Heat resistance of Bacillus spores
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Spores of *Bacillus thermoamyloivorans* with very high heat resistances germinate poorly in rich media despite the presence of *ger* clusters, but efficiently upon non-nutrient Ca-DPA exposure.

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Chapter 5

Abstract

High heat resistance of spores of *Bacillus thermoamylovorans* poses challenges to the food industry as industrial sterilization processes may not inactivate such spores, resulting in food spoilage upon germination and outgrowth. In this study, the germination and heat resistance properties of spores of four food-spoiling isolates were determined. Flow cytometry counts of spores were much higher than their counts on rich medium (maximum 5%). Microscopic analysis revealed inefficient nutrient-induced germination of spores of all four isolates despite the presence of most known germination-related genes, including two operons encoding nutrient germinant receptors (GRs), in their genomes. In contrast, exposure to non-nutrient germinant calcium-dipicolinic acid (Ca-DPA) resulted in efficient 50 to 98% spore germination. All four strains harbored *cwlJ* and *gerQ* genes, which are known to be essential for Ca-DPA-induced germination in *Bacillus subtilis*. When determining spore survival upon heating, low viable counts can be due to spore inactivation and an inability to germinate. To dissect these two phenomena, the recoveries of spores upon heat treatment were determined on plates with and without pre-exposure to Ca-DPA. The high heat resistance of spores as observed in this study ($D_{120^\circ C}$ 1.9 ± 0.2 and 1.3 ± 0.1 minutes, z-value 12.3 ± 1.8°C) is in line with survival of sterilization processes in the food industry. The recovery of *B. thermoamylovorans* spores can be improved via non-nutrient germination, thereby avoiding gross underestimation of their levels in food ingredients.
**Introduction**

*Bacillus* endospores (or spores) are widely present in nature and may contaminate food ingredients and food products. Due to the intrinsic stability of spores, which allows them to withstand environmental insults, sufficient inactivation of spores in commercially sterile food products is a major challenge for the food industry (6, 53, 54, 63).

*Bacillus thermoamylovorans* produces spores with high heat resistance (54), and the spores are known to survive industrial food sterilization processes. The organism is facultatively anaerobic and has the ability to grow at temperatures between 40°C and 58°C (11, 31). In our experience, strains of *B. thermoamylovorans* are able to grow at 37°C but not at 30°C. The organism was first described as a non-sporogenous species (10, 11), but in an amended species description the formation of spores was reported (13). The occurrence of *B. thermoamylovorans* has been reported in a gelatin production plant and at dairy farms (14, 54). The genome sequence of one non-food-related *B. thermoamylovarans* strain from a biogas plant was published recently (31). Overall, the species has not been well characterized and little is known about the spore properties that are important for control in foods, including spore resistance to various processing conditions and germination of spores that survive.

When spores exit dormancy via germination, food spoilage can occur upon outgrowth. These processes have been well studied in *Bacillus subtilis* (38, 42, 56, 57). Germination can be induced both by nutrient and non-nutrient triggers, called germinants. Nutrients can initiate germination via interaction with germinant receptors (Ger receptors, GRs) that are localized in the inner membrane of the spore and consist of three or four different subunits (A, B, C and D) (41, 47, 51, 60). The responsiveness of GRs to nutrient triggers can be enhanced by exposure of spores to sub-lethal temperatures during a so-called heat activation step (30, 56). In contrast, germination via the non-nutrient germinant dipicolinic acid chelated with Ca²⁺ ions (Ca-DPA) occurs by direct activation of the cortex lytic enzyme (CLE) CwlJ, thereby bypassing the requirement of GRs (40). Activated CwlJ then hydrolyzes the protective peptidoglycan cortex resulting in rehydration of the spore core (9). Ca-DPA-induced germination has been reported to be independent of a heat activation treatment (8). The germination behavior of spores is a heterogeneous process (17), which is reflected by varying germination kinetics and/or the emergence of so-called superdormant spores that do not respond to the applied germination trigger (22, 23, 44, 50). For *B. subtilis* it has previously been described that spores superdormant to nutrients harbor lower numbers of germination receptor proteins (21), whereas spores that were superdormant to Ca-DPA, showed decreased levels of CwlJ (44).
Improved understanding and control of bacterial food spoilage can be facilitated by combining experimental findings with in silico analysis of genome content (48). In this study, spore germination of four food isolates of *B. thermoamylovorans* (isolated from either acacia gum or milk) was investigated in response to nutrient and non-nutrient triggers, and the genome sequences of the strains were determined (32). The strain-specific spore germination data were linked with presence or absence of important germination-related genes. In addition, spore heat resistance kinetics were determined using standard plating techniques, with and without a Ca-DPA pre-treatment, based on the insights into germination of this species obtained in this study. This approach led to a more accurate assessment of viable spore counts and heat resistance properties of spores of this species.

**Materials and methods**

**Strains**

Four strains of *B. thermoamylovorans* isolated from different sources were used in this study for characterization of the spore properties. Strains B4064 and B4065 were isolated from acacia gum, whereas strains B4166 and B4167 were isolated from milk. For all strains the genome sequences were determined (32).

**Spore preparation**

Spores of *B. thermoamylovorans* were prepared as previously described for *B. subtilis* (4, 52) with slight modifications. The strains were pre-cultured for 16 hours at 45°C in Brain Heart Infusion Broth supplemented with 1 mg/L vitamin B$_{12}$ (BHI-B, Merck) and subsequently spread on Schaeffer sporulation agar plates supplemented with 1 mg/L vitamin B$_{12}$ (54). These plates were incubated at 45°C for 7 days, and spores were harvested and washed successively in sterile water, as described before (4). Prior to experiments, spore suspensions were stored at 4°C for 2 to 4 weeks. The purity of the spore suspensions (>95 % phase-bright spores) was checked using phase-contrast microscopy (see below). For each strain, three independent spore crops were prepared.

**Spore quantification**

Spore suspensions were enumerated in two ways, namely by plate counting and flow cytometry. The spore counts were assessed by plate counting as follows. Spore suspensions were heat-activated at 80, 90 and 100°C for 10 minutes, followed by pour-plating in BHI-agar (BHI-A) plates supplemented with 1 mg/L vitamin B$_{12}$ (in duplicate). Plates were incubated for 5 days at 45°C, after which colony forming units (CFUs) were enumerated. An increase in the heat activation temperature (up to 100°C
Germination and heat resistance of B. thermoamylovorans spores

...for 10 minutes) did not affect the CFU counts, therefore 80°C for 10 minutes was used routinely to assess the spore CFU counts. Based on the initial counts obtained, the spore suspensions were diluted to a working spore suspension of approximately 10⁸ CFU/mL, in phosphate buffered saline (PBS), with a pH of 7.4.

Absolute spore counts were also determined by flow cytometry using a BD FACSAria II flow cytometer operated with BD FACSDiva Software (version 6.0, BD Biosciences). Spore suspensions were diluted 100 times in sheath fluid (BD FACSFlow, BD Biosciences) to obtain event rates below 2000 events s⁻¹, while at least 20,000 events were measured for each spore crop (19). A predetermined amount of reference beads (Microsphere standard (ø 6 µm) Live/Dead BacLight Bacterial Viability and Counting Kit L34856) was added to each spore suspension, corresponding to 5 x 10⁵ beads per mL. For each strain, three independent spore crops were measured in duplicate.

**Spore germination**

Spore germination was studied and quantified using phase-contrast microscopy (see below). Prior to the experiments, spore crops were washed with ice-cold sterile Milli-Q water. If not stated otherwise, spores were heat-activated at 80°C for 10 minutes or at 70°C for 30 minutes and subsequently cooled on ice and washed again with cold water. Heat-activated spores were diluted to a final OD₆₀₀ of 1 in 200 µl of BHI or Luria-Bertani (LB) medium supplemented with vitamin B₁₂ (1 mg/L) and chloramphenicol (7.5 mg/L) to prevent outgrowth of vegetative cells (26, 59). Alternatively, spores were diluted in mixtures of various nutrient-based germinants dissolved in 25 mM Tris-HCl, pH=7.4: i) 100 mM L-alanine; ii) L-asparagine, D-fructose, D-glucose, KCl (all 50 mM); iii) L-alanine, L-arginine, L-asparagine, aspartic acid, L-cysteine-HCl, glutamic acid, L-glutamine, glycine, L-histidine, inosine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonyne, L-tryptophan, L-valine (all 10 mM); iv) L-alanine, L-arginine, L-asparagine, aspartic acid, L-cysteine-HCl, glutamic acid, L-glutamine, glycine, L-histidine, inosine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-valine, D-fructose, D-glucose, KCl (all 10 mM), chloramphenicol (7.5 mg/L). For non-nutrient-induced germination experiments, non-heat-activated spores were diluted in equimolar mixtures of 20, 40, 60, 80 mM CaCl₂ and DPA (pH = 7.4). A preliminary analysis indicated that 40 mM Ca-DPA was the most efficient concentration to trigger germination (data not shown) and this concentration was used in further experiments. As negative controls, both non-heat-activated and heat-activated spores were diluted in 25 mM Tris-HCl, pH=7.4. Spore dilutions were then incubated at 42°C while shaking (220 rpm). After 3, 6 and 24 hours, the transition of phase-bright dormant spores to phase-dark germinated spores was monitored using phase-contrast microscopy. Microscopic
imaging was performed using an IX71 microscope (Olympus) with a CoolSNAP HQ2 camera (Princeton Instruments), using a 100x phase contrast objective, and DeltaVision softWoRx 3.6.0 (Applied Precision) software. Images were taken using 32% APLLC White LED light and 0.3 s exposure. The pixel size was 0.0643 µm and binning was set to 1x1. Images obtained were analyzed using Fiji software (http://fiji.sc/Fiji (55)). For quantification of ratios of germinated and dormant spores, a minimum of 300 spores per condition was examined. All experiments were performed in duplicate using two independent spore crops.

**Spore heat inactivation**

Spore heat inactivation was determined using capillary tubes using two independent spore crops for each of the four strains, as previously described (4, 64). For all strains the spore working suspensions (10^8 spores/mL in PBS) were heated at 110°C, at ten different time points up to 23 minutes. For strain B4064 the inactivation experiments were additionally performed at 115°C and 120°C to allow for detailed inactivation kinetics determination. Upon heat treatment, one part of the spore suspension was 10-fold serially diluted in peptone water and appropriate dilutions were pour-plated in duplicate in BHI-agar plates supplemented with 1 mg/L vitamin B12. The other part of the heated spore suspension was exposed to 40 mM Ca-DPA in sterile peptone water for 3 hours at 45°C, followed by pour-plating 10-fold serial dilutions (made in peptone water) in duplicate in BHI-agar plates supplemented with 1 mg/L vitamin B12. Per experiment, plating was performed in duplicate. After incubation for 5 days at 45°C, CFUs were enumerated and recovery of spores determined.

The survivor count was plotted against the inactivation time, and based on the shape of the inactivation plot a model was selected for fitting. Model fitting was performed with Microsoft Excel using the Solver Add-in.

For the experiments that included an incubation step with Ca-DPA prior to plating, the data were fitted with the log-linear model where the \( D \)-value was determined, as presented in equation 1.

\[
1: \quad \log N(t) = \log N(0) - \frac{t}{D}
\]

With \( N_i \) being the surviving spore count at time \( t \), \( N_0 \) being the initial spore concentration, \( t \) the time (time unit), and \( D \) the decimal reduction time.

The inactivation plots of the experiments that did not include a Ca-DPA incubation step prior to plating showed the presence of a heat sensitive and a heat resistant population; therefore these were fitted with the biphasic Geeraerd model as described in Equation 2 (20).
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2: \[ \log \left( \frac{N_t}{N_0} \right) = \log \left( (1 - f) \cdot e^{k_{\text{sen}} \cdot t} \cdot \frac{e^{k_{\text{sen}} \cdot S}}{1 + (e^{k_{\text{sen}} \cdot S} - 1) \cdot e^{-k_{\text{sen}} \cdot t} + f \cdot e^{k_{\text{res}} \cdot t}} + \frac{e^{k_{\text{res}} \cdot S}}{1 + (e^{k_{\text{res}} \cdot S} - 1) \cdot e^{-k_{\text{res}} \cdot t}} \right) \]

Where \( N_t \) is the survivor count at time \( t \), \( N_0 \) is the initial spore count, \((1-f)\) and \( f \) the heat sensitive and heat resistant fraction, respectively, \( k_{\text{sen}} \) and \( k_{\text{res}} \) the inactivation rates (time unit\(^{-1}\)) of the sensitive and the resistant populations, respectively, \( t \) the time (time unit) and \( S \) the duration of the shoulder (time unit). The \( D \)-value, was calculated by dividing the reciprocal of the inactivation rates by the natural logarithm of 10.

For the experiments with strain B4064 that included incubation with Ca-DPA prior to plating, additionally the \( z \)-value, the increase in temperature required to achieve an additional log unit reduction, the reference \( D \)-value \( (D_{\text{ref}}) \) at reference temperature \( (T_{\text{ref}}) \) 121.1°C, and the 95% prediction interval (PI) were calculated as previously described (62).

\textit{Genome mining}

For all of the predicted protein sequences of the four \textit{B. thermoamylovorans} strains and reference strain \textit{B. subtilis} 168, an orthology prediction was performed using Ortho-MCL (33) (Supplementary Dataset 1). To find potential functional equivalents for a selection of germination-related genes (Table 1), corresponding protein sequence alignments were made using MUSCLE (16), followed by construction of a hidden Markov model (HMM) that was subsequently used to scan all genomes (29). For selected proteins found in this manner, maximum likelihood trees were constructed using the maximum likelihood phylogeny program PHYML (25). Phylogenetic trees were manually inspected for evolutionary relatedness of the proteins. Additionally, the genomic context was manually verified after visualization in the SEED Viewer (39) on the RAST annotation server (2). Prediction of binding sites for sporulation sigma factors (18) upstream of selected genes was performed with use of the database of transcriptional regulation in \textit{B. subtilis} (DBTBS; http://dbtbs.hgc.jp) (37). Schematic visualization of the predicted operon structures of genes: \textit{ger(x1)ABC, ger(x2)ABC, spoVAA-AF, cwlJ, gerQ, cwlJ2, gerQ2} (Figure 3) was made with the draw context tool on the Genome2D server (http://genome2d.molgenrug.nl) (3).

\textit{Results}

\textit{Quantification of spores}

Spores, prepared on Schaeffer agar plates, were characterized with respect to spore germination and heat resistance. The number of spores in 1 mL of the working spore
suspension was quantified using flow cytometry or CFU enumeration. The obtained numbers of spores per mL were strikingly different depending on the quantification technique used (Figure 1). Using flow cytometry, the absolute number of spores in spore suspensions of strains B4064, B4065, B4166 and B4167 were 1.9, 1.3, 1.8 and 1.6 log units higher, respectively, than when enumerated using plating in BHI-A with vitamin B$_{12}$. Since CFU plate counting enumeration depends on spore germination and outgrowth, this discrepancy indicates that only a small fraction (1.3% ± 1.0%, 5.3% ± 2.4%, 1.6% ± 0.9%, and 2.5% ± 2.1% for spores of strains B4064, B4065, B4166, and B4167, respectively) of the absolute number of spores undergoes germination and subsequent outgrowth on the BHI-A plates.

**Germination with nutrient germinants**

To establish whether the discrepancy between absolute spore counts and CFU counts was caused by inefficient germination, the germination efficiency of the heat-activated spores in the nutrient-rich BHI medium was assessed using phase-contrast microscopy. The analysis showed that the fraction of spores that germinated in BHI did not exceed 2.6% ± 0.8%, 13.6% ± 3.6%, 4.8% ± 0.5% and 5.8% ± 2.1% for strains B4064, B4065, B4166 and B4167, respectively (Figure 2). These numbers hardly exceeded the percentage of phase-dark spores in the negative controls (2.2% ± 0.7%, 6.2% ± 0.5%, 2.7% ± 2.2% and 4.8% ± 2.3%, respectively) (Figure 2). Moreover, the percentage of germinated spores did not increase significantly in time (Figure 2). This implies that spores of *B. thermoamylovorans* germinate very poorly in BHI. In addition, spore germination was
assessed in LB and simple nutrient mixtures: i, ii, iii, and iv (details in Materials and Methods), which resulted in similar observations (data not shown). Likewise, altering or omitting the heat-activation treatment did not increase germination efficiency in rich medium (data not shown). Altogether, these results indicated that the tested nutrient germinants were not triggering germination of *B. thermoamylovorans* spores efficiently.

**Germination with Ca-DPA**

Besides germination of spores in response to nutrients, which requires the presence of GRs in the spores (42, 56), germination can also be induced by non-nutrient germinants, for instance Ca-DPA, or by very high hydrostatic pressure (400–800 MPa) via mechanisms that are independent of GRs (5, 15, 42, 56). A weak germination response of *B. thermoamylovorans* spores was observed for nutrient triggers (Figure 2A). In addition, the germination of spores in response to addition of the non-nutrient germinant Ca-DPA was assessed; this type of germination does not require GRs (40). Exposure of spores to 40 mM Ca-DPA for 3 hours resulted in very efficient spore germination for strains B4064 and B4065 (75.3% ± 7.2% and 95.7% ± 2.1% of germinated spores, respectively) and moderately efficient germination for strains B4166 and B4167 (32.8.7% ± 5.4% and 43.0% ± 9.4%, respectively) (Figure 2). After 24 hours of incubation, spore germination increased to 95.6% ± 3.0 % and 97.6% ± 1.2% for strains B4064 and B4065, respectively, whereas it reached 49.7% ± 5.5% and 58.3% ± 5.6% for strains B4166 and B4167, respectively (Figure 2). Altogether, these results indicate that Ca-DPA is an efficient germination trigger for *B. thermoamylovorans* spores, but the germination responses varied between the different isolates.

**Genome mining for germination genes**

To explain the observed germination phenotypes, i.e. inefficient germination in response to nutrient triggers, and different responses to Ca-DPA between strains, the presence of germination genes was evaluated in the genomes of the four *B. thermoamylovorans* isolates through genome mining (Table 1). In *B. subtilis*, nutrient-induced germination requires specific GRs that bind nutrient germinants (41, 51) and is facilitated specifically by several proteins, such as GerD (43), and GerPABCDEF (7). The analysis of the genomes of the four *B. thermoamylovorans* strains revealed the presence of GR genes which are deemed important for sensing nutrient germinants (Table 1), but despite their presence, only weak germination of spores was observed in the presence of rich media and various nutrients. The GR genes included two complete tri-cistronic operons, referred to further as *ger*(x1)ABC and *ger*(x2)ABC, both encoding putative GRs (Table 1 and Figure 3). Both *ger* operons are predicted to be preceded by single (in the case of *ger*(x1)ABC) or double (in the case of *ger*(x2)ABC) binding sites for the sporulation...
Figure 2. Quantification of spore germination efficiency using phase-contrast microscopy. Spores were either heat-activated (HA) or not (n-HA) and exposed to BHI plus vitamin B12 (A), Ca-DPA (B) or Tris buffer (control). Germination was calculated as the percentage of phase-dark spores on phase-contrast microscopic images made after 3, 6 and 24 hours of incubation with germinant (images are shown for 24h only). Mean percentages of two independent experiments were plotted, including error bars based on standard deviations. Scale bar, 2 μm.
Germination and heat resistance of *B. thermoamylovorans* spores

Sigma factor SigG (Figure 3). In addition, the following genes encoding proteins that are expected to facilitate responses to nutrients (7, 28, 43) were found in the genomes of all four stains: *gerD, gerF, gerPA, gerPB, gerPC, gerPD, gerPE, gerPF* (Table 1).

Genes other than the ones directly involved in sensing nutrients, but which play a role in subsequent germination events, were also found in the *B. thermoamylovorans* genomes (Table 1). These included the *cwlJ, sleB, gerQ* and *ypeB* genes which encode proteins that are important for lysis of the protective cortex layer, and nearly all of the *spoVA* genes (*spoVAA, spoVAB, spoVAC, spoVAD, spoVAEb, spoVAF*), some of which encode proteins that are responsible for release of DPA from the spore core (42, 56). The germination gene *spoVAeA* was absent in the four sequenced *B. thermoamylovorans* strains but *SpoVAeA* is considered to play only a minor role in germination (45). Interestingly, some *spoVA* genes, namely *spoVAC, spoVAD, spoVAEb*, occurred in multiple copies in the genome of the sequenced strains of *B. thermoamylovorans* (Table 1). Thus, besides single *spoVAA* and *spoVAB* genes, all strains possessed three *spoVAC* and *spoVAD* genes as well as two *spoVAEb* and two *spoVAF* genes. The *spoVA* genes of *B. thermoamylovorans* were found in five different operons: i) the *spo(VA1)* operon comprising *spoVAA-spoVAB-spoVAF*; ii) *spo(VA2)* consisting of *spoVAC-spoVAD* genes; iii) *spo(VA3)* and iv) *spo(VA4)* operons, both containing *spoVAC-spoVAD-spoVAEb* genes; and v) *spoVA5*, which comprises a single *spoVAF* gene (Table 1 and Figure 3).

In *B. subtilis*, Ca-DPA initiates germination by direct activation of the cortex lytic enzyme CwlJ (36, 40), which requires GerQ for proper localization in the spore coat (46). Strains B4166 and B4167 contain a single *cwlJ* gene and *gerQ* gene, whereas strains B4064 and B4065 both carry two copies of *cwlJ* (further referred to as *cwlJ* and *cwlJ2*) and two copies of *gerQ* (referred to as *gerQ* and *gerQ2*) (Table 1). Both *cwlJ* and *gerQ*, as well as *cwlJ2* and *gerQ2* are adjacent to each other on the chromosome, possibly forming an operon preceded by the predicted SigE and SigK binding sites (Figure 3). Pairwise amino acid alignments revealed 81% sequence identity between the CwlJ and CwlJ2 proteins. Moreover, both CwlJ and CwlJ2 contain the probable key catalytic glutamate 21 residue (E21) (Supplementary Figure 1) (34). The same holds true for GerQ and GerQ2, which also exhibit high sequence identity (61%).

**Spore heat inactivation and modelling**

The heat inactivation of spores at 110°C was assessed for all strains, without a Ca-DPA treatment and with a Ca-DPA treatment to trigger non-nutrient germination before plating. The inactivation curve of spores that were not treated with Ca-DPA prior to plating showed bi-phasic behaviors with tailing, and data were fitted with a biphasic model from which the shoulder parameter was omitted (Figure 4) (20). For these data
Table 1. Presence and absence of germination genes in four *B. thermoamylovorans* isolates. The table shows locus tags of the genes present in each *B. thermoamylovorans* strain that belong to the orthologous groups (OG) corresponding to different germination genes. For reference, also a model laboratory strain, *B. subtilis* 168 was included in the set together with locus tags and names of its germination genes. Empty cells indicate absence of genes in the respective strains. In some cases, multiple genes, listed by different locus tags, belong to one OG in the individual strains. For multiple spoVA genes, which occur in five different operons in *B. thermoamylovorans* genomes, the numbers that indicate operon affiliation of individual genes have been arbitrarily added to the gene names. An asterisk (*) indicates that these genes were combined in one OG based on the multiple sequence alignment and phylogenetic tree. Abbreviations: N.A. – not applicable.

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<th>Gene name</th>
<th>Locus tag <em>B. subtilis</em> 168</th>
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<td>Germinant receptor subunit B (41, 51)</td>
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<td>OG_1063</td>
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<td>gerD</td>
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<td>OG_1032 BSU10680</td>
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<td>spoVAa</td>
<td>B4064_0505 B4065_0471 B4166_0596 B4167_0663 spo(VA)1A</td>
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<td>spoVAc</td>
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sets, the $D_{110°C}$ values ranged from 0.7 (standard error ± 0.1) min to 3.3 ± 0.5 min for the heat sensitive fraction of the spores, and from 9.3 ± 1.9 min to 33.7 ± 2.0 min for the heat resistant fraction (Supplementary Table 1). In contrast, tailing was not observed for any of the strains when spores were treated with Ca-DPA prior to dilution and plating. These inactivation curves were fitted with a log-linear model. For these data sets, the $D_{110°C}$ values ranged from 9.7 ± 0.5 min to 26.1 ± 3.2 min (Supplementary Table 1). For strain B4064, the inactivation kinetics were determined at 110°C, and in addition also at 115°C and 120°C. This allowed for the determination of the $z$-value (12.2 ± 1.8°C) and calculation of a $D_{\text{ref}}$ (Tref = 121.1°C) of 1.4 min (upper 95% prediction interval 2.9 min) (Supplementary Table 1).

**Discussion**

Spores of *B. thermoamylovorans* can pose problems in commercially sterile foods due to their high heat resistance and unpredictable germination. To improve our understanding of this problematic species and identify possible leads for spoilage control, we combined a phenotypic characterization of the germination behavior of four different food-related isolates with *in silico* analysis of their genome sequences (32). Based on our new insights into the germination properties of spores of this species, we subsequently determined heat resistance properties of spores of individual strains.

This study has shown that poor recovery of spores of *B. thermoamylovorans* on standard rich cultivation media leads to a significant underestimation of the spore load that is actually present: enumeration of spores in spore suspensions using flow cytometry and plating on BHI-A showed that only a few percent (1.3%-5.0%) of *B. thermoamylovorans* spores formed colonies (Figure 1). Increase of the activation temperature did not improve spore counts (data not shown). This low number of colonies on BHI plates resulted mainly from inefficient spore germination in response to nutrients, as only 2.6%-13.6% of spores germinated in the BHI broth (Figure 2). Notably, the germination of *B. thermoamylovorans* spores was also very limited in the presence of LB broth and a variety of tested nutrient germinants, including L-alanine, a combination of L-asparagine, D-glucose, D-fructose, KCl, a mixture of 19 individual amino acids and inosine with or without D-fructose, D-glucose and KCl (data not shown). Based on these observations, the absence of genes encoding one or more germination proteins would provide a plausible explanation for a weak germination response of *B. thermoamylovorans* spores to nutrients. In *B. subtilis* it is known that the initial stages of nutrient germination require at least one functional germinant receptor plus the GerD protein (43), and the germination process is facilitated by the GerP proteins (7). At a later stage, some of the SpoVA proteins enable release of Ca-DPA from the spore core to the environment and finally, at least one of the two lytic enzymes, CwlJ or SleB, is required for hydrolysis of the spore protective cortex layer (42, 56). *In silico* analysis of the genome sequences of
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Figure 3. Schematic visualization of the predicted operon structures of: ger(x1)ABC and ger(x2)ABC encoding putative germinant receptors (A); cwlJ gerQ and cwlJ2 gerQ2 involved in Ca-DPA germination (B); five spoVA operons: spo(VA1), spo(VA2), spo(VA3), spo(VA4) and spo(VA5) (C). The sigma factor binding sites, together with the threshold p-values used for their prediction, are indicated with black arrows. The asterisk (*) indicates that cwlJ2 and gerQ2 are present only in strains B4064 and B4065 (A). The spoVA operons 2, 3 and 4: spo(VA2), spo(VA3) and spo(VA4) next to spoVA genes contain also genes encoding hypothetical proteins, indicated with light gray arrows, and predicted internal sigma factor binding sites (C). Operon spo(VA2) containing spo(VA2)C and spo(VA2)D genes is located on the edge of the contig in genomes of all B. thermoamylovorans food isolates. Thus, the nucleotide sequence, and predicted promoters, upstream of the two genes that encode hypothetical proteins indicated as "h**" are unknown. However, as the nucleotide sequence of the two h** genes encoding hypothetical proteins (h**) in the operon spo(VA2) is highly similar to the sequence of the h** gene in front of the spo(VA3)C gene in the operon spo(VA3), it is probable that the sequences upstream of the operons spo(VA2) and spo(VA3) are similar (C). Scales below each part of the figure indicate distances in nucleotide base pairs.
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*B. thermoamylovorans* showed that all known germination-specific genes, with the only exception of *spoVAEa*, were present in the genomes of the four strains, some of them in multiple copies (Table 1).

Two tri-cistronic operons encoding putative GRs were found on the chromosome, as well as the *gerD* gene (Table 1), indicative of a potential of *B. thermoamylovorans* spores to respond to nutrient germination triggers. Despite the fact that one of the GR operons in strains B4064 and B4065 encoded proteins that belong to the same orthologous group as the *B. subtilis* GerK receptor subunits, spores of *B. thermoamylovorans* displayed very little or no response to a nutrient mixture known to specifically trigger GerK (namely L-asparagine, fructose, glucose and potassium ions). In *B. subtilis*, activation of the GerK receptor in response to this mixture is also linked with the GerB receptor (1); our analysis indicated that subunits of the GerB receptor are absent in *B. thermoamylovorans*, which may explain the lack of germination in response to this mixture. Also, it is known from literature that even small changes in a GR subunit sequence can alter or abolish activity of the GR complex in response to certain nutrients (9, 12, 35). For *B. thermoamylovorans*, no specific response could be detected in any of the nutrient combinations tested. The genomic sequences of the germinant receptor operons showed intact genes with predicted binding sites for the SigG transcription factor, which typically regulates expression of GR genes (18), upstream of the tri-cistronic operons. Assuming that these genes are expressed during sporulation and that the GR proteins are functional in the spore, the specificity of the two GRs present in *B. thermoamylovorans* remains to be determined.

All other key genes related to germination, including the *spoVA* operon (required for release of Ca-DPA from the core upon germination in *B. subtilis* (42, 56)), as well as those encoding the cortex lytic enzymes, *sleB* and *cwlJ*, were found in all four strains (Table 1). The only gene that was missing was *spoVAEa*, but the absence of this gene is in fact not uncommon for spore-forming species of the Bacillales and Clostridiales orders (45). In *B. subtilis*, deletion of *spoVAEa* has been associated with a slower nutrient-induced germination phenotype (45), but this fairly moderate decrease does not fully explain the dramatic loss in germination efficiency in *B. thermoamylovorans* (Figure 2). In contrast, some other *spoVA* genes, namely *spoVAC* and *spoVAD* and *spoVAEb*, were found in multiple copies in the genomes (Table 1). The impact of this duplication is so far unclear; although it may alter the release of Ca-DPA from the spore core upon germination. On the whole, the poor nutrient germination response of *B. thermoamylovorans* cannot be linked directly to absence of key germination genes. Other explanations for the observed inefficient germination may be a weak penetration of nutrients through the coat layers, poor binding of nutrients to the GRs, lack of GR functionality or lack of adequate signal transduction downstream of the germination receptors (42, 56).
Interestingly, despite very weak germination responses to nutrients, spores of all four *B. thermoamylovorans* strains germinated well in response to a non-nutrient germinant, namely exogenous Ca-DPA. Ca-DPA is known to directly activate the cortex lytic enzyme CwlJ (40), which requires the GerQ protein for localization in the spore coat (46). CwlJ and GerQ have been shown to be essential for Ca-DPA-induced germination in *B. subtilis* and *B. megaterium* (40, 46, 55). Assuming that the germination process of spores of *B. thermoamylovorans* is similar to *B. subtilis*, our results suggest that *B. thermoamylovorans* nutrient germination is not impaired at the stage of peptidoglycan degradation and downstream events, but at the stage preceding cortex hydrolysis. However, a clear difference in germination efficiency in response to Ca-DPA was observed between the strains, with germination of spores of B4064 and B4065 being highly efficient, and B4166 and B4167 being moderately efficient.

Analysis of the four genomes revealed the presence of two *cwlJ* and *gerQ* genes in strains B4064 and B4065, and single *cwlJ* and *gerQ* genes in strains B4166 and B4167. CwlJ and CwlJ2 on the one hand, and GerQ and GerQ2 on the other hand displayed high amino-acid sequence similarity (Supplementary Figure 1), suggesting that both copies of each protein potentially play similar or identical roles in spore germination of strains.
B4064 and B4065. Spores of B4064 and B4065 showed higher germination efficiencies in response to Ca-DPA than spores of B4166 and B4167 (Table 1), but a direct link between the higher Ca-DPA germination efficiency in strains B4064 and B4065 than in strains B4166 and B4167 and the presence of two *cwlJ* and *gerQ* genes remains to be established.

Limited germination in response to nutrients has implications for counts obtained using standard plating techniques on rich media, as colony formation from single spores relies on efficient germination of spores and subsequent outgrowth. We demonstrated that enumeration on BHI plates strongly underestimates the number of viable spores. More efficient germination was observed following non-nutrient germination in response to Ca-DPA. To establish heat resistance, spores were subjected to heat treatments at 110°C and plated directly or after a Ca-DPA treatment. For all four strains, much higher recoveries were observed upon Ca-DPA exposure compared with direct plating (up to 3.4 log higher counts for spores of strain B4065), and tailing effects were absent. Heating was also performed at 115°C and 120°C for strain B4064, and at these temperatures, similar effects were observed (see Figure 4).

Interestingly, the differences in viable spore counts with or without Ca-DPA exposure prior to plating were more prominent for strains B4064 and B4065, than for spores of strains B4166 and B4167. The latter two strains harbor only a single copy of the *cwlJ* and *gerQ* genes, and their spores showed less efficient germination with Ca-DPA than spores of strains B4064 and B4065, each harboring two *cwlJ* and *gerQ* genes (Figure 2). Following heating at 110°C, the differences in recoveries with and without Ca-DPA treatments were less prominent for the strains harboring the single *cwlJ* and *gerQ* copies, which is likely due to the fact that spore germination was not complete for these spores, even following Ca-DPA exposure (Figure 4). Even for spores that germinated best in response to Ca-DPA, germination was not 100% after incubation for 3 hours with Ca-DPA (Figure 2), indicating that counts on plates might still be underestimated.

*B. thermoamylovorans* was shown to produce highly heat resistant spores when compared to other spore-forming *Bacillus* spp. The decimal reduction times at 120°C ($D_{120°C}$) were 1.9 min ± 0.3 and 1.3 min ± 0.1 for two independent spore crops of strain B4064 (Supplementary Table 1) obtained with an additional Ca-DPA treatment prior to plating. This is comparable with reported D-values of *B. subtilis* strain A163, which is known to produce highly heat resistant spores ($D_{120°C}$ of 1.8 min ± 0.1 and 1.6 min ± 0.1) (4). The spore heat resistance of the *B. thermoamylovorans* strains is only slightly lower than that of *B. sporothermodurans*, which is known to survive UHT processing and has reported $D_{120°C}$ values of 2.25 min (27). When spores of strain B4064 were directly plated on BHI, a heat resistant fraction (tailing) was observed, with $D_{120°C}$ values of 2.9
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± 0.3 min and 2.7 ± 0.5 min (Supplementary Table 1). The calculated $D_{140^\circ C}$ for spores of strain B4064, based on the inactivation data obtained after plating preceded by a Ca-DPA treatment, was 2.3 s (upper 95% PI 5.0 s) which is very high, but still slightly below that of B. sporothermodurans spores, with reported $D_{140^\circ C}$ of 4.7 s and 5.0 s (27, 53). When comparing the heat resistance of spores of B. thermoamylovorans with spores of G. stearothermophilus, the $D_{\text{ref}}$ of 1.4 minutes calculated for strain B4064 is lower than the $D_{121.1^\circ C}$ of 3.3 minutes that has been reported for G. stearothermophilus based on literature data of 430 $D$-values of this species (49).

Based on the data obtained in this study, it can be concluded that the spores of B. thermoamylovorans are highly resistant, and are potentially able to survive UHT treatments. When conventional plating techniques are used to determine the initial spore concentration and to estimate spore heat resistance, it is likely that predictions are not accurate, especially for non-characterized species and strains. The lack of efficient nutrient germination of spores can lead to strong underestimations of counts, both of initial levels and of surviving spores after a heat treatment. When applied in a food processing setting, such large underestimations of the initial spore concentration can have detrimental effects on the safety boundaries of such processes.

In summary, we have demonstrated that spores of B. thermoamylovorans do not germinate efficiently upon nutrient-induced germination, despite the presence of the genes encoding two GRs. Spore germination was triggered upon exposure to Ca-DPA. Our results clearly show the importance of determining spore germination and outgrowth conditions prior to characterization of spore properties, including heat resistance, to avoid strong underestimation of viable spores that fail to germinate in response to regular nutrient germinants. The improved estimations of spore heat resistance obtained in this study will aid efforts in the food processing environment towards better control of spores of B. thermoamylovorans and assuring sterility of food products.

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**Supplementary Dataset 1**

Gene presence/absence matrix of B. thermoamylovorans strains B4064, B4065, B4166 and B4167 and B. subtilis 168 based on the OrthoMCL analysis.
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References


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### Supplementary materials

**Supplementary Table 1.** Calculated inactivation kinetics for the *B. thermoamylovorans* strains B4064, B4065, B4166 and B4167, pour-plated in Brain Heart Infusion Agar supplemented with 1mg/L vitamin B₁₂, with prior exposure to Ca-DPA (condition Ca-DPA) and without prior exposure to Ca-DPA (condition normal plating). For the Ca-DPA condition, a log-linear model was fitted to the entire spore population expressed in decimal reduction times (*D*-value). For the normal plating, a bi-phasic model was fitted to the data and resulted in *D*-values for the sensitive and resistant subpopulations. For strain B4064, additionally, the temperature increase required to achieve one extra log reduction, the *z*-value, the reference *D*-value (*D*<sub>ref</sub>), and the 95% prediction interval (PI) were calculated. Abbreviations: S.E. – standard error, *D*<sub>ref</sub> – reference *D*-value, *T*<sub>ref</sub> – reference temperature, N.A. – not applicable, PI – prediction interval.

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<th>S.E.</th>
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Germination and heat resistance of B. thermoamylovorans spores

Supplementary Figure 1. Multiple-sequence amino-acid alignment of the CwlJ (A) and GerQ proteins from the four B. thermoamylovorans food isolates (B4064, B4065, B4166, B4167). As mentioned in the text, strains B4064 and B4065 potentially encode two CwlJ and GerQ proteins referred to as CwlJ, CwlJ2 and GerQ, GerQ2, respectively. Conserved catalytic glutamate 21 residue (E21) in CwlJ proteins is marked above the alignment.