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Comparative analysis of *Bacillus weihenstephanensis* KBAB4 spores obtained at different temperatures

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Running title: Characterization of *B. weihenstephanensis* KBAB4 spores

ABSTRACT

The impact of *Bacillus weihenstephanensis* KBAB4 sporulation temperature history was assessed on spore heat resistance, germination and outgrowth capacity at a temperature range from 7 to 30°C. Sporulation rate and efficiency decreased at low temperature, as cells sporulated at 12, 20 and 30°C with approximately 99% efficiency, whereas at 7°C and 10°C, a maximum 15% of sporulation was reached. Spores formed at 30°C showed the highest wet heat resistance at 95°C, with spores formed at 7 and 10°C displaying only survival of 15 min exposure at 70ºC, indicating their low level heat resistance. RT-PCR analysis revealed expression of sporulation sigma factor *sigG*, and germinant receptor operons *gerI*, *gerK*, *gerL*, *gerR*, *gerS*, and (plasmid-located) *gerS2* to be activated in all sporulation conditions tested. Subsequent germination assays revealed a combination of inosine and L-Alanine to be very efficient, triggering over 99% of the spores to germinate, with spores obtained at 30°C showing the highest germination rates (99%). Notably, spores obtained at 12, 20 and 30°C, germinated at all tested temperatures, showing >70% spore germination even at temperatures as low as 5°C. Less than 5% of spores obtained at 7 and 10°C showed a germination response. Furthermore, spores produced at 12, 20 and 30°C showed similar outgrowth effiency at these temperatures, indicating that low temperature sporulation history does not improve low temperature outgrowth performance. Insights obtained in sporulation and germination behaviour of *B. weihenstephanensis* KBAB4, in combination with the availability of its genome sequence, may contribute to our understanding of the behaviour of psychrotolerant spoilage and pathogenic Bacilli.

KEYWORDS: sporulation, wet heat resistance, germination, spore outgrowth, psychrotolerant *Bacillus cereus*.

INTRODUCTION

The presence of bacterial spores is one of the main problems for food quality and safety, because of their high resistance compared to vegetative cells (Gould, 2000). These spores could survive hygienization treatments, germinate, multiply and thus cause problems in food, such as food spoilage and food poisoning (van Netten et al., 1990; Gould, 2000; Granum and Baird-Parker, 2000). *Bacillus weihenstephanensis* is a member of the *Bacillus cereus* group of species, comprising psychrotolerant strains that grow at 7ºC or below (Lechner et al., 1998). Occurrence of psychrotolerant *B. cereus* species spores in food products could limit food shelf-life (Anderson Borge et al., 2001) because of their ability to survive heat treatments (Carlin et al., 2010), to germinate and subsequently grow at refrigeration temperatures and to produce toxins (Anderson Borge et al., 2001; Stenfors et al., 2002).

Factors contributing to spore resistance include low water content in the core, the intrinsic stability of spore proteins and saturation of DNA with Small Acid-Soluble Proteins (SASP) (Setlow, 2006). Previous research indicated several factors to affect spore characteristics including sporulation temperature (Palop et al., 1999), divalent cation availability and chemical agents, such as hydrogen peroxide or Betadine (Melly et al., 2002). Sporulation history may also affect germination efficiency, an important determinant of outgrowth capacity of spores in foods (Raso et al., 1998; Cortezzo and Setlow, 2005; Gounina-Allouane et al., 2008).

Spore germination has been defined as those events that result in the loss of the spore-specific properties, such as heat resistance, core hydration and expansion or loss of dormancy (Setlow, 2003). Spores use sensing systems, the so-called germinant receptors, that can monitor the availability of nutrients, such as ribonucleosides and amino-acids in the surrounding environment, thus triggering germination at the

appropriate moment (Setlow, 2003; Moir, 2006). Germinant receptors, located in the inner membrane of the spore, are multicomponent sensors for nutrients and are generally encoded by tricistronic operons, i.e., *ger* operons (Moir et al., 2002). After spore germination, the outgrowth phase occurs, when macromolecular synthesis converts the germinated spore into a growing cell (Paidhungat et al., 2002).

Not only nutrients are able to trigger germination events, but also mechanical treatments, such as abrasion or high pressures, presumably by inducing conformational changes in relevant spore membrane and/or cortex enzymes (Raso et al., 1998; Nicholson et al., 2000). High Pressure (HP) is an alternative mild food preservation method that allows for maintenance of sensory, nutritional and functional properties of food (Mañas and Pagán, 2005). Although HP-induced inactivation of spores is not very efficient, its alternative use in spore germination activation is receiving increased interest, because germination makes the spores more sensitive to subsequent food preservation stresses (Black et al., 2007).

Although sporulation and germination has been studied quite extensively in recent years in Bacilli, including representatives from the *Bacillus cereus* group, i.e. *Bacillus cereus* and *Bacillus anthracis* (Hornstra et al., 2006; Setlow, 2006; Senior and Moir, 2008; Carr et al., 2010), only limited information is available about these processes in psychrotolerant representatives. Available information includes germination responses with spores obtained at 15 and 37 °C from psychrotolerant *B. cereus* strains (Gounina-Allouane et al., 2008) and sporulation and germination responses of *B. weihenstephanensis* KBAB4 at 30 °C (Voort et al., 2010). In addition, Anderson Borge et al. (2001) investigated toxin profiles, growth, sporulation and germination of eleven strains of *Bacillus cereus* isolated from milk and meat products, including strains that grew at low temperature (4-7 °C). Spore germination was found

to be faster for the two strains that grew at 6 °C than for the other nine strains in milk at 7 and 10 °C. Thorsen et al. (2009) studied the impact of Modified Atmosphere Packaging on germination and growth at 8 °C on BHI agar and in a meat model, with spores obtained at 30 °C of emetic toxin producing *B. weihenstephanensis* strains.

Therefore, the objective of this study was to investigate the effect of temperature (7, 10, 12, 20 and 30°C) on growth, sporulation, and spore characteristics, including wet heat resistance, germination and outgrowth capacity, of the psychrotolerant *B. weihenstephanensis* strain KBAB4. Based on the available genome sequence of this strain (Lapidus et al., 2008), phenotypic responses could be coupled to expression analysis of sporulation sigma factor sigmaG and genes encoding germinant receptors.

MATERIALS AND METHODS

Strain and culture conditions

The *Bacillus weihenstephanensis* KBAB4 strain used in this research was kindly provided by Dr. Vincent Sanchis from Institut National de Recherche Agronomique and cultured routinely on Luria Broth (LB, Merck, Germany) in a shaking incubator at 30°C with rotary shaking at 200 rpm. Spores were prepared in a nutrient-rich, chemically defined sporulation medium designated MSM medium, which contained the following components (final concentrations): nutrient broth (NB, Difco, the Netherlands, 8 g/l), maltose (10 mM), $CuCl_2$ (12.5 µM), $ZnCl_2$ (12.5 µM), $MnSO_4$ (66 µM), $MgCl_2$ (1 mM), $(NH_4)_2SO_4$ (5 mM), Na_2MoO_4 (2.5 µM), $CoCl_2$ (2.5 µM), $Ca(NO_3)_2$ (1 mM) and $FeSO_4$ (1 µM) (Sigma Aldrich., the Netherlands). 500-ml Erlenmeyer flasks containing 50 ml of MSM medium were inoculated with LB overnight-subcultures to a final concentration of 0.5%. These cultures were incubated in a shaking incubator at 7, 10, 12, 20 and 30°C with rotary shaking at 200 rpm. Cultures were monitored by the increase in OD_{600} to determine the exponential growth-phase duration and the entry in stationary phase; and by phase-contrast microscopy to check the appearance and proportion of phase-bright spores for determination of the sporulation rate. When sporulation was finished, spores were harvested, washed repeatedly, and stored as previously described (de Vries et al., 2005). The sporulation rate was determined by use of a phase-contrast microscope. The numbers of phase-bright spores and vegetative cells were estimated in at least three separate fields of view (20-50 spores each). The extent of sporulation is expressed as a percentage, relative to the number of phase-bright spores, with the

number of vegetative cells at every time. The data presented were the result of three independent experiments.

Spore properties

Spore surface hydrophobicity was measured according to the method described by Rosenberg et al. (1980). Spores were suspended in water, to an OD_{660} of 0.4 to 0.5 (OD before), whereafter 0.1 ml of *n*-hexadecane (Sigma Aldrich, the Netherlands) was added to 2 ml of spore suspension in a plastic tube. This mixture was vortexed for 1 min, after which the phases were allowed to separate for 15 min. Then, the OD_{660} of the aqueous phase was determined (OD after), and the percent transfer to the *n*-hexadecane was calculated by the formula 100 – [(OD after/ OD before) x 100]. Hydrophobicity was determined as mean values obtained from at least two independent experiments.

For the heat resistance assay, aliquots of 100 µl spores suspended at a concentration of 10^4 spores/ml in phosphate buffer pH 7.4 were sealed in 1 mm-diameter micropipettes (Brand, Germany), placed in a water bath calibrated to 95°C, and cooled after a set time in ice-cold water. Because of the small diameters of the micropipettes and the small volume of spore suspension, we assume that the heating and cooling of the spore suspensions were instantaneous. Samples were plated onto Brain Heart Infusion broth (BHI) (Difco, the Netherlands) solidified with 1.5% agar (Difco). Colonies were counted after overnight incubation at 30°C. D_{95} values were calculated as the negative reciprocals of the slopes of the regression lines plotted with the values of the survival curves ($\log_{10}$ population versus time at 95ºC). D_{95} values were determined in duplicate. Survival counts were based on mean values obtained

from at least two independent experiments. The data presented indicate the mean values and mean standard deviations for the data points.

In order to determine spore size, flow cytometry was performed with a FACSCalibur (Becton Dickinson, USA.) equipped with an air-cooled 15-mW argon ion laser operating at 488 nm. Spores were stained with 0.1 μM of 4'6-diamidino-2-phenylindole (DAPI) (Molecular Probes BV, The Netherlands) to better visualize the spores. 20000 events were acquired at the low rate, and the cell concentration was adjusted to maintain a count of 500 to 600 events/s. The data were analyzed with the Cyflogic software (CyFlo Ltd, Finland). The Flow Cytometry Size Calibration Kit (Molecular Probes BV, the Netherlands) was used to correlate Forward Scatter parameters (FSC) with size values.

RNA isolation and real-time PCR

The expression of the genes encoding the GerA-component of the germination receptors (*gerI, gerK, gerL, gerR, gerS, gerS2*) and of *sigG* encoding the sporulation sigma factor σ^G was monitored by use of real-time reverse transcription (RT)-PCR, performed as described earlier (van Schaik et al., 2005) by use of 500 ng of total RNA, a mix of reverse primers relevant for the specific strain and Superscript III reverse transcriptase (Invitrogen, Breda, the Netherlands). Quantitative PCR was performed with the synthesized cDNAs by using an ABI Prism 7700 with SYBR Green technology (PE Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). The level of expression was related to the expression of the reference genes *rpoA*, *tufA* and 16S *rRNA* expression. Expression of the *ger* genes and *sigG* in the exponential phase and at the first point indicated in Figure 2 were similar, therefore expression at this first point was set to be zero (no regulation). Cell samples were taken at preset

intervals during incubation at 12, 20 or 30ºC. Samples were snap frozen in liquid N_2 for RNA isolation. After thawing on ice, samples were centrifuged at 13,000 rpm and resuspended in TriReagent (Ambion, Huntingdon, UK). Subsequently, after bead-beating, RNA was isolated according to the TriReagent protocol and residual DNA was removed using Turbo DNAse free (Ambion, Huntingdon, UK) .

All the samples in this communication were handled in exactly the same way to enable a good comparison of the gene expression in the three conditions tested. Real-time RT-PCRs were carried out in duplicate and analyzed with REST-MCS v.2.0 (Pfaffl et al., 2002).

Bioinformatics analysis

The genome sequence data for *B. weihenstephanensis* KBAB4 (Lapidus et al., 2008) was accessed via the ERGO database (Overbeek et al., 2003). From this genome sequence all *B. weihenstephanensis* KBAB4 operons encoding germination receptors were identified by homology searches. Genome context was visualized using the ERGO Bioinformatics Suite (http://ergo.integratedgenomics.com/ERGO). In addition, 150 bp upstream promoter sequences of the germination receptor operons were analyzed using DBTBS (http://dbtbs.hgc.jp) for identification of putative binding sites of σ^G binding (threshold 5%). Upstream regions, for which DBTBS could not identify a σ^G binding site, were screened manually for sequences resembling σ^G binding sites. A multiple sequence alignment by use of Muscle 3.6 (Edgar, 2004) was performed with the putative binding sites obtained for the germination receptors. Subsequently, the obtained consensus sequence for σ^G binding was visualized using Weblogo (Crooks et al., 2004).

Germination assays

Spore germination was measured by the drop in OD_{600} of spore suspensions produced at 7, 10, 12, 20 and 30°C by using a Spectramax Plus^{384} plate reader (Molecular Devices, USA). Spores were suspended at an OD_{600} of 0.5 to 1.0 in phosphate buffer, and after the addition of the germinants, the OD_{600} was followed with intermittent shaking to prevent settling of the spores. Spores were germinated at different temperatures, 5, 10, 12, 20 and 30ºC. The reduction in the OD_{600} reflects the number of germination events in the whole spore population by a change in refractivity of the spores from phase-bright to phase-dark. The percentage of germination was determined following calculations by Hornstra et al. (2006). Since 62% drop of the initial OD_{600} reduction reflected 100% germination, the other germination responses were related to this maximum response to calculate % germination. Spores were routinely checked for their germination behavior by phase contrast microscopy. The concentrations of the germinants used were: 12.5 mM inosine, 25 mM L-Alanine, 1 mM and 20 mM of the L-Amino acids Glycine (Gly), Valine (Val), Leucine (Leu), Isoleucine (Ile), Aspartic acid (Asp), Glutamic acid (Glu), Asparagine (Asn), Glutamine (Gln), Lysine (Lys), Arginine (Arg), Histidine (His) and Proline (Pro); 1 and 5 mM of the L-Amino acids: Phenylalanine (Phe), Tryptophan (Trp), Tyrosine (Tyr), Cysteine (Cys), Methionine (Met), Serine (Ser), and Threonine (Thr); and 20 mM Calcium-Di-Picolinic Acid (CaDPA) (Sigma Aldrich., the Netherlands).

Germination was also assayed in model food products including UHT milk (Campina, the Netherlands), meat bouillon (Knorr, the Netherlands) and cooked rice

water (Lassie BV, the Netherlands). These products were prepared as described by Hornstra et al. (2005).

Where indicated, spores were heat-activated by incubation at 70ºC for 15 min in phosphate buffer, washed and resuspended to appropriate numbers in the germination assays.

High Hydrostatic Pressure treatment

1 ml of a spore suspension with an OD_{600} of 0.6-1.0 in phosphate buffer pH 7.4 was transferred to a sterile plastic stomacher bag (Seward, United Kingdom) and heat-sealed while avoiding air bubbles in the bag. Pouches with spore suspensions were pressurized in a high-pressure unit (Resato, the Netherlands) containing glycol at 20°C as the compressing fluid. Spore suspensions were exposed to 150 MPa for 30 sec and 500 MPa pressure for 2 min. Due to temperature control, adiabatic heating only caused a transient temperature rise of 7°C at 150 MPa and 14°C at 500 MPa. After the pressure treatment, the OD_{600} reduction was taken as measure of spore germination.

Outgrowth and growth assays

Spores were suspended at an OD_{600} of 0.1 to 0.2 in BHI, and the OD_{600} was followed as described above in the germination assays and incubated at 12, 20 or 30ºC. To simplify the figure, the part corresponding to the germination process has been omitted. The lowest point after germination was set to 100%. Subsequent increases in this percentage represent spore development and multiplication.

Statistical analysis

t-test or ANOVA analysis were used to detect statistical differences between the samples. The statistical significance of each attribute considered was calculated at the (p = 0.05) level. All statistical analyses were carried out using GraphPad PRISM (GraphPad Software, Inc., San Diego, USA).

RESULTS

Growth and sporulation

The incubation temperature used largely affected growth and sporulation behaviour of *Bacillus weihenstephanensis* (Figure 1). Entry into stationary phase increased from 9h at 30ºC, and 21h at 20ºC to 50h at 12ºC. At all incubation temperatures, vegetative growth to a cell density at 600nm of approximately 5.5 preceded sporulation. The initiation time for sporulation in stationary phase increased from 6h at 30ºC, to 7h at 20ºC and to 50h at 12ºC. Another valuable parameter to consider is the elapsed time from the detection of the first phase-bright spore to the detection of more than 99% of cells harbouring a phase-bright spore. Figure 1 shows that this time decreased from 17h at 12ºC to 6h when the sporulation temperature was 20ºC and to only 2h when the temperature was 30ºC. The high degree of sporulation (more than 99%) facilitated the spore purification, since no vegetative cells were detected in the spore suspensions. Notably, *B. weihenstephanensis* KBAB4 cells also sporulated at 7 and 10ºC. However, a low sporulation degree of only 10-15% hampered their purification, and these spore suspensions were therefore only used in selected experiments as described later.

Spore properties

The obtained spores at 12, 20 and 30ºC remained phase-bright over time when resuspended in washing phosphate buffer. Hydrophobicity characteristics of the spore batches tested were similar ($p<0.05$), showing these spores to be highly hydrophobic, with around 90% of the spores being transferred from water to the *n*-hexadecane phase in the BATH assay (Rosenberg et al., 1980). The average spore size differed as

spores obtained at 12ºC and 30ºC had a similar size of approximately 1.5 μm (p>0.05), whereas spores produced at 20ºC were somewhat larger, 1.8 μm (p<0.05). The difference in sizes was corroborated by Scanning Electron Microscopy (data not shown).

Wet heat resistance parameters are given in Table 1. Comparing the D_{95} values, spores produced at 30ºC were 12-fold more heat resistant than those produced at 12ºC, and 5-fold more resistant than spores produced at 20ºC. Notably, spores obtained at 7 and 10ºC did not survive heat-challenge experiments of 1 min at 95ºC, although survival of these spores was observed after a 15 min treatment at 70ºC (data not shown).

Consensus sequence for σ^G binding

The sequenced genome of *B. weihenstephanensis* KBAB4 was analysed and 6 *ger* operons were identified. Subsequently, these *ger* operons by homology were named *gerI*, *gerK*, *gerL*, *gerR*, *gerS* and *gerS2*. Five of these *ger* operons were identified to be on the bacterial chromosome, with *gerS2* located on a plasmid (Lapidus et al., 2008). For five of six promoter sequences of the different *ger* operons, a putative σ^G promoter binding site could be identified using DBTBS (Sierro et al., 2008) (Table 2). In addition, a putative sixth σ^G promoter binding site for the *gerL* operon was identified by a manual search (Table 2). The putative consensus binding site for σ^G was deduced from the individual binding sites (Table 2).

Transcription levels of the *ger* operons at different incubation temperatures

At all incubation temperatures, relative transcription levels of the *ger* operons and *sigG* were measured from the entry into stationary phase until the detection of the

first phase-bright spores. Transcription of the *ger* operons and *sigG* started and reached its maximum at different times depending on the incubation temperature. As seen in Figure 2A, at 30ºC expression was observed at an incubation time of 11h, 2h after the culture entered stationary phase, and was prolonged for at least the next 4h. Interestingly, expression of the *ger* operons and *sigG* occurred at the same time, indicating a simultaneous activation of these genes. At the time of the appearance of the first phase-bright spores, *sigG* expression was highly activated. At 20ºC (Figure 2B), the induction of expression started 2h after the start of stationary phase and lasted for at least the next 8h. At 12ºC, expression of *sigG* and the *ger* operons occurred 10h after entry into the stationary phase and was kept for over 40h (Figure 2C). This points to delayed and extended activation of expression of these spore parameters at low temperatures.

Germination characteristics of *Bacillus weihenstephanensis* KBAB4 sporulated at different temperatures

a) Effect of germinant molecules

To assess the germination characteristics of spores prepared at 12, 20 and 30ºC, spore germination assays were performed using 20 L-Amino-acids, the purine ribonucleoside inosine and exogenous CaDPA as germinant molecules. In the absence of germinants, no germination was observed (Figure 3A). Exposure to inosine resulted in a delayed germination response after 90 min. Germination triggering capacity of L-amino-acids was tested, but not one of the L-amino-acids was able to initiate a clear germination response. In contrast, analysis in combination with a non-triggering concentration of inosine (0.1mM), identified eleven L-amino-acids (Ala, Phe, Gly, Val, Leu, Ile, Cys, Met, Ser, Thr, and Gln) to be able to trigger a germination response

(data not shown). A combination of inosine with L-Alanine was shown to induce a quick and strong germination response of spores, resulting in more than 50% germination within 15 min (Figure 3A). Moreover, addition of CaDPA could not trigger a germination response in not-heat activated *B. weihenstephanensis* spores (data not shown).

b) Effect of Heat activation

A heat-activation (70ºC/15 min) stimulated spore germination. As shown in Figure 3B (grey symbols), heat activated spores exposed to inosine initiated a slow germination response within 30 min. In addition, the combination of inosine with L-alanine induced a quicker and stronger germination response of spores (Figure 3B). However, the other tested germinants did not show increased germination responses after heat activation (data not shown). Germination of the spores was also tested in (model)foods, such as meat bouillon, rice water or sterilized milk, but only low level germination (<5%) was observed under these conditions after 2h of incubation at 30ºC (data not shown).

c) Effect of sporulation temperature

Heat-activated spores obtained at the different temperatures were tested for their germination efficiency with the most powerful germinant molecules identified. When spores were resuspended in inosine at 30ºC and analysed after 90 min, the spores obtained at 30ºC germinated more efficiently than those obtained at 20 or 12ºC (Figure 3A). The combination of inosine and L-Alanine triggered spore germination to the same extent (>99%) after 90 min (Figure 3A), independently of their sporulation temperature. However, the rate in OD_{600} decrease differed as a function of the sporulation temperature. Thus, after 10 min at 30ºC in contact with inosine and L-alanine, the germination rate was >99%, 85% and 65% for heat-activated-spores

produced at 30, 20 and 12ºC, respectively (Figure 3B). In contrast, the sporulation temperature had a different effect on the germination efficiency of the not-heat-activated spores. After 10 min the germination rate was 85%, 55% and 65% for not-heat-activated-spores produced at 30, 20 and 12ºC respectively (Figure 3A). So, if no heat-activation was applied, spores produced at 12ºC germinated faster than spores produced at 20ºC. It is also noticeable that germination after 10 min of spores produced at 12ºC was not influenced by a heat-activation. In addition, the spores obtained from cultures incubated at 7 and 10ºC showed no detectable germination (<5%) under any of these conditions (data not shown).

d) Effect of germination temperature

The impact of germination temperature ranging from 5ºC to 30ºC, was assessed using heat-activated spores. After 90 min of exposure to the germinants inosine and L-alanine, all the tested spore batches showed germination at all the temperatures (Figure 4). Final germination percentages ranged between 74% for spores obtained at 12ºC and germinated at 5ºC, and >99% for spores obtained at 30ºC and germinated at 30ºC. Generally, spores formed at higher temperatures germinated more efficiently at all temperatures tested. Notably, spores produced at 12ºC showed a clear optimum in germination capacity at this same temperature of 12ºC.

e) Germination of spores by HP

Figure 5 shows the influence of sporulation temperature on the germination of *B. weihenstephanensis* spores obtained at 12, 20 and 30ºC triggered by two HP treatments of different intensity. Germination of the spores was observed to be induced by all these treatments, however to a different extent. Pressurization at 150 MPa for 30 sec showed 50%, 15% and >99% germination of the spores obtained at 12, 20 and 30ºC, respectively. Treatment of the spores at 500 MPa for 2 min, resulted

in 35% of germination, independently of the temperature of sporulation. Remarkably, for spores obtained at 20ºC, it was shown that at the highest pressure applied, the germination capacity increased.

(Out)growth capacity

Heat-activated spores were incubated in BHI at different temperatures and outgrowth capacity was monitored by following the OD_{600} increase, displaying biphasic graphs (Figure 6). The first part of the graph shows a lag phase for the germinated spores, identified to be the outgrowth or spore development phase. After an increase of 20% in the initial OD_{600}, the exponential-phase part of the graph represents the growth and multiplication of the vegetative cells.

The duration and kinetics of the outgrowth lag phase were dependent on the incubation temperature but showed to be independent of the temperature at which the spores were obtained. Incubation at 30, 20 and 12ºC showed similar performances for the three different spore batches, and OD_{600} values increased 2-fold in approximately 120, 300 and 660 min, respectively.

DISCUSSION

B. weihenstephanensis KBAB4 vegetative cells were shown to grow and sporulate slower when the temperature was decreased. Spores were obtained with sporulation percentages close to 100% at 12, 20 and 30ºC, signifying that MSM is a very effective liquid culture medium for sporulation. *B. weihenstephanensis* KBAB4 spores had diameters varying from 1.5 to 1.8 µm and showed a high hydrophobicity independently of the sporulation temperature. Spore surface hydrophobicity is a major determinant for adhesion capacity to hydrophobic surfaces (Dickson and Koohmaraie, 1989), an important factor in recontamination of foods (Kumar and Anand, 1998).

Sporulation temperature had a significant impact on *B. weihenstephanensis* KBAB4 spore heat resistance properties, with cells sporulated at low temperatures showing significantly lower thermoresistance. This is in agreement with previous observations that showed sporulation temperature to be an important determinant in the wet heat resistance of spores (Palop et al., 1999; Melly et al., 2002). Although *B. weihenstephanensis* KBAB4 can grow and sporulate efficiently at low temperatures, the spores obtained at these conditions could be inactivated more efficiently than spores obtained at higher temperatures. Interestingly, *B. weihenstephanensis* KBAB4 also grew and sporulated at 7 and 10ºC, but with a decreased and heterogeneous sporulation efficiency, leading to suspensions comprising phase-bright and phase-dark spores and vegetative cells. Notably, these spores did not survive exposure to 95ºC, but they could survive exposure for 15 min at 70ºC, suggesting that such spores may survive pasteurisation treatments and grow out in these foods stored in refrigeration conditions. Reduced stability and heat resistance was previously noted for *Bacillus subtilis* spores that had reduced capacity to accumulate dipicolinic acid (DPA)

(Setlow et al. 2006; Magge et al. 2008). Whether reduced accumulation of DPA, or other mechanisms play a role in the reduced spore maturation efficiency and the reduced heat resistance capacity of *B. weihenstephanensis* KBAB4 spores produced at 7 and 10ºC in the conditions tested, remains to be elucidated.

Bioinformatic analysis allowed us to deduce a putative consensus σ^G promoter binding site for all the *ger* operons similar to that deduced for *B. subtilis* (Wang et al., 2006), indicating that the function of σ^G in *B. subtilis* is conserved among other members of the genus *Bacillus* and supporting its role in activation of expression of the *ger* operons. Furthermore, it was observed that, at 12, 20 and 30ºC, the induction of all the *ger* operons present in *B. weihenstephanensis* KBAB4 and *sigG* occurred at representative times in the cellular differentiation process, indicating that σ^G possibly plays a role in the final stages of sporulation by regulating not only synthesis of germinant receptor proteins but also other spore proteins such as SASPs, as shown in *B. subtilis* (Helmann and Moran, 2002).

Germination of psychrotolerant *B. cereus* strains has been studied before in comparison to mesophilic *B. cereus* strains, showing the germination of psychrotolerant *B. cereus* strains to be more efficient at low temperature (Anderson Borge et al., 2001). However, the influence of the sporulation temperature on germination of psychrotolerant *B. cereus* strains had not been extensively studied (Gounina-Allouane et al., 2008). Detailed germination studies at different temperatures revealed the combination of inosine and L-Alanine to be the most powerful germinant for spores obtained at 12, 20 and 30ºC. Not-heat-activated KBAB4 spores did not germinate with L-Amino-acids as single germinants or with CaDPA, previously shown to act as germinants for other *Bacillus* species (Hornstra et al., 2006; Paidhungat et al., 2001), however germination in combination with inosine

was shown for 11 L-Amino-acids. Germination of strain KBAB4 spores was highly stimulated after heat activation, conceivably by facilitating access to the germinant receptors for the germinants (Alimova et al., 2006; Leuschner and Lillford, 1999). Furthermore, it was observed that the higher the sporulation temperature, the faster the germination by the combination of inosine and L-Alanine, with the final spore germination efficiency similar for the different types of spores. A similar observation was made for *C. botulinum* spores produced at temperatures of 20 and 30ºC (Peck et al., 1995).

B. weihenstephanensis KBAB4 spores could also be germinated by low-(100-150 MPa) and high-(500-800 MPa) pressure treatments (Wuytack et al., 1998). Germination of *B. weihenstephanensis* KBAB4 spores by low-pressures showed significant differences as a function of the sporulation temperature, since spores obtained at 20ºC showed lower germination capacity than 12ºC and 30ºC spores. By increasing the pressure, the same final germination efficiency was obtained independently of the sporulation temperature. This observation confirms that mechanisms of germination by high-pressures differ from those activated by low-pressures (Black et al., 2007). Since low pressure-induced germination is assumed to involve activation of germinant receptors, whereas high pressure-induced germination is not (Paidhungat et al., 2002; Black et al., 2007), this would point to lower germinant receptor activity and/or triggering capacity in strain KBAB4 spores produced at 20ºC. This is in line with the observed lower germination activation of the 20ºC-derived spores by the combination of Alanine/inosine in nutrient-induced germination assays in comparison to the 30ºC-derived spores (Figure 4). Whether the larger size, conceivably due to a thicker cortex of the spores formed at 20ºC has a role in this, remains to be elucidated. Spores derived at 12ºC showed reduced germination

in comparison to 30ºC- and 20ºC-derived spores, especially at the highest (30ºC) and lowest (5ºC) germination temperature tested. This indicates low-temperature sporulation to influence the germination properties of the spores. In contrast, spores obtained at 12, 20 and 30ºC were observed to germinate, grow out and grow with similar kinetics in nutrient rich BHI at different temperatures. Noticeably, these kinetics varied as a function of the incubation temperature, with slower outgrowth and growth at lower temperatures, indicating the food preservation temperature to be a more important determinant for germination and outgrowth in (model)foods, than the sporulation temperature. In contrast to the general behaviour identified for vegetative cells where low temperature growth history stimulates subsequent performance at these temperatures (Hebraud and Potier, 1999), low temperature sporulation memory is not maintained or not effective in stimulating subsequent germination and outgrowth at these temperatures.

In conclusion, spores of *B. weihenstephanensis* KBAB4 were characterized according to several important factors for the food industry, such as heat-resistance, germination and outgrowth properties. The sporulation temperature was shown to influence a range of relevant spore properties including size, wet heat resistance and nutrient and pressure-induced germination capacity. Obviously, *B. weihenstephanensis* KBAB4 spores with different sporulation temperature histories can germinate and grow out with similar efficiencies at refrigeration temperature. Insights obtained in this study with *B. weihenstephanensis* KBAB4 may, together with the information contained in its genome sequence, contribute to the understanding of sporulation and germination behaviour of psychrotolerant Bacilli, and at the end supply tools for their enhanced control in foods.

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FIGURE CAPTIONS

Figure 1. Influence of incubation temperature (○: 30ºC, □: 20ºC and △: 12ºC) on OD_{600} (closed symbols) and percentage of sporulation (open symbols) in *Bacillus weihenstephanensis* KBAB4 cells.

Figure 2. Influence of sporulation temperature (A: 30ºC; B: 20ºC and C: 12ºC) on transcription levels of *ger* operons (▲: *gerI*, ▼: *gerK*, ◇: *gerL*, ●: *gerR*, □: *gerS* and △: *gerS2*) and *sigG* (■). Arrow indicates the moment of the occurrence of the first bright-phase spores. The results shown are the averages of duplicate experiments performed with two independent spore batches.

Figure 3. Germination at 30ºC of *B. weihenstephanensis* KBAB4 spores in phosphate buffer pH 7.4 (open symbols), with 12.5 mM Inosine (grey symbols) and a combination of 12.5 mM inosine and 25 mM L-Alanine (closed symbols). Spores were produced at different temperatures (○: 30ºC, □: 20ºC and △: 12ºC). Spores were germinated without (A) and with heat activation (B). The results shown are the averages of duplicate experiments completed with two independent spore batches.

Figure 4. Final germination percentage of *Bacillus weihenstephanensis* KBAB4 spores after 90 minutes of incubation at different temperatures with a combination of 12.5 mM inosine and 25 mM L-Alanine. Spores were produced at different temperatures (■: 30ºC,■: 20ºC and □: 12ºC).

Figure 5. Final germination percentage of *Bacillus weihenstephanensis* KBAB4 spores after High Hydrostatic Pressure treatments: LP (Low-Pressure: 150 MPa for 0.5 min) and HP (High-Pressure: 500 MPa for 2 min). Spores were produced at different temperatures (■: 30ºC, ■: 20ºC and □: 12ºC).

Figure 6. OD_{600} changes of germinated *Bacillus weihenstephanensis* KBAB4 spores in BHI incubated at different temperatures (circles, 30ºC; squares, 20ºC; triangles, 12ºC). Spores used were produced at different temperatures (black symbols, 30ºC; grey symbols, 20ºC; white symbols, 30ºC).

Figure 1

Figure 1

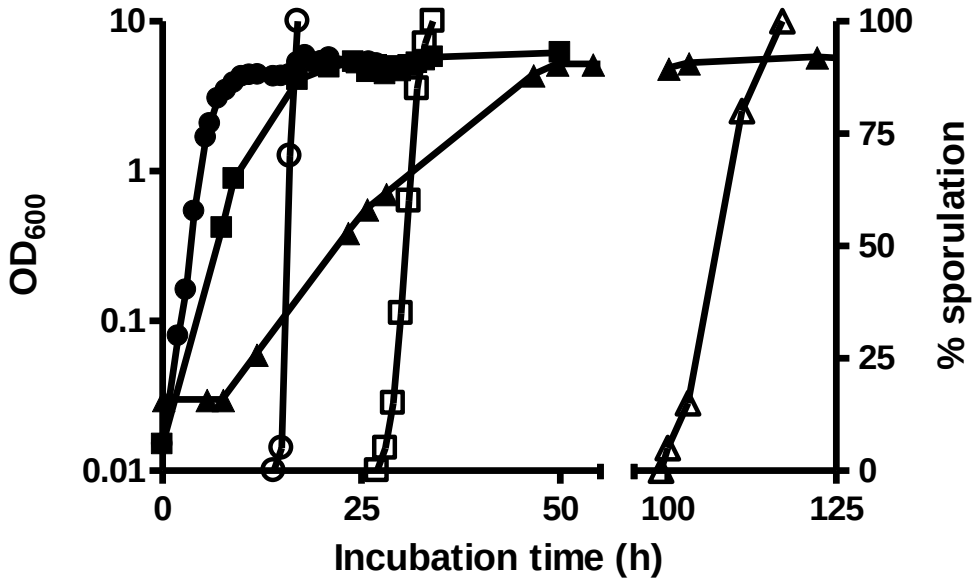

Table 1

Table 1: Properties of *Bacillus weihenstephanensis* KBAB4 spores obtained at different temperatures.

Sporulation temperature	Hydrophobicity (% transfer hexadecane)	Size (μm)	D_{95} (min)
12ºC	87.43±0.46 [a]	1.48±0.19 [a]	0.91±0.10 [a]
20ºC	91.04±2.29 [a]	1.82±0.14 [b]	4.80±2.53 [b]
30ºC	90.05±4.05 [a]	1.53±0.22 [a]	12.61±0.98 [c]

[a–c]: Any two means in the same column followed by the same letter are not significantly different (p<0.05).

Table 2

Table 2: Putative binding site for σ^G and consensus promoter sequences for *ger* operons in *Bacillus weihenstephanensis* KBAB4 and their chromosomal or plasmid location.

ger operons	Location	Putative binding site for σ^G
gerI	Chromosome	GAATAA-AATTCAAACATATAAAAATAATA
gerK	Chromosome	GCATAATTTTTTCATAAAAAGCAAAAATTA
gerL	Chromosome	GTATATATTTTCTTCTATTAGCGGAATCTA
gerR	Chromosome	GTATAA-ATTCCCGTCTTTCCCAAAAACTA
gerS	Chromosome	GGATAT-TTTTTCTTACTATATGCATACTA
gerS2	Plasmid	GAATAA-TATACAAAATTAGCCACAAAATA
consensus sequence		GNATA wwT ww AwwNTA