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Die another day: Fate of heat-treated *Geobacillus stearothermophilus* ATCC 12980 spores during storage under growth-preventing conditions

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ABSTRACT

Geobacillus stearothermophilus spores are recognized as one of the most wet-heat resistant among aerobic spore-forming bacteria and are responsible for 35% of canned food spoilage after incubation at 55 °C. The purpose of this study was to investigate and model the fate of heat-treated survivor spores of *G. stearothermophilus* ATCC 12980 in growth-preventing environment. *G. stearothermophilus* spores were heat-treated at four different conditions to reach one or two decimal reductions. Heat-treated spores were stored in nutrient broth at different temperatures and pH under growth-preventing conditions. Spore survival during storage was evaluated by count plating over a period of months. Results reveal that *G. stearothermophilus* spores surviving heat treatment lose their viability during storage under growth-preventing conditions. Two different subpopulations were observed during non-thermal inactivation. They differed according to the level of their resistance to storage stress, and the proportion of each subpopulation can be modulated by heat treatment conditions. Finally, tolerance to storage stress under growth-preventing conditions increases at refrigerated temperature and neutral pH regardless of heat treatment conditions. Such results suggest that spore inactivation due to heat treatment could be completed by storage under growth-preventing conditions.

1. Introduction

Bacillus species spores can survive a variety of stress factors such as wet and dry heat, UV and gamma radiation, desiccation and toxic chemicals, which kill rapidly vegetative cells (Setlow, 2006). *Geobacillus stearothermophilus* spores are recognized as one of the most wet-heat resistant ($D_{121.1°C}$ = 3.3 min; $CI_{95\%}$ = [0.8; 9.6]; Rigaux et al., 2013) among aerobic spore-forming bacteria (Guizelini et al., 2012; Zhou et al., 2013). Presence of survivors after sterilization is responsible for 35% of canned food spoilage after incubation at 55 °C (André et al., 2013; Durand et al., 2015). Although wet-heat treatment is probably the oldest and the most common method used to inactivate spores in food process, the specific mechanism for wet-heat killing of spores is not completely accounted for. According to Setlow (2006), many factors linked to spore structure may contribute to wet-heat resistance. The major factor determining spore enzymatic dormancy and resistance is core water content (Cowan et al., 2003). Among *Bacillus* species, higher spore resistance is obtained for lower core water content (ranging from 27 to 55% of wet weight; Setlow, 2006). Besides core water content, other factors may be involved in moist-heat resistance of spores: (1) Accumulation of pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) and its chelated divalent cations (Ca^{2+}) in the spore core contribute to the reduction of the core water content during sporulation and to the increase in wet-heat resistance (Coleman et al., 2007; Setlow, 2006; Zhang et al., 2010a); (2) The nature of the mineral cation associated with DPA (higher wet-heat resistance is associated with a high level of Ca^{2+}) (Setlow, 2006); and (3) Saturation of spore DNA with the α/β-type SASP (small, acid-soluble spore proteins) provides protection and resistance

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proprieties to heat, chemical and UV radiation (Nicholson et al., 2000; Setlow, 2000).

Over the last decade, many studies have investigated the specific mechanism and targets whereby *Bacillus* species spores are inactivated by moist-heat. The events leading to inactivation of spores during wet-heat treatment can be summarized as follows: As moist-heat treatment proceeds, some spore proteins are damaged or denatured (Coleman et al., 2010; Zhang et al., 2011, 2010a). When loss of crucial proteins and enzymes takes place, inactivation occurs. These inactivated spores can retain CaDPA for a short period (Coleman et al., 2007). However, further exposure to heat leads to more protein damage. This presumably leads to the breakdown of the spore inner membrane, which represents the major permeability barrier to small molecules. At this stage, a rapid CaDPA release occurs (Zhang et al., 2010a).

It is well known that damage repair of heat-injured spores occurs probably during germination and outgrowth, which require rich complex media (Coleman and Setlow, 2009; Cronin and Wilkinson, 2008; Hornstra et al., 2009; Kell et al., 1998; Setlow, 2006; Stringer et al., 2011). Sub-optimal conditions of incubation or media containing selective agents such as high organic acids or salt concentrations affect the viability of heat-damaged spores (Coleman and Setlow, 2009; Mtimet et al., 2015). These supplemented stress factors act synergistically to enhance the inactivation effect of wet-heat treatment (Leistner, 2000; Leistner and Gould, 2002).

Almost all information concerning spore inactivation and heterogeneity observed during damage repair (germination and outgrowth) is provided during metabolic resumption under growth conditions (Smelt et al., 2008; Ter Beek et al., 2011; Warda et al., 2015). However, few studies have investigated the outcome of wet heat-treated spores during incubation in adverse conditions that prevent their germination and outgrowth. Canned food is generally stored at room temperature (between 20 °C and 30 °C), which represents a preventing-growth condition for strict thermophilic bacteria such as *G. stearothermophilus* (Mtimet et al., 2015). According to Leistner (1995), injured heat-treated spores cannot overcome storage stress under no growth conditions, thus leading to inactivation of survivors during storage. It should be considered that these growth-preventing conditions could act as another hurdle leading to further inactivation after a heat treatment.

The aim of this work is to explore the fate of injured heat-treated *G. stearothermophilus* spores during incubation under growth-preventing conditions. The effect of heat treatment and storage in lethal environments was investigated.

2. Materials and methods

2.1. Bacterial strain

G. stearothermophilus ATCC 12980 (DSM 22) was obtained from the American Type Culture Collection. It is the type strain which was originally isolated from spoiled canned corn and beans (Coorevits et al., 2012; Nazina et al., 2001). Aliquots of vegetative cell suspension at a concentration of 6.0 $\log_{10}$ CFU/ml were frozen at −20 °C in nutrient broth (NB, Biokar Diagnostics, Beauvais, France) supplemented with 50% (v/v) glycerol.

2.2. Assessment of growth of G. stearothermophilus ATCC 12980 for evaluation of growth/no growth interface

Pre-culture from 1 ml frozen aliquots was performed in 100 ml of nutrient broth at 57 °C for 5 h, to reach the late exponential phase (Mtimet et al., 2015). Concentration in vegetative cells was approximately 6.0 $\log_{10}$ (CFU/ml). Incubation was performed in 250 ml flasks by shaking (100 rpm).

To assess the growth of *G. stearothermophilus* ATCC 12980 at different pHs, nutrient broths were adjusted with HCl or NaOH (1 or 5N). The medium was sterilized by filtration (Stericup Filter Unit, 0.22 μm, Millipore, Darmstadt, Germany) and the final pH values were measured using a pH-meter (PHM210, MeterLab, Radiometer, Copenhagen, Danemark).

As described by Tienungoon et al. (2000), 2 ml of adjusted pH broth were pipetted into wells of 24-well plates (4 wells by 6 wells, Nunc Nunclon Delta 24-Well, Thermo-Fisher Scientific, Denmark). Each 24-well plate was used to assess growth/no growth in five conditions with four replicates. 100 μL of pre-culture were added to each well containing 2 ml of modified nutrient broth to reach a standardized initial concentration of approximately 5.0 $\log_{10}$ (CFU/ml) (Le Marc et al., 2005). Two wells served as negative controls (NB, pH 7.1 non-inoculated), while the two remaining wells served as positive controls (NB, pH 7.1 containing 100 μl of the inoculum) (Tienungoon et al., 2000). Growth abilities were assessed at seven different temperatures, and well-plate incubation was performed by shaking (100 rpm).

Well-plate cultures were checked daily to assess growth/no growth for 45 incubation days. Three cases were expected: an obvious growth when the culture turns into turbid, no change in turbidity, or doubtful. Cultures no showing obvious growth were mixed each week, and 50 μL of culture were used to confirm growth (or not) by enumeration on nutrient agar pH 7.1 (NA, Biokar Diagnostics, Beauvais, France) using an automated spiral plater (WASP1, AES Chemunex, Biomérieux, Marcy l'Etoile, France). Plates were incubated aerobically for 48 h at 57 °C, after which colonies were counted by an automatic colony counter (Scan® 1200, Interscience, Saint-Nom-la-Bretèche, France). An increase of at least one log compared to the initial concentration confirms growth. However, conditions not supporting growth cause a decline in cell number.

2.3. Evaluation of the effect of heat treatment conditions and incubation under growth-preventing conditions on heat-treated spore viability

2.3.1. Preparation of spore suspension

Late exponential phase cells (described above) were inoculated on nutritive agar adjusted at pH 6.7 with phosphate buffer 20 mM (K_2HPO_4/KH_2PO_4) and supplemented with $MnSO_4$, $7H_2O$ (5.0 mg L^{-1}) and $CaCl_2$, $2H_2O$ (5.0 mg L^{-1}) (Sigma–Aldrich, Saint Quentin Fallavier, France). Inoculated nutritive agar petri dishes were packaged in plastic bags to avoid drying, and were incubated at 57 °C. In these conditions, a high sporulation ratio and a high heat-resistance can be achieved (Mtimet et al., 2015). When more than 95% of free refractive spores were observed (phase-contrast microscopy: Olympus BX50, Olympus Optical, Germany; 10 × 100 Oil Ph3), plates were flooded with sterile distilled water, and spores were harvested by scraping the agar surface. Spores were harvested by centrifugation, washed 5 times (8000 g for 30 min) in sterile ultrapure water (Millipore Simplicity Water Purification System, Germany) and were stored at 4 °C for one month at least before using in experiments. To evaluate the variability, three spore batches were performed independently as described above. These three batches were used to replicate 8 of the 29 conditions of the experimental design.

2.3.2. Heat treatment

Heat treatments were performed according to the capillary tube method (200 μL glass capillary tubes, Hirschman Laborgeräte, Germany) (Mtimet et al., 2015). Spore stock suspensions were