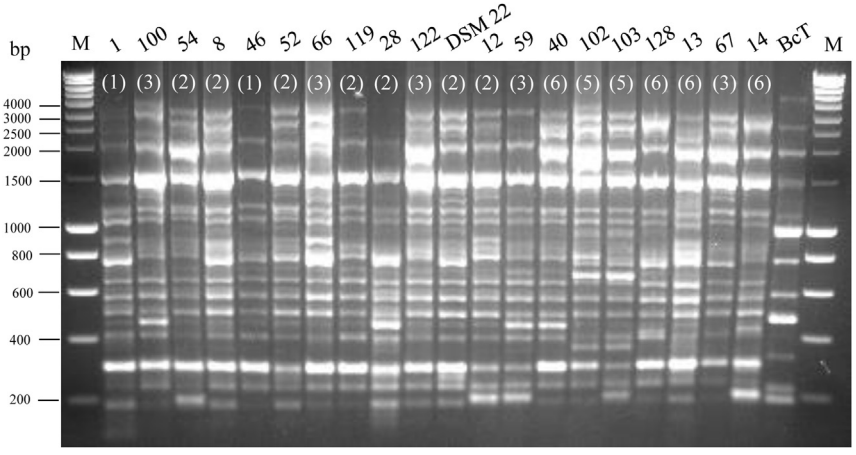
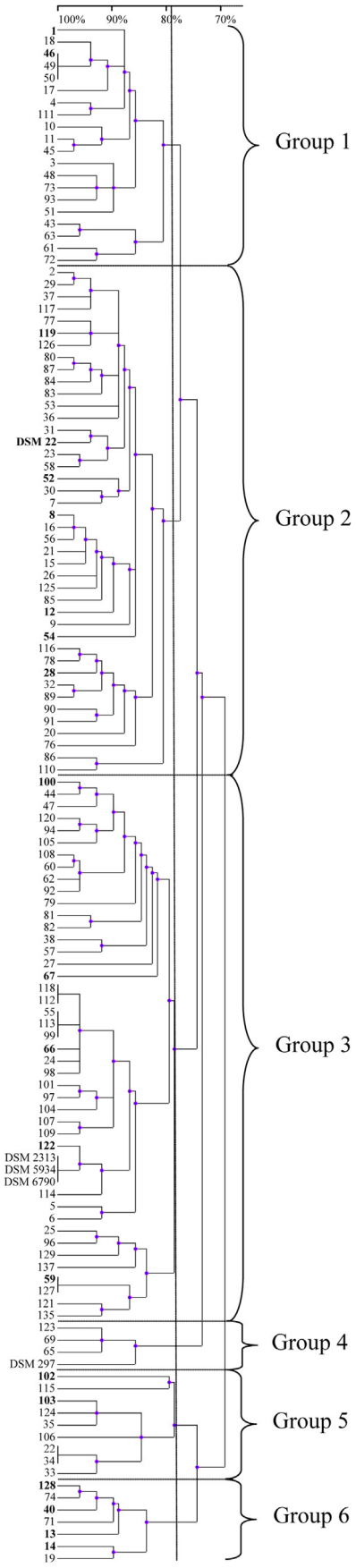


Fig. 1. M13-PCR banding profile of 20 *G. stearothermophilus* strains. M: DNA molecular size marker Smart ladder SL (Eurogentec). Strain numbers are indicated at the top of each lane, with group numbers between brackets. BcT, *B. cereus* ATCC 14579 positive control.



assayed in triplicate by independent PCR, electrophoresis and electrophoretic profiles analysis. Six closely related groups among the 127 isolates of *G. stearothermophilus* were obtained at a similarity threshold of 79%, using Dice's coefficient and UPGMA clustering methods (Fig. 2 and Supplementary Table 1). The groups differed by the number of isolates: 84% of strains belonged to the first three groups, named 1 (16%), 2 (33%) and 3 (35%). The other three groups, named 4, 5 and 6, contained four (3%), nine (7%) and seven (6%) strains respectively. Three DSM collection strains DSM 2313, DSM 5934 and DSM 6790 were found in Group 3. DSM 22 and DSM 297 were respectively in groups 2 and 4. Despite the great differences between origins of strains (geographic area, food product, Food Company and year of isolation, see Supplementary Table 1), the similarity between M13-PCR profiles was high (Figs. 1 and 2), reaching more than 75% similarity for 107 strains (84% of strains) and more than 69% for all strains. This emphasized the low intra-specific diversity suggested in *G. stearothermophilus* by the high *panC* sequence identity percent. However, M13-PCR had success in differentiating closely related strains. In particular, some strains exhibited one or two specific bands in their profile that could allow recognizing them. Thus, this may be an important tool to trace *G. stearothermophilus* spoilers on the food processing chain.

3.2. Growth of selected G. stearothermophilus strains at a range of temperature, pH and NaCl concentrations and rates of sporulation

Several strains were chosen on the basis of their different origin and their different genetic profile (groups 1, 2, 3, 5 and 6 in the present work) to estimate the phenotypic diversity among *G. stearothermophilus* strains isolated from canned food. A set of 19 strains that differed by their isolation origin and the type strain DSM 22 were tested for their ability to grow at pH between 4.5 and 6.5 and between 37 °C and 75 °C (Table 1). All strains exhibited growth in less than 48 h at 50 °C at the three initial pH values of 6.5, 6.0 and 5.5, as well as at 55 °C and 70 °C, but only for initial pH values of 6.5 and 6.0. At an initial pH value of 5.0, only six strains grew at 50 °C, only three at 55 °C, and none at 70 °C. No strain was able to grow at pH 4.8 and below at 50 °C within 14 days. The most low-pH-tolerant strains were isolate #122 and the type strain DSM 22. At the most favorable initial pH of 6.5, growth was undetected for all strains at 72 °C, and 75 °C. At this pH, the lowest temperature that allowed growth for 12 isolates was 40 °C. Strains #28 and #67 grew in 48 h at 40 °C. None of the 20 tested strains was able to grow at 39 °C and 37 °C. At pH 5.5, growth ability at 70 °C differed according to the strain: only strains #28, #46, #102, #103 and #128 grew, and that growth was observed within 48 h. Interestingly, isolate #28 exhibited a rapid growth at 40 °C pH 6.5, at 70 °C pH 6.5 and at 50 °C pH 5. Conversely, temperature and pH ranges of growth were restricted for isolate #119.

Growth in the presence of NaCl was assayed for the same strains in a concentration range of 1–5% (Table 2). The results showed that all strains grew at 1% and 2%, but only 12 and three strains (#40, #100 and #102) were able to develop at 3% and 4% NaCl respectively (Table 3).

All strains grew at 50 °C and 55 °C and pH 6.0 and 6.5, including in the presence of 2% NaCl. Many of our observations are in accordance with characters reported in the taxonomic revision of the genus *Geobacillus* (Coorevits et al., 2012; Ronimus et al., 2003):

Fig. 2. Dendrogram of *G. stearothermophilus* genotypes obtained with the M13-PCR method. The percentage similarities were calculated using Dice's coefficient and UPGMA clustering methods. The groups were formed with a similarity level at 79% similarity (represented by a vertical scale). Strains with M13-PCR profiles in Fig. 1 are indicated with bold characters.

Table 1
Growth of *G. stearothermophilus* strains at different initial pH and temperatures.

Strain number (M13 group)	Time to growth at indicated pH and temperature (°C)[a]										
	pH 6.5			pH 6.0		pH 5.5				pH 5.0	
	50 55 70[b]	45	40	50 55 70[b]	45	70	55	50	45	55	50
#1 (1)	2	4	>4	2	4	ng	3	2	ng	ng	ng
#46 (1)	2	4	ng	2	4	2	3	2	ng	ng	ng
#8 (2)	2	4	ng	2	4	ng	4	2	ng	ng	ng
#12 (2)	2	4	>4	2	4	ng	3	2	ng	ng	ng
#28 (2)	2	2	2	2	4	2	3	2	ng	4	4
#52 (2)	2	4	4	2	4	ng	3	2	4	ng	3
#54 (2)	2	4	4	2	4	ng	3	2	4	ng	ng
#119 (2)	2	ng	ng	2	ng	ng	3	2	ng	ng	ng
DSM 22 (2)	2	4	4	2	4	ng	3	2	ng	4	2
#59 (3)	2	4	ng	2	4	ng	4	2	ng	ng	ng
#66 (3)	2	4	4	2	4	ng	3	2	4	ng	ng
#67 (3)	2	2	2	2	4	ng	3	2	ng	ng	ng
#100 (3)	2	4	4	2	4	ng	3	2	ng	ng	ng
#122 (3)	2	4	4	2	4	ng	3	2	ng	4	2
#102 (5)	2	4	ng	2	4	2	3	2	ng	ng	ng
#103 (5)	2	4	ng	2	4	2	3	2	ng	ng	ng
#13 (6)	2	4	ng	2	ng	ng	3	2	ng	ng	>4
#14 (6)	2	4	4	2	4	ng	3	2	ng	ng	ng
#40 (6)	2	4	ng	2	4	ng	ng	2	ng	ng	>4
#128 (6)	2	4	4	2	4	2	3	2	ng	ng	ng

[a] 2, growth after 2 days incubation; 3, growth after 3 days incubation; 4, growth after 4 days incubation; >4, growth after more than 4 days incubation; ng, no growth observed for 14 days.
[b] Same results were obtained for the temperatures 50, 55 and 70 °C.

maximal temperature for growth not exceeding 70 °C, variable minimal temperature for growth in the range 30 °C–45 °C and no growth at 5% NaCl. In these studies, most of the strains can grow between 40 °C and 60 °C. In our study, growth temperature and pH range suitable for growth were close for all strains whatever their origin, although some differences could be noted. By contrast, strains of *B. cereus* have distinct growth characteristics depending on their genetic groups (Guinebretiere et al., 2008). It has been shown that *B. cereus* strains phenotypic traits are related according to their toxicity, but not according to their origin (Carlin et al., 2006).

Table 2
Growth of *G. stearothermophilus* strains incubated at different NaCl concentrations.

Strain number (M13-group)	Time to growth (days) at NaCl concentrations (wt/vol) (a_w)		
	2% (0.973)	3% (0.971)	4% (0.966)
#1 (1)	2	3	ng
#46 (1)	2	ng	ng
#8 (2)	2	ng	ng
#12 (2)	2	ng	ng
#28 (2)	2	2	ng
#52 (2)	2	2	ng
#54 (2)	2	2	ng
#119 (2)	3	ng	ng
DSM 22 (2)	2	2	ng
#59 (3)	2	3	ng
#66 (3)	2	ng	ng
#67 (3)	2	3	ng
#100 (3)	2	2	3
#122 (3)	2	2	ng
#102 (5)	2	3	3
#103 (5)	2	ng	ng
#13 (6)	2	ng	ng
#14 (6)	2	2	ng
#40 (6)	2	2	3
#128 (6)	2	ng	ng

ng, no growth observed after 14 days.

Table 3
Sporulation of *G. stearothermophilus* strains from different genotypic groups.

Strain number (M13 group)	% of sporulation after incubation at different temperature (°C) and pH conditions[a]						
	70 °C		55 °C			45 °C	40 °C
	pH 7.0	pH 7.0	pH 6.0	pH 5.5	pH 5.0	pH 7.0	pH 7.0
#1 (1)	12	100	90	80	nt	7	2
#46 (1)	5	100	95	95	nt	0	nt
#8 (2)	0	45	2	30	nt	90	nt
#12 (2)	0	70	95	10	nt	0	0
#28 (2)	1	50	20	50	1	95	0
#52 (2)	80	90	72	15	nt	20	1
#54 (2)	35	80	10	10	nt	80	0
#119 (2)	0	80	70	40	nt	nt	nt
DSM 22 (2)	0	95	60	nt	0	5	0
#59 (3)	0	70	60	15	nt	0	nt
#66 (3)	0	95	95	90	nt	95	4
#67 (3)	1	99	80	10	nt	90	0
#100 (3)	0	70	60	65	nt	0	0
#122 (3)	0	75	70	60	60	30	7
#102 (5)	0	90	80	20	nt	23	nt
#103 (5)	5	40	10	2	nt	2	nt
#13 (6)	0	50	45	40	nt	0	nt
#14 (6)	0	85	15	10	nt	5	7
#40 (6)	0	90	60	nt	nt	85	nt
#128 (6)	2	60	10	0	nt	90	0

nt, not tested.
[a] The number is the percentage of spores among total cells formed on sporulation medium after 21 days.

The percentage of spores produced as a function of initial pH and incubation temperature was determined for the same 20 strains (Table 3). The most efficient condition for spore production was pH 7.0 and 55 °C for all strains except for three isolates (#8, #28 and #128), which formed more spores at 45 °C, and isolate #12, which formed more spores at pH 6.0. Only isolate #52 produced 85% of spores at 70 °C, whereas all the others produced less than 35% or none.

3.3. Heat-resistance of selected G. stearothermophilus strains

Survival curves were followed on 3.5 to >6 log reduction. A satisfactory fitting was obtained with at least one of the survival models, as shown by low RSS (Table 4) and by visual examination of graphs (data not shown). The t_{5D} value (time necessary to inactivate 5-log of population) at 120 °C was used to compare heat-resistance of spores produced at pH 7.0 and 55 °C (Table 4). Strains #28, #52, #67 and DSM 22 exhibited a t_{5D} value above 10 min. The t_{5D} values were between 6.4 and 8.9 min for seven strains and below 4.9 min for six strains. The lowest t_{5D} value, 1.9 min, was obtained for isolate #119. Lastly, strains #8, #14 and #46 exhibited log-reduction greater than 7.0 in 0.5 min and t_{5D} was arbitrarily set at <0.5 min. The spore wet heat-resistance differed significantly between strains (no overlap in the 95% confidence intervals of many strains). These data were compared to those recorded in a meta-analysis of *G. stearothermophilus* heat-resistance parameters (Rigaux et al., 2013). At 120 °C and pH 6.5 to 7.0, *D* values range between 1.3 min and 16.7 min with a median at 3.0 min, which gives t_{5d} between 6.5 min and 84 min, with a median at 15.0. The t_{5d} values reported in this work, although not all based on log-linear reduction, are clearly in the lower range of these previous observations.

Three out of the four most heat-resistant strains (t_{5D} > 10 min) were able to grow at the lowest temperature (40 °C), at the lowest initial pH (pH 5.0) or with 3% NaCl. The fourth strain could grow in the same conditions except at the lowest initial pH. Thus spore heat-resistance was related to the ability to grow under the most

Table 4
Heat resistance at 120 °C of *G. stearothermophilus* spores produced at 55 °C and pH 6.5.

Strain number (M13-group)	t_{5D} value (min)	95% confidence interval	RSS[a]	log-model[b]
#1 (1)	8.0	[7.7:8.4]	0.147	Biphasic
#46 (1)	<0.5			
#8 (2)	<0.5			
#12 (2)	6.6	[6.3:7.0]	0.708	Weibull
#28 (2)	11.0	[10.8:11.3]	0.011	Biphasic
#52 (2)	12.1	[11.5:13.1]	0.197	Weibull
#54 (2)	8.1	[7.8:8.4]	0.079	Biphasic
#119 (2)	1.9	[1.8:2.1]	0.032	Weibull
DSM 22 (2)	12.4	[11.8:13.0]	0.234	Linear
#59 (3)	3.6	[3.4:3.9]	1.097	Linear
#66 (3)	7.1	[6.7:7.6]	0.400	Biphasic
#67 (3)	10.7	[10.4:11.0]	0.260	Weibull
#100 (3)	2.7	[2.6:2.9]	0.141	Linear
#122 (3)	4.9	[4.8:5.1]	0.162	Weibull
#102 (5)	4.5	[4.3:4.6]	0.269	Weibull
#103 (5)	3.8	[3.7:3.8]	0.031	Weibull
#13 (6)	8.9	[8.3:9.7]	0.273	Biphasic
#14 (6)	<0.5			
#40 (6)	6.4	[6.0:7.0]	0.099	Weibull
#128 (6)	6.6	[6.5:6.7]	0.006	Biphasic

[a] RSS, Residual Sum of Squares.
[b] Models: "linear" when log-reduction was linear with time, "Weibull" when a shoulder was observed at the beginning, and "biphasic" when curves displayed a linearity with latency.

extreme temperature and pH values, although the relationship was not reciprocal. This relationship was also observed among emetic *B. cereus* strains. The highest heat-resistance of spores was combined with a shift of growth limits towards high temperature (Warth, 1978).

3.4. Relationship between genotypic group and isolate origin or phenotypic diversity

The possible relationship between genotypic group and strain characteristics or strain origin was investigated by multiple correspondence analyses (MCA) on several sets of *G. stearothermophilus* isolates tested in this work.

Several data set were analyzed in each of the three MCA performed: (i) variables "food category", "food product", "food company", "area of isolation", "year of isolation" and "genotypic group" applied to 127 strains (Supplementary Table S1), (ii) variables "vegetable production area", "isolation year" and "genetic group" applied to 51 isolates from canned vegetables (Supplementary Table S1), or (iii) variables "minimal pH of growth", "maximal pH of growth", "minimal temperature of growth", "maximal temperature of growth", "maximal sodium chloride concentration of growth" and "genetic group" applied to 20 isolates (Table 1, Table 2). Genetic grouping in each MCA was unrelated to food category or product, year or area of isolation, or food company, or phenotypic features (data not shown).

3.5. Conclusions

Regarding the strains tested in our study, the low phenotypic diversity observed may be explained by two hypotheses: either phenotypic diversity is truly weak within the species giving a monomorphic status for *G. stearothermophilus*, or the poor diversity observed is related to the common features shared by most of the strains, *i.e.* spoiled canned food after prolonged storage at 55 °C. However the M13-PCR typing should be an efficient tool to trace *G. stearothermophilus* in food processing plant. From a practical point of view, and although recommendations should be modulated by the possible presence of other spore-forming bacteria with higher temperature tolerance such as *Moorella* spp. (André et al., 2013), some process and product conditions regarding *G. stearothermophilus* as a spoilage hazard in the storage of high-temperature canned food can be proposed. For instance, as *G. stearothermophilus* can grow and sporulate at temperatures between 40 °C and 70 °C, processing line temperatures should be maintained above 70 °C. Similarly, salt addition below 3% does not exhibit a significant effect, while pH decrease to 5.0 considerably hinders the growth of most strains. Although an increase in salt concentration cannot be used for organoleptic and nutritional reasons, a decrease in food pH could be investigated, and should result in a lower frequency of long-term canned food spoilage caused by *G. stearothermophilus*. Lastly, heat-treatment process settings could be re-assessed in the light of a 20-fold range of variability for t_{5D} value at 120 °C. Strains #28, #52 and DSM 22 were the most tolerant regarding growth conditions, and exhibited high heat resistance at 120 °C: they can be proposed as test strains in canned food processing design.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.01.015.

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