**Geobacillus stearothermophilus** ATCC 7953 spore chemical germination mechanisms in model systems

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**A B S T R A C T**

Bacterial endospores through their strong resistance to both chemical and physical hurdles constitute a risk for the food industry. Inactivation strategies are based on thermal and/or chemical treatments but rely on incomplete knowledge of the mechanisms of inactivation. Alternative strategies were suggested to achieve food safety while improving product quality. One of them relies on the successive germination and inactivation by pasteurization of bacterial spores. However, to date, a gap of knowledge on bacterial spore germination remains and hinders such an application for food sterilization. *Geobacillus stearothermophilus* ATCC 7953 spore germination mechanisms were investigated by in situ fluorometry combined with plate counts. *G. stearothermophilus* spores’ inner membrane was stained with Laurdan fluorescent dye. While nutrient pathways showed no strong germination with the combinations tested, successful germination up to 3 log10 could be achieved using 60 mmol l−1 calcium-dipicolinic acid (CaDPA) at 55 °C for 2 h. A model for the CaDPA germination mechanism in two phases could be derived which suggested a potential key role of cortex fragments in the germination path, before completion of the cortex degradation. Additionally, it was confirmed that the germination potential of CaDPA, which does not rely on nutrient receptors, is a widespread germination trigger across spore formers. Understanding germination mechanisms and the limitations of different germination paths is important for the development of multi-hurdle approaches to achieve commercial sterility with reduced thermal load.

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1. Introduction

Many members of the gram positive bacterial orders are able to survive starvation by forming dormant, resistant spores. Bacterial endospores through their strong resistance to both chemical and physical hurdles constitute a risk for the food industry (Esty & Meyer, 1922; Heinz & Knorr, 1996; Sale, Gould, & Hamilton, 1970; Setlow, 2000, 2006). Inactivation strategies for food and equipment surfaces have been based on thermal and chemical treatments but rely mostly on incomplete knowledge of the mechanisms at stake, leading to over processing and/or low product quality (Georget et al., 2013). Alternative hurdles have been suggested to achieve food safety while improving product quality, which do not solely depend on very strong thermal treatments (Knorr & Heinz, 2001). One of them is based on germination and successive inactivation by pasteurization of bacterial spores (Gould, 2006). Even being dormant, these spores constantly monitor their environment and under conditions advantageous for growth, in particular the presence of nutrients, they can germinate, outgrow and ultimately become growing vegetative cells (Setlow, 2003). Germination is an early and important step in this process and is of high interest since it weakens the bacterial spore to pasteurization treatments through core partial rehydration. Unfortunately, the inability to stimulate full germination of spores is still limiting the implementation of this approach for food sterilization. In particular, the incomplete knowledge of the pathways at stake during germination has been mentioned (Abel-Santos, 2012) and hinders the validation of this approach for the food industry (Mathys, 2008; Reineke, 2012).

Nutrient germinants of low molecular weight include amino acids, purine derivatives, and sugars. These nutrient germinants
usually trigger the germination of bacterial spores in the environment through what is speculated to be stereospecific binding to nutrient receptors localized on the inner spore membrane (Paidhungat & Setlow, 2000; Paredes-Sabja, Setlow, & Sarker, 2011). Most commonly, L-Alanine in combination with Bacillus subtilis spores has been extensively investigated (Stewart, Johnstone, Hagelberg, & Ellar, 1981). There is some evidence that specific spore coat proteins make it easy for such exogenous germinants to pass through the outer layers of Bacillus spores, but how this is accomplished is not known (Behravan, Chirakkal, Masson, & Moir, 2000; Carr, Janes, & Hanna, 2010). This germination path is mostly referred to as nutrient or physiological germination. To date, the best established fact is that the initial interaction of the germinant with the spore forms the trigger reaction and irreversibly commits the spore to undergo the complex series of germination events (Setlow, 2003; Stewart et al., 1981). Moreover, sub lethal heat treatments also were suggested to enable what was described as “heat activation” which increases the rate and extent of nutrient germination (Curran & Evans, 1945; Finley & Fields, 1962; Stewart et al., 1981). While activation is seen as a reversible process (Keynan, Evenchik, Halvorson, & Hastings, 1964), the mechanism of spore activation is still not fully understood (Aiba & Toda, 1966; Paidhungat & Setlow, 2002). Most clearly, the inner spore membrane appears as one of the key spore structures where the current knowledge gap is critical. The inner membrane of bacterial spores was reported to have a composition very close to the cytoplasmic membranes of growing cells (Cortezzo & Setlow, 2005) where the proportion of proteins is expected to be between 20% and 35% by comparison to the Fluid Mosaic Model (Singer & Nicolson, 1972; Stevens & Arkin, 2000). These proteins are localized in a lipid environment and the inner membrane carries 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 expected to be largely immobile in this environment (Setlow, 2008b). It was indeed suggested that the unusual highly compact and impermeable state of this membrane impacts on the proteins’ mobility as well as functionality (Cowan, Koppel, Setlow, & Setlow, 2003; Cowan et al., 2004). However, in situ studies are still very scarce.

Non-nutrient germination pathways also exist which do not require nutrient receptors. The best characterized is exogenous dipicolinic acid and its 1:1 calcium chelate (CaDPA), a substance required nutrient receptors. The best characterized is exogenous exogenous scarce. It is expected to be largely immobile in this environment (Setlow, 2008a). This led to wonder whether the initiation of cortex degradation might trigger germination through activation by the resulting peptidoglycan fragments, before mechanical rehydration occurs due to full cortex degradation (Setlow, 2008a).

A literature overview has finally shown that in recent times, little attention has been given to the investigation of Geobacillus steareothermophilus spore germination mechanisms (Campbell & Williams, 1953; Cheung et al., 1982; O’Brien & Campbell, 1957). Most of the studies on going in this field have focused on different strains and thus, the current state of the art in terms of mechanistic studies of spore germination was related to spores of B. subtilis, Bacillus cereus, Bacillus megaterium or spores the Clostridium genus (Nerandzic & Donskey, 2013; Paredes-Sabja et al., 2011; Pelczar & Setlow, 2008; Ramirez & Abel-Santos, 2010). Some of these studies in particular suggested the use of combined germination and decontamination either through antimicrobials or pasteurization as a mean of sterilization (Nerandzic & Donskey, 2013; Worthington, 2011). In order to validate such approaches, a sufficiently universal substance needs to be identified in order to eradicate a large spectrum of spores of different strains and families.

With regard to the above introduced facts, further investigations of the mechanisms of spore germination are needed to understand and control this process. Applications of this knowledge could be to heat-sensitize spores in food matrices or, conversely, to prevent germination to take place, targeting the control of spores rather than inactivation. In this work, chemical germination of G. steareothermophilus ATCC 7953 spores, an industrial thermal sterilization indicator (European Pharmacopoeia Commission, 1997), was investigated at 55 °C (optimal growth temperature of the indicator) by nutrient and non-nutrient chemicals. A series of in and ex situ analyses previously used for this bacterium (Georget et al., 2014) was applied to achieve the in situ analysis of the inner membrane, a key player in germination pathways, during germination.

2. Material and methods

2.1. Bacterial strain

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

2.2. Sporulation method

Sporulation of G. steareothermophilus was achieved in less than a week following the method described elsewhere (Georget et al., 2014). A single colony of G. steareothermophilus grown on nutrient agar overnight was used to inoculate 10 ml of TSB medium (Tryptic Soy Broth Fluka T8907-500G from Sigma–Aldrich Company, Germany — 30 g l−1 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 OD600 was checked every 30 min until the required density was reached (1.6—1.8) with cell density meter Ultrospec 10 from Amersham Biosciences GmbH, Germany). A 200 µl aliquot of the culture was then spread onto Difco sporulation plates (Schaefler, Millot, & Aubert, 1995). The plates were sealed in plastic bags and incubated at 35 °C for a minimum of three days. Sporulation was monitored by controlling the phase brightness with a transmitted-light microscope daily. When more than 95% sporulation was observed by Thoma chamber, plates were removed from the bags and left at room temperature for drying. The plates were monitored 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 4900 g, 4 °C for 30 min until the supernatant was clean. Following cleaning, the spores were suspended in distilled water and kept at −20 °C until further use within a time window of six months.
2.3. Laurdan staining

Lipid packing/order can be quantified by fluorometry of an environment-sensitive fluorophore such as 6-Dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan) (Molina-Höppner, Doster, Vogel, & Gänzle, 2004; Sezgin et al., 2012). The fluorescent naphthalene moiety of the Laurdan molecule possesses an electrical dipole moment, which increases upon excitation, and may cause reorientation of the surrounding solvent dipoles. Solvent reorientation results in a decrease of the excited state energy of the probe, which is reflected in a red shift of the probe’s emission spectrum (Sánchez, Tricerri, Gunther, & Gratton, 2007).

Based on an adaptation of the method developed previously (Hofstetter, Denter, Winter, McMullen, & Gänzle, 2012; Hofstetter, 2012), Laurdan fluorescent staining of G. stearothermophilus spores’ inner membrane was achieved with the sporulation method described above and as explained elsewhere (Georget et al., 2014). Laurdan (Sigma Aldrich, Hamburg, Germany) was selected as fluorescent dye because of its non-cytotoxicity (Owen, Rentero, Magenau, Abu-Sinijeh, & Gaus, 2012), enabling further evaluation of the physiological state of spores by plate count, for instance.

A single batch of stained spores of G. stearothermophilus was used for this study. Using a Thoma chamber, the spore percentage was estimated to be 94.1% ± 1.5% (average of four counts and corresponding standard deviation). Additionally, and to establish a reference in terms of membrane fluidity, vegetative cells of G. stearothermophilus were stained with Laurdan adapting a method described elsewhere (Molina-Höppner et al., 2004). Overnight cell culture in TSB broth were centrifuged at 2700 g for 5 min and suspended in sterile 0.9% saline twice. Laurdan suspended in ethanol was added to the cell suspension to a final concentration of 40 μM. The staining was conducted for 30 min at 55 °C in the dark following which cells were washed twice by centrifuging at 2700 g for 5 min and finally re-suspended in sterile saline 0.9%.

2.4. Chemical germination

2.4.1. Nutrient germination

Previous genomic analyses underlined the presence of homologs of the B. subtilis germinant receptors genes as well as gerD and spoVAB, C, D genes in other strains of the Geobacillus genus, thus suggesting potential for germination of G. stearothermophilus spores with l-Alanine or AGFK (Paredes-Sabja et al., 2011; Pelczar & Setlow, 2008).

Therefore, the potential of individual amino acids to trigger G. stearothermophilus spore germination was assessed. 16 l-amino acids (l-Alanine, l-Aspartic acid, obtained from Fluka, Sigma Aldrich, Hamburg, Germany and l-Leucine, l-Arginine, l-Lysine, Glycine, l-Histidine, l-Methionine, l-Proline, l-Threonine, l-Isoleucine, l-Tryptophan, l-Tyrosine, l-Glutamine, l-Valine, l-Phenylalanine, obtained from Carl Roth, Karlsruhe, Germany) were stained with Laurdan adapting a method described elsewhere (Molina-Höppner, Doster, Vogel, & Gänzle, 2004). Over-night cell culture in TSB broth were centrifuged at 2700 g for 5 min and suspended in sterile 0.9% saline twice. Laurdan suspended in ethanol was added to the cell suspension to a final concentration of 40 μM. The staining was conducted for 30 min at 55 °C in the dark following which cells were washed twice by centrifuging at 2700 g for 5 min and finally re-suspended in sterile saline 0.9%.

was within the precision of the method. Heat activated and non-heat activated spore suspensions were distributed between the Eppendorf tubes, centrifuged at 4800 g and 4 °C for 5 min and re-suspended each with the respective nutrient solutions to be tested. All samples were germinated for 2 h at 55 °C. During germination, the Eppendorf tubes were shaken to prevent sedimentation. After germination, the CFU ml−1 for each sample was determined by plate count before and after heat inactivation at 80 °C for 20 min to quantify the germinated population.

Furthermore, additional experiments were conducted with combined l-Alanine and inosine, previously underlined as co-germinant (Dodatko et al., 2009; Luu, Akochere, Patra, & Abel-Santos, 2011; Pinzón-Arango, Nagarajan, & Camesano, 2010), as well as with AGFK which consisted of 100 mmol l−1 l-Asparagine, 10 mmol l−1 D-glucose-monohydrate, 10 mmol l−1 D-fructose and 50 mmol l−1 KCl (AGFK) in PBS buffer, pH 7 (Cabrera-Martinez, Tovar-Rojo, Vepachedu, & Setlow, 2003; Paredes-Sabja, Torres, Setlow, & Sarker, 2008; Pelczar, Igarashi, Setlow, & Setlow, 2007; Ramirez & Abel-Santos, 2010; Wuytack, Soons, Poschet, & Michiels, 2000).

For l-Alanine and inosine, different heat activation temperatures and incubation durations were tested (80 or 100 °C) taking over earlier work on G. stearothermophilus spores (Finley & Fields, 1982; Foerster, 1983) and incubation up to 24 h. In all cases, germination was assessed by the plate count method with or without heat inactivation post germination and by the measurement of the optic density (OD) during the germination. For OD controls, the trials were conducted in reagent tubes with the spore suspension set at OD =1 in the preheated nutrient solutions. All samples were germinated during 2 h at 55 °C and the OD of samples was measured at 600 nm along time (Ultrspec 10 Cell Density Meter, classic, GE Healthcare, Germany).

2.4.2. Non-nutrient germination by dipicolinic acid and its chelates

CdDPA, NaDPA and pure DPA were considered as potential germinant for G. stearothermophilus spores following suggestions of previous studies (Fields & Frank, 1969; Perez-Valdespino et al., 2013).

DPA (Sigma—Aldrich, Hamburg, Germany) and chloride salts (CaCl2, NaCl Carl Roth, Karlsruhe, Germany) were diluted with Tris buffer at a concentration of 60 mmol l−1 and adjusted to the pH level 8.0 following the optimized conditions described elsewhere (Ghosh & Setlow, 2010; Gould, 1971; Magge et al., 2008). The potential effect of heat activation was evaluated for each condition by treating in parallel non-heat activated spores and spore suspensions that had been heated at 80 °C for 30 min and cooled down on ice. Heat activated and non-heat activated spore suspension were centrifuged at 4800 g, 4 °C for 5 min and re-suspend with the 60 mmol l−1 DPA chelate solutions tested. All samples were germinated during 2 h at 55 °C and were plated before and after heat inactivation of germinated spores at 80 °C for 20 min.

The CdDPA germination kinetic was determined through heat inactivation after different incubation times. In order to determine the kinetic of phase I germination by CdDPA as suggested in the Section 3 (CdDPA release and partial core hydration leading to heat sensitization resulting from activation by cortex fragments), a small sample (80 μl) was taken out of the spores’ suspension at given time intervals and subjected to rapid inactivation at 80 °C during 20 min in preheated tubes and then cooled and kept in ice, with subsequent plate count. Additionally, the samples used for this work were Laurdan stained and monitoring the generalized polarization (GP) signal enabled to follow the second suggested phase of CdDPA germination (inner membrane extension upon completed cortex degradation). A sample was extracted from the experimental spore suspension at given time intervals for spectra acquisition.
All graphical representations in this work were plotted with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA). The bars in the figures represent the standard deviations associated to triplicates.

### 3. Calculation

#### 3.1. Generalized polarization

As introduced in the Section 2.3, Laurdan emission spectra exhibits a red shift in the liquid-crystalline fluid-like phase of lipid membranes compared with the ordered gel phase which is characterized by a dehydration of the lipid’s upper chain region, due to dense lipid packing. For Laurdan, these two extreme conditions of lipid bilayers have been studied in detail and the wavelength maxima for the two extremes were found to be at 440 nm for ordered gel phases and at 490 nm for disordered liquid-crystalline phases (Harris, Best, & Bell, 2002; Owen et al., 2012; Parasassi, De Stasio, Ravagnan, Rusch, & Gratton, 1991). Since wavelength displacements may be challenging to characterize only by peak wavelengths, the GP presents a more precise parameter to quantify the polarity change (i.e., shift of the emission maximum). The detailed concept of GP was introduced elsewhere (Parasassi, De Stasio, d’Ubaldino, & Gratton, 1990) and the reader is referred to this work for additional details. GP is defined by Eq. (1) (Parasassi et al., 1991) and was used to characterize the lipid bilayer state in the bacterial spore membrane during the different treatments.

\[
GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}
\]

\(I_{440}\) and \(I_{490}\) correspond to the fluorescence emission intensities measured at 440 nm and 490 nm, respectively, following excitation at 360 nm. The GP values are limited between +1 and −1 but practically, values range from −0.3 to 0.6 (Hofstetter, 2012; Parasassi, Krasnowska, Bagatelli, & Gratton, 1998).

An FP-8500 Fluorescence Spectrometer (Jasco, Germany) was used in this study. Excitation light (at 360 nm) was obtained with a Xe lamp source and emission spectra collected from 400 to 550 nm. Laurdan-stained spores where suspended to a final OD_{600} of 0.5.

#### 3.2. Modeling approach for CaDPA germination

A two phase kinetic model was tested to represent the CaDPA isothermal germination kinetics of G. stearothermophilus spores. The aim was to assess whether a full cortex degradation (and corresponding core and inner membrane extension) was occurring before or after the release of CaDPA and partial core hydration. In this model, the first step (termed phase I) corresponds to the release of CaDPA from the core and partial core hydration as could be assessed by inactivation at 80 °C 20 min post germination and plate counts. The second step (termed phase II) accounts for the full cortex degradation, resulting in a core swelling and inner membrane extension upon further hydration, as could be monitored through Laurdan GP kinetics. Although these two stages are by now accepted for nutrient induced germination, CaDPA germination (a non-nutrient triggered germination) might follow a different mechanism which has to date not been fully elucidated (Bassi, Cappa, & Cocconcelli, 2012). It was previously suggested that the cortex degradation might be an initiating stage leading to rehydration (Cowen et al., 2003) and the reverse hypothesis is being considered in this work. The objective was to derive and compare the different kinetic rates for these two phases and test whether the heat sensitization of spores occurs before or after the complete cortex degradation. Based on the \(in\) and \(ex\) \(situ\) data collected, a multi-response kinetic model was tested to derive a global model for CaDPA germination. The two step model in Eq. (2) was suggested, based on a mechanistic model for chemical reactions, and solved with a non-linear multi-response regression. The set of the differential equations used was Eqs. (3)–(5), with \(k_a\) and \(k_b\) as rate constants.

\[
\begin{align*}
dormant - k_a & \rightarrow \text{germinated phase I} \\
\text{germinated phase I} - k_b & \rightarrow \text{germinated phase II}
\end{align*}
\]

\[
\frac{d[\text{dormant}]}{dt} = -k_a[\text{dormant}]
\]

\[
\frac{d[\text{germinated I}]}{dt} = k_a[\text{dormant}] - k_b[\text{germinated I}]
\]

\[
\frac{d[\text{germinated II}]}{dt} = k_b[\text{germinated I}]
\]

The set of differential equations (3)–(5) was solved with the differential equation solver Berkeley Madonna (Version 8.0.1, R.I. Mackey & G.F. Oster, University of California at Berkeley, CA, USA) and the rate constants optimized based on the experimental data obtained from plate counts and Laurdan GP. The modeled equations and source data were plotted with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA).

The final form of the integrated differential set of equations can be seen in Eqs. (6)–(8).

\[
[\text{dormant}] = [\text{dormant}]_0 \cdot e^{-k_at}
\]

\[
[\text{germinated I}] = \frac{k_a}{k_b - k_a} \cdot [\text{dormant}]_0 \cdot (e^{-k_at} - e^{-k_{bt}})
\]

\[
[\text{germinated II}] = [\text{dormant}]_0 \cdot \left[1 - \frac{k_b \cdot e^{-k_{bt}} - k_a \cdot e^{-k_at}}{k_b - k_a}\right]
\]

### 4. Results

#### 4.1. Laurdan staining of G. stearothermophilus spores’ inner membrane and impact on the heat resistance

Laurdan staining allowed for the assessment of the inner membrane state in the non-treated spores of G. stearothermophilus. It was achieved as described in previous work (Georget et al., 2014). In this work, it was compared to the one of the vegetative cells of the same strain (Fig. 1).

To confirm the location of the stain in the inner membrane, decoating trials were conducted using an adaptation of a method described elsewhere (Fitz-James, 1971) with all incubations conducted at 55 °C. Decoating removes the outer layers of the spores including the outer membrane which would also be expected to contain Laurdan. To measure the efficiency of the decoating, the inactivation of decoated spores by a lysozyme treatment at 55 °C was assessed as described elsewhere (Klobuchar, Ragkossi, & Selitow, 2006) and a complete inactivation of decoated spores compared to non-decoated spores was achieved. Finally, the spectra obtained for vegetative cells of G. stearothermophilus are also shown and give a benchmark for the liquid crystalline membrane of this organism (Fig. 1).

Using equation (2), the Laurdan GP value calculated before and after decoating amounted to 0.68 and 0.68 respectively (average of two repetitions). GP of vegetative cells of G. stearothermophilus...
amounted to 0.31 which corresponds to GP expected in lipid liquid crystalline-like phases (ranges from −0.3 to +0.3). A comparison of the decimal reduction time (D) and z values of stained and non-stained spores of *G. stearothermophilus* at sterilization temperature was conducted using the capillary method as described elsewhere (Mathys, 2008). A linear Arrhenius-type equation was used to fit the experimental rate constants \( k(T) \) obtained at 110, 121 and 130 °C in equation (1) and derive the energy of activation \( E_a \).

\[
\ln k(T) = -\frac{E_a}{RT} + \ln k_0
\]

The aim was to ensure that the staining protocol applied did not compromise the spore characteristics, notably its inner membrane properties, which could have resulted in biased further results. The results presented in Table 1 show no major difference in the D, z-value and \( E_a \) between stained and non-stained spores and if any would only suggest a slight increased resistance of stained spores at higher temperature.

### 4.2. Germination of *G. stearothermophilus* spores by single or combined nutrients

A broad range of individual L-amino acids and one purine base were tested for their potential to trigger nutrient germination of *G. stearothermophilus* spores. However, regardless of the use of heat activation or not, no individual amino acid or purine base tested had a significant advantage over the reference treatment (PBS buffer alone) (Fig. A1 appendix). In the best case, between 0.5 and 1 log\(_{10}\) germination could be achieved. Although significant from a microbiological perspective, these results remained largely insufficient for any food application and failed at underlining an individual component which could induce a full nutrient germination when included in a food matrix for instance. Combined L-Alanine and inosine at different heats of activation (80 °C or 100 °C) also showed no benefits in terms of germination and increase in the overall incubation duration up to 24 h also did not show any increased germination. The OD decrease was not different in the PBS reference and in the samples incubated with L-Alanine (with and without inosine) reached a maximum of approximately 25% OD reduction which matches the <0.2 log\(_{10}\) germination established by plate counts. Finally, germination with the AGFK mixture over 2 h led to a germination of <0.2 log\(_{10}\) which was once more not sufficient for the potential application considered (data not shown).

### 4.3. Germination of *G. stearothermophilus* spores with DPA chelates

DPA chelates (Ca\(^{2+}\) and Na\(^+\)) and pure DPA (60 mmol l\(^{-1}\)) were prepared in Tris buffer pH 8.0, as described previously (Ghosh & Setlow, 2009; Paidhungat & Setlow, 2000). Fig. 2 shows the results of germination with DPA and DPA chelates during 2 h. Germination with CaDPA and successive thermal inactivation gave the best results and reached about 3 log\(_{10}\) spore reduction following inactivation of the heat sensitized population. The difference between heat activated and non-heat activated spores was not significant. CaDPA having shown the most complete germination, it was further investigated for the determination of germination kinetics as described in Section 3.

Fig. 3 shows the normalized fluorescent signal intensity as function of the wavelength in nanometers. The highest intensity laid in the boundaries of 420 and 440 nm in agreement with previous data (Fig. 1). Along with germination, the relative intensity at 490 nm increased in time and for easier data analysis, these results are presented in terms of Gerian GP vs. time of incubation in Fig. 4. Fig. 4 presents the log\(_{10}\)-reduction corresponding to the germinated population in time as well as the Laurdan GP value in time of the spore batch being germinated. In model lipid membranes, GP

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**Table 1**

Comparison of D and z-value of stained and non-stained spores of *G. stearothermophilus* at sterilization temperatures 121 and 130 °C (standard deviation under brackets).

<table>
<thead>
<tr>
<th>( T ) (°C)</th>
<th>121</th>
<th>130</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D</strong> (s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stained</td>
<td>89.7 (10.1)</td>
<td>5.1 (1.5)</td>
</tr>
<tr>
<td>Stained</td>
<td>128.7 (33.4)</td>
<td>10.5 (0.3)</td>
</tr>
<tr>
<td><strong>( E_a )</strong> (kJ mol(^{-1}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stained</td>
<td>386.4 (16.9)</td>
<td></td>
</tr>
<tr>
<td>Stained</td>
<td>445.7 (40.6)</td>
<td></td>
</tr>
<tr>
<td><strong>z-value</strong> (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stained</td>
<td>7.6 (0.3)</td>
<td></td>
</tr>
<tr>
<td>Stained</td>
<td>8.4 (1.4)</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2.** Germination with 60 mmol l\(^{-1}\) CaDPA [●], NaDPA [–], DPA [□] and Tris reference trial [●] during 2 h at 55 °C (initial concentration of spores: 10\(^7\) CFU ml\(^{-1}\)) and heat inactivation step at 80 °C for 20 min. The logarithmic reduction shows the germinated heat sensitized spores. A: non-heat activated spores; B: heat activated spores at 80 °C for 30 min.
values, which are equal or higher than 0.5, correspond to a gel phase of the lipids in the inner membrane. Values which are below 0.3 imply a liquid crystalline phase (Sánchez et al., 2007). These values were used as benchmark to assess the changes observed in this work.

As previously described, dormant spores are characterized by the presence of a gel phase of the phospholipids, while the lipid structure of the vegetative cells represents the liquid crystalline phase (Fig. 1) (Cowan et al., 2004; Georget et al., 2014). After 20 min already about 90% of the spores have become heat sensitive (~1 log10 reduction), and a part of this population had also fully degraded its cortex (decrease of the GP value parallel to the log reduction). The GP, which is an average of the signal emitted by all spores, starts decreasing from ~5 to 8 min onwards after the beginning of germination and it can be inferred that this is due to a fraction of spores whose relative emission has shifted towards 490 nm, due to completed cortex degradation and resulting inner membrane extension. The reason why the absolute GP value is still in the gel phase domain is because only a limited fraction of the spores have shifted to germination phase II while the others have not yet. These results suggest a strong heterogeneity of the spore population in germination commitment within the first 20 min of germination. After 20 min already about 90% of the spores have completed the first phase of germination (~1 log10 reduction), and it is noticeable that a part of this population has also begun to transit to phase II of the germination (decrease of the GP value parallel to the log reduction). After 30 min no further shift in Laurdan GP is measured and it can be concluded that >90% of spores have also completed the full cortex degradation and full core hydration/inner membrane extension. This occurs about 10 min after reaching the first log of heat sensitized spores, and suggests that the hydration of the core takes place before the full cortex degradation.

In order to model the kinetics of the suggested germination phase I and II and based on the information above, the data had to be put on a comparative scale and were represented as percentages. Typically, the log reduction could be expressed in percent of remaining spores after germination and inactivation of heat sensitized spores, which corresponds to the dormant population. The Laurdan GP values were normalized and expressed as percentages of the highest GP value vs. time. This percentage was associated with the fraction of spores that were still dormant and/or spores that had not yet fully degraded their cortex and reflected their proportion in the sample at t. The complementary population of spores emitting a lower GP i.e. the spores in phase II of germination could thus be deduced. Finally, using the percentage of dormant spores (plate count) and the percentage of dormant spores and spores in phase I (Laurdan GP), the population in phase I could be estimated. The two-step kinetic model, based on differential chemical equations and presented in section 3, was used to derive a global model from the data generated. This model, used in Fig. 5, fits the experimental data extracted from the Laurdan GP values and plate counts with the optimized constants k2 = 0.11 min−1 and k0 = 0.07 min−1.

5. Discussion

5.1. Laurdan staining of G. stearothermophilus spores’ inner membrane

GP in gel lipid phases generally ranges from 0.5 to 0.6 (Parasassi et al., 1998). Values of 0.77 and 0.73 were reported for Clostridium beijerinckii and Clostridium sporogenes spores (Hofstetter et al., 2012). This indicates that the range obtained in this study, though higher than for conventional gel phases, is in agreement with the results obtained on spores of other genera. These GP values emphasize the high degree of order and
compressed state of the lipids in the inner spore membrane pointed out in other studies (Cowan et al., 2004; Sunde, Setlow, Hederstedt, & Halle, 2009).

The D, z-value and E₅₀ results were comparable between stained and non-stained spores (Table 1), and if any, would suggest only a minor increased resistance of stained bacterial spores over non-stained ones at high temperatures. This could however also be due to a batch to batch variability. D and z-value determination for the same strain of G. stearothermophilus was done in another study using certified NAMSA spores (NAMSA, Northwood, Ohio, USA) and showed D₁₂₂ = 126 s and D₁₃₀ = 12.4 s, z-value = 7.6 °C and E₅₀ = 389.5 kJ mol⁻¹ (Mathys, 2008) which confirmed that the range found above both for stained and non-stained spores is in agreement with the expected thermal resistance of these spores and suggests that the presence of Laurdan in the inner membrane is not affecting its properties, in particular its impermeability and capacity to retain the CaDPA core dehydrated.

5.2. Germination of G. stearothermophilus spores by individual and combined nutrients

None of the approaches tested to trigger nutrient germination of G. stearothermophilus spores were successful to induce a strong germination response, which could be used in the perspective of a decontamination treatment. Commonly investigated germinants in previous studies, for either clostridial spores or spores of the Bacillus genus, proved to lead to limited germination of spores of G. stearothermophilus within the combination tested and by comparison to PBS alone. Individual amino acids, combinations of inosine and L-Alanine, as well as complex mixes (AGFK) applied at comparison to PBS alone. Individual amino acids, combinations of inosine and L-Alanine, as well as complex mixes (AGFK) applied at the optimal growth temperature of the indicator (55 °C) for up to 24 h failed at showing germination >1 log₁₀. This confirmed the results obtained in a recent study on single G. stearothermophilus NGB101 spores using L-Valine and AGFK, in spite of different heat activation (or respectively triggered superdormancy) is high-bacterial spores of seemed to effectively impact on the overall germination of the different spore heat activation (or respectively triggered superdormancy) is high-

5.3. Germination of G. stearothermophilus spores with DPA chelates

It was expected that CAHPA might lead to a more complete spore germination prior to a comparatively mild decontamination regimen (Perez-Valdespino et al., 2013). However, up to now, few studies investigated the impact of DPA chelates on G. stearothermophilus spore germination (Fields & Frank, 1969; Zhou et al., 2013) and it was of interest to find out whether this germination pathway is commonly spread among different strains and families of spore formers. Based on the results of this work, it seems reasonable to suppose that for this germination pathway, heat activation does not play any role, in agreement previous work for other spore formers (Ghosh & Setlow, 2010; Keynan & Halvorson, 1962; Paidhungat, Ragkousi, & Setlow, 2001; Setlow, 2003).

Simultaneous measurements of the log₁₀-reduction post heat inactivation of the germinated spores and the GP value in time enabled the observation of the two stage germination kinetic suggested in this work (Fig. 4). The first stage was conditioned by the excretion of potassium and hydrogen ions, the excretion of CaDPA, partial water uptake and the loss of heat resistance. The second stage was characterized by the full cortex hydrolysis, core water uptake and expansion, restoration of inner membrane lipid mobility and restoration of core protein mobility (Setlow, 2003) as traced by variations in GP.

A first observation based on the Laurdan GP shift and absolute value as described in Section 4.3, suggested that within one spore batch, the germination commitment varied strongly. While some spores had already proceeded towards phase II (cortex degradation), a large subpopulation had not yet gone through phase I. However, once initiated, the succession between phase I and II occurred rapidly confirming at the scale of the population the results obtained on single spores by Zhou et al. (2013).

It appears that the phase preceding the start of germination phase II (cortex completed degradation/inner membrane extension) is a very short one. Indeed, k₉ is nearly twice the value of k₈, confirming that the cortex full hydrolysis occurs very rapidly after the initiation of germination (Dowd, Orsburn, & Popham, 2008) but is still preceded by another short step leading to the heat sensitization of the spores, likely partial hydration. How this hydration is triggered can only be hypothesized at this stage of the work but might be the result of a receptor activation on the inner membrane, sensing cortex segments as was suggested before (Setlow, 2008a).

An alternative hypothesis would be that upon CaDPA activation of a cortex lytic enzyme and corresponding initiation of cortex degradation, the pressure on the inner-membrane and spore core is locally reduced and leads to the release of ions and CaDPA by
stimulating mechano-sensitive proteins in the inner membrane. This second hypothesis however raises a number of questions, and in particular the need for a preferential degradation of the cortex from the inside towards the outside to allow for an early activation of these mechano-sensitive proteins permitting the release of the core CaDPA depot shortly before the completion of the cortex degradation.

On the other hand, the activation of germination by peptidoglycan fragments was already shown using fragments released by vegetative cells and it was shown that this is an extremely sensitive path, with picograms per milliliter being sufficient to activate it (Shah et al., 2008). The germination potential of spore cortex fragments was however not assessed to date. Dowd et al. (2008) characterized the muropeptides released during germination and cortex degradation but did not attempt germination using these extract. They found a total of 38 muropeptides in Bacillus anthracis of which most were already present in the dormant spores. However, some novel muropeptides associated with germination-specific cortex hydrolysis were found. It seems thus plausible that upon initiation of the cortex degradation following CaDPA activation of a cortex lytic enzyme, the spore inner membrane could rapidly detect the presence of the novel peptidoglycanic fragments in its vicinity and in that way be the first steps towards germination before completion of the cortex degradation.

Additionally, individual spores of B. cereus and B. subtilis were investigated (Kong et al., 2011; Kong, Zhang, Yu, Setlow, & Li, 2010). The kinetics of germination with CaDPA were observed by uptake of the nucleic acid dye SYTO 16. The dormant spore core could not be penetrated by SYTO 16. However, when spores were completely germinated, SYTO 16 could cross the spores’ inner membrane, bind nucleic acids, and exhibit strong green fluorescence. It was shown that during CaDPA germination of B. subtilis there was a 4–7 min delay after time of CaDPA release (first stage) before fluorescence of dye began to increase (second stage) which would match the findings of this work on G. stearothermophilus spores.

This would also be in agreement with the results obtained in another work looking at CaDPA germination of single G. stearothermophilus spores. There, it was shown via differential interference contrast microscopy that germination of individual spores followed a two stage mechanism, namely CaDPA release followed by full cortex degradation (Zhou et al., 2013). The results presented in this study allow extending this mechanistic hypothesis at the level of an entire spore population.

5.4. Conclusions

A non-nutrient path of germination, through DPA chelates proved to be successful, in particular with CaDPA as was observed for G. stearothermophilus spores and for spore formers of other families (Paidhungat et al., 2001; Setlow, 2003). Up to 3 log₁₀ of spores could be germinated and subsequently inactivated. This work underlined two main points:

- Spores of G. stearothermophilus could be germinated by CaDPA, which confirmed that the CaDPA activation mechanism is broadly spread across different species and families, possibly as was suggested, through a cortex lytic enzyme (possibly CwlJ) which would be CaDPA activated. This is in agreement with the presence of orthologues of CLE CwlJ of B. subtilis in several other strains of the Geobacillus genus (Paredes-Sabja et al., 2011).
- Either the mechanism or the enzyme would seem to be widely spread among spore formers of different species.
- Within one spore batch, the germination kinetics between phase I and II varied. While some spores had already proceeded towards phase II (cortex degradation), a large subpopulation had not yet gone through phase I. However, once initiated, the succession between phase I and II occurred rapidly.

As far as food sterilization goes, it is finally relevant to consider that CaDPA is not a GRAS ingredient, it is therefore, to date, not usable in food systems and toxicological validation studies would be needed prior to use in food. Future work would also need to investigate whether individual, purified cortex peptidoglycan fragments could trigger strong germination and thus replace CaDPA as germination trigger. Also, the questions around the super-dormancy remain. While 3 log₁₀ could be germinated and inactivated, a remaining population was not affected by this non-nutrient germinant, perhaps due to an absence of necessary cortex lytic enzyme as was suggested by Perez-Valdespino et al. (2013). Therefore, this non-nutrient germination might benefit from a combination with an additional hurdle leading to nutrient like germination, for instance moderate high pressure germination (Reineke, Mathys, Heinz, & Knorr, 2013).

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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.foodcont.2014.08.044.

References
