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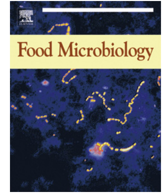

In situ investigation of *Geobacillus stearothermophilus* spore germination and inactivation mechanisms under moderate high pressure

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A B S T R A C T

Bacterial spores are a major concern for food safety due to their high resistance to conventional preservation hurdles. Innovative hurdles can trigger bacterial spore germination or inactivate them. In this work, *Geobacillus stearothermophilus* spore high pressure (HP) germination and inactivation mechanisms were investigated by *in situ* infrared spectroscopy (FT-IR) and fluorometry. *G. stearothermophilus* spores' inner membrane (IM) was stained with Laurdan fluorescent dye. Time-dependent FT-IR and fluorescence spectra were recorded *in situ* under pressure at different temperatures. The Laurdan spectrum is affected by the lipid packing and level of hydration, and provided information on the IM state through the Laurdan generalized polarization. Changes in the $-CH_2$ and $-CH_3$ asymmetric stretching bands, characteristic of lipids, and in the amide I′ band region, characteristic of proteins' secondary structure elements, enabled evaluation of the impact of HP on endospores lipid and protein structures. These studies were complemented by *ex situ* analyses (plate counts and microscopy). The methods applied showed high potential to identify germination mechanisms, particularly associated to the IM. Germination up to 3 $\log_{10}$ was achieved at 200 MPa and 55 °C. A molecular-level understanding of these mechanisms is important for the development and validation of multi-hurdle approaches to achieve commercial sterility.

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1. Introduction

Bacterial endospores constitute a risk for food processors due to their strong resistance to both chemical and physical hurdles (Esty and Meyer, 1922; Heinz and Knorr, 1996; Sale et al., 1970; Setlow, 2000, 2006). Presence of bacterial spores in a food matrix or on processing equipment may lead to food (re)contamination following germination, and jeopardize consumer safety as well as product quality. Exemplarily, bacteria such as *Clostridium botulinum*, which produces botulin toxin (LD50 of 1 ng/kg), highlight the necessity of inactivating bacterial spores prior to commercialization (Black et al., 2007; Esty and Meyer, 1922).

To counter this problem, inactivation strategies both for food and equipment surfaces have been developed based on thermal or a combination of thermal and chemical treatments (Georget et al., 2013). Though effective, these strategies often rely on incomplete knowledge of the mechanisms at stake leading to over processing and/or low product quality.

Alternative, innovative hurdles have been suggested to achieve food safety while improving product quality (Koutchma et al., 2005). Notably, high pressure represents a promising alternative for pasteurization (Palou et al., 1999). It has been implemented for several years now in different sectors of the food industry (Mújica-Paz et al., 2011). However, it was highlighted from early on that high pressure alone is insufficient to achieve bacterial spore inactivation, a prerequisite to sterilization and ambient temperature storage (Basset and Macheboeuf, 1932; Hite et al., 1914; Larson et al., 1918). The potential of high pressure as a sterilizing technology was highlighted only later on through its capacity to trigger bacterial spore germination (Gould and Sale, 1970; Heinz, 1997; Heinz and Knorr, 1998; Reineke, 2012) or inactivation when combined with temperature (Ananta et al., 2001; Heinz and Knorr, 1998; Knoerzer

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et al., 2007; Mathys, 2008; Mathys et al., 2009). The inability to stimulate full germination and equipment limitations for high pressure thermal sterilization are still limiting the implementation of HP[1] technology for food sterilization. In particular, the incomplete knowledge of the pathways at stake during HP germination, a key step in reducing spore thermal resistance, has been pointed out (Abel-Santos, 2012) and hinders the validation of this technology for the food industry (Hendrickx and Knorr, 2002; Mathys, 2008; Reineke, 2012).

To date, most studies conducted to improve the understanding of germination mechanisms could only be conducted via *ex situ* analyses and little is known about the transformations occurring during the high pressure process itself. Additionally, most of the knowledge gathered in the literature focuses on germination of *Bacillus subtilis* (Black et al., 2005; Heinz, 1997; Reineke, 2012; Setlow, 2003, 2007; Vepachedu et al., 2007; Wuytack, 1999), which is one of the best known strains, but not necessarily the most relevant for the food industry.

Reviews of the current knowledge on bacterial spore germination have strongly emphasized the key role played by the inner membrane in the regulation of the associated mechanisms even though many unknowns remain, in particular regarding the first phase of the germination (Paidhungat and Setlow, 2002; Setlow, 2003; Stewart et al., 1981; Yi et al., 2011). Considering the importance of the inner membrane in germination, the current lack of knowledge about this structure is a limitation.

The inner membrane of bacterial spores was reported to have a composition very close to the cytoplasmic membranes of growing cells (Cortezzo and Setlow, 2005). One of the key structural models established so far is the Fluid Mosaic Model which represents the biological membrane essentially as a two-dimensional fluid with embedded proteins (Singer and Nicolson, 1972). The proportion of proteins in such membranes is between 20% and 35% (Stevens and Arkin, 2000) and these proteins are localized in a lipid environment. Besides its unique properties, the inner membrane has a set of unique proteins, including the nutrient receptors and proteins, such as those encoded by the *spoVA* operon, possibly involved in movement of core molecules across the inner membrane. As these proteins function while being localized in the inner membrane, they are likely to be largely immobile in this environment (Setlow, 2008). Membrane lipids and proteins may influence each other directly as a result of their biochemical nature and in response to environmental changes (Winter and Jeworrek, 2009). This scenario is particularly relevant for bacterial spores' inner membrane, where the presence of proteins plays a key role in the germination process under pressure (Setlow, 2007). It has been suggested that the highly and unusual compact and impermeable state of this membrane impacts on the proteins' mobility as well as functionality (Cowan et al., 2003, 2004). However, *in situ* pressure studies are still very scarce. The investigation of the pressure effects on Na^+, K^+-ATPase reconstituted into phospholipid bilayers showed that HP above 220 MPa irreversibly changed the protein conformation, probably because of the dissociation and partial unfolding of the subunits (Powalska et al., 2007), highlighting the need for further studies *in situ* of the bacterial spores' inner membrane under pressure.

In this work, we investigated the germination mechanisms of *Geobacillus stearothermophilus* spores, an industrial thermal sterilization indicator, by means of high pressure (200 MPa) and temperature up to 55 °C, using a combination of *in* and *ex situ* analyses to improve the understanding of the phenomenon at stake under high pressure conditions. A series of methods was developed or adapted to this bacterium to achieve the *in situ* analysis under pressure of the inner membrane, a key player in germination pathways, as well as of the protein secondary structures.

2. Material and methods

2.1. Bacterial strain

The strain used in this work was *G. stearothermophilus* ATCC 7953 obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, and stored at −80 °C at the German Institute of Food Technologies (DIL).

G. stearothermophilus spores were obtained following successful development of a sporulation method for this indicator enabling >95% spores, as described hereafter.

2.2. Sporulation method

By modifying a method described elsewhere for *B. subtilis* (Nicholson and Setlow, 1990; Paidhungat and Setlow, 2000) and adapting it to *G. stearothermophilus*, a high sporulation ratio could be achieved. Repeatable sporulation was achieved in less than a week. A single colony of *G. stearothermophilus* grown on nutrient agar over night was used to inoculate 10 mL of TSB medium (Tryptic Soy Broth Fluka T8907-500G from Sigma–Aldrich Company, Germany – 30 g/L in distilled water). The TSB cultures were incubated at 55 °C and 250 rpm in a shaking incubator for a minimum of 5 h. After 5 h, the OD_{600} was checked every 30 min until the required density was reached (1.6–1.8/control with cell density meter Ultrospec 10 from Amersham Biosciences GmbH, Germany). A 200 μL aliquot of the culture was then spread onto Difco agar plates (for 1 L distilled water: pepton 5 g, meat extract 3 g, Agar 20 g, KCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.12 g, 1 M $Ca(NO_3)_2$ 1 mL*, 0.01 M $MnCl_2$ 1 mL*, 1 mM $FeSO_4$ 1 mL*[2]). The plates were sealed in plastic bags and incubated at 55 °C for a minimum of 3 days. Sporulation was monitored via phase brightness with a transmitted-light microscope daily. When more than 95% sporulation was observed, plates were removed from the bags and left at room temperature for drying. The plates were controlled under phase contrast microscope each day until remaining vegetative cells were dried out; following which the spores were collected using 4 °C distilled water and cleaned by repeated centrifugation at 4800 g, 4 °C for 30 min until the supernatant was clean. Following sporulation, spores were stored at −80 °C until further use.

2.3. Laurdan staining of the inner membrane

Based on the method previously developed by Hofstetter et al. (Hofstetter et al., 2012; Hofstetter, 2012) detailing the use of the fluorescent probe Laurdan to label and measure inner membrane fluidity of endospores of *Clostridium* spp., fluorescent staining of *G. stearothermophilus* spore inner membrane was conducted with the sporulation method described above. Laurdan was selected as fluorescent dye because of its non-cytotoxicity (Owen et al., 2012), enabling further evaluation of the physiological state of spores by plate count, for instance.

Endospores containing Laurdan were generated by plating 400 μL of saturated Laurdan (6-Dodecanoyl-N,N-dimethyl-2-naphthylamine, purchased from Sigma–Aldrich, Germany) suspended in 100% ethanol onto Difco agar plates, allowing the ethanol

[1] FIB: Focused Ion Beam FT-IR: Fourier Transform Infrared GP: Generalized Polarization HP: High Pressure SEM: Scanning Electron Microscopy.

[2] Components marked with an * were sterile filtered and added post autoclaving.

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