Diversity of spore germination in response to inosine and L-alanine and its interaction with NaCl and pH in the Bacillus cereus group

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Diversity of spore germination in response to inosine and L-alanine and its interaction with NaCl and pH in the *Bacillus cereus* group

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Running head: diversity of *B. cereus* spore germination

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Aims: Our aim was to assess the diversity of the nutrient germination response of *Bacillus cereus* spores.

Methods and Results: *Bacillus cereus* spore germination was monitored by decrease in optical density using a Bioscreen C analyser in response to the major germinant substances inosine and L-alanine. Spores of a set of twelve strains taken to illustrate the diversity of the *B. cereus* group showed ranging germination capacities. Two strains never germinated in the presence of L-alanine, at any of the germinant concentrations tested. Both the extent and rate of spore germination were affected by low pH and high NaCl concentration, but differently according to the strain.

Conclusions: A broad diversity was observed in nutrient-triggered spore germination among the members of the *B. cereus* group. Spore germination of some strains occurred at low concentrations of inosine or L-alanine, suggesting high receptor sensitivity to germinants. The activity of these receptors was also affected by pH or high NaCl concentration.

Significance and impact of the study: The greater ability of some strains to germinate in response to L-alanine and inosine is one criterion among others for *B. cereus* strain selection in food processing or storage studies, before confirmation in complex food or laboratory media. The diversity in response to germinants found among the *B. cereus* strains suggests a differential expression and (or) absence of some germination genes involved in the response, mainly to L-alanine.

Keywords: nutrient spore germination, *B. cereus*, strain diversity, L-alanine, inosine, NaCl, pH

INTRODUCTION

Bacillus cereus is an endospore-forming pathogenic bacterium frequently isolated from food (Nguyen-the and Broussolle 2005). Spores can survive food processing and vegetative cells generated from spores will adapt to environmental conditions such as temperature, pH, or a_w. Spore germination and growth of *B. cereus* during storage can cause food spoilage and (or) food poisoning. In food, some *B. cereus* strains can produce cereulide, a cyclic peptide causing vomiting, and in the host small intestine several enterotoxins can be produced, causing diarrhoea (Granum 2007). Spore germination is a key event in the development of spore-forming foodborne bacterial pathogens and is triggered by germinants, ribosides or amino acids, that differ among species (Gould 1969). For *B. cereus* and *B. thuringiensis*, major germinants are inosine and L-alanine. Spore germination of the closely related *B. anthracis* is triggered by L-alanine alone at high concentration, whereas inosine is only efficient as a co-germinant with other aminoacids (Ireland *et al.* 2002). In the germination process, germinants first interact with specific receptors located in the inner spore membrane (Hudson *et al.* 2001; Paidhungat and Setlow 2001; Alberto *et al.* 2005). This interaction then triggers the release of spore components, *ie* cations and dipicolinic acid, and their replacement by water. These events lead to the hydrolysis of the spore cortex by specific lytic enzymes, the expansion of the germ cell wall and finally the resumption of the spore metabolism and macromolecule synthesis that turns a germinated spore into a growing cell. The mechanism of spore germination has been extensively studied in *B. subtilis* and more recently in *B. cereus*, with a special focus on the nutrient germination receptors (Moir 2006; Setlow and Johnson 2007). Seven operons encoding these receptors have been identified in the type strain *B. cereus* ATCC 14579 genome and their respective roles in nutrient-induced germination have recently been studied (Hornstra *et al.* 2006b). Three of these receptors were also described in the *B. cereus* strain ATCC 10876 (Clements and Moir 1998; Barlass *et al.*

2002). These earlier studies to determine the specific germinants for *B. cereus* and the germination mechanism at a molecular level were restricted to one or two model strains, not necessarily representative of the intra-species diversity. Diversity in *B. cereus* virulence factors (Moravek *et al.* 2006), heat resistance and growth characteristics (Carlin *et al.* 2006) has been extensively studied. In contrast, information on spore germination is scarce. The aim of the present study was to investigate the diversity in the germination capacities of spores of the *B. cereus* group *sensu lato* (Guinebretière and Sanchis 2003; Guinebretière *et al.* 2007) in twelve reference and food strains, in response to inosine and L-alanine in various conditions of pH and NaCl, which may interfere with the spore germination process (Clements and Moir 1998; Southworth *et al.* 2001) and subsequent growth.

MATERIALS AND METHODS

B. cereus strains and production of spores

The bacterial strains used were *B. cereus* ATCC 14579 (American Type Culture Collection, Manassas, VA, USA), *B. cereus* TL811 and *B. cereus* TZ415, *B. cereus* INRA-5, *B. cereus* INRA-32 (INRA, Avignon, France), *B. cereus* I3, *B. cereus* I11, *B. cereus* I32 (ADRIA Normandie, Villers Bocage, France), *B. cereus* F4430/73 (gift of Prof. P.E. Granum, Norwegian School of Veterinary Science, Oslo, Norway), *B. cereus* DSM 4282 (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Germany). *B. mycoides* CIP 103472 (Collection Institut Pasteur, Paris, France), *B. weihenstephanensis* WSBC 10204 (gift of Prof. S. Scherer, Technische Universität München, Freising, Germany) were added to extend the study to strains of the *B. cereus sensu lato* group (Guinebretière and Sanchis 2003; Guinebretière *et al.* 2007)

Spores were prepared on fortified nutrient agar (FNA) plates containing (all per litre) peptone 5 g, beef extract 3 g, agar 20 g, NaCl 3 g, glucose 0.1 g, $MnSO_4.H_2O$ 0.05 g, $CaCl_2$

0.06 g, $(NH)_4SO_4$ 0.08 g, $MnCl_2.4H_2O$ 0.008 g, $CuSO_4.5H_2O$ 0.005 g, $ZnSO_4.7H_2O$ 0.005 g, final pH adjusted to 7.0 (Fernandez *et al* 1999) spread with an overnight culture of *B. cereus* in J-Broth medium and incubated at 30 °C. Spores were harvested when the cultures contained more than 95% of free phase-bright spores, which required 5-21 days. They were pasteurised for 15 min at 70°C to kill vegetative cells and highly purified on an Urographin gradient (Plowman and Peck 2002). Microscopic examination confirmed that spores were >95% phase-bright. Spores were then counted on J-Broth agar plates, diluted in cold water to a final concentration of 10^9 spores/ml and stored at -20°C until use.

Bacterial growth from 10^4-10^5 cfu/ml spore inocula was tested for 7 days in J-Broth medium (Claus and Berkeley 1986) at 30 °C. The pH values (3.8, 4.4, 5.0, 5.6, 6.2, 6.8, 7.4) were adjusted using 2 N HCl, and media were supplemented with NaCl (0.06, 0.25, 0.5, 1, 2, 3.5 and 5% wt/vol final concentrations). After inoculation, tubes were inspected daily and the time by which a visible bacterial growth could be detected was defined as time to turbidity.

Solutions for germination assays

Solutions of inosine (50 µM to 1 mM) and L-alanine (1 to 200 mM) were prepared in distilled water and stored at 4 °C until required. Effects of germinants and NaCl were tested using sterile 10 mM Tris-HCl/ 10 mM NaCl pH 7.4 buffer. Effects of pH on spore germination were tested in citrate-phosphate buffer prepared to obtain the desired pH (3.8 to 7.4). In these assays, inosine and L-alanine solutions were also prepared in the same buffer. D-cycloserine was used at 20 mM in L-alanine germination assays to inhibit the spore alanine racemase activity (Gould *et al.* 1966). Tris-HCl buffer was supplemented with NaCl to test the effect of different concentrations of salts (0.06 % to 5 %, wt/vol) on spore germination.

Spore germination

Spores, non-heat-activated or heat-activated at 70 °C for 10 min, were added to the germination buffer to an optical density at 600 nm (OD_{600}) of approximatively 1.0 in honeycomb microplates and incubated for 15 min at 30 °C. After addition of the germinant to a final volume of 350 µl, the germination process at 30 °C was followed by monitoring the OD_{600} with a Bioscreen C analyser system (Labsystems, Uxbridge, UK). To prevent spores settling, microplates were vigorously shaken before reading the optical density every 2 min. Three replicate wells were tested for each combination of germinant. For each condition, a negative control was made up consisting of spores in buffer alone.

The extent of germination was expressed as the percentage decrease in OD_{600} obtained using the formula: $[1 - \{OD_t - (OD_{Ct} - OD_{C0})\}/OD_0] \times 100$ where OD_t and OD_{Ct} are the OD_{600} values measured after incubation time t for the test sample and the negative control (without germinant) respectively, and OD_0 and OD_{C0} are the initial OD_{600} values measured for the sample and the negative control respectively. At the end of each experiment, the proportion of phase-dark/grey (germinated) spores and phase-bright (ungerminated) spores were estimated under a phase contrast microscope by counting approximately 100 spores in each of three to five observation fields. The percentage mean deviation (% difference between extreme values and the mean) calculated for 49 sets of replicate experiments (same strain, germinant and NaCl concentration, experiments performed with two spore preparations) was less than 15% for 44 out of 49 sets of data and marginally higher for the remaining data.

Statistical analyses were carried out using variance analysis using the ANOVA procedure (Systat version 9, SPSS, Chicago, IL, USA). Germination curves were modelled using the following equation (McCormick 1965):

$$OD_t - (OD_{Ct} - OD_{C0})/OD_0 = 100 \times [1 - (1-\alpha) \times (e^{-kt})^{-c}] \qquad (1)$$

where OD_t and OD_{Ct} are the OD_{600} values measured after an incubation time t for the test sample and the negative control respectively, and OD_0 and OD_{C0} the initial OD_{600} of the test sample and of the negative control respectively, and α, k, and c are constants. The maximum germination rate V_{max} was taken as the value of the first derivative of equation (1) at t_{max}, the time at which the second derivative of equation (1) is equal to 0, given by

$$t_{max} = (kc/1+c)^{1/c} \qquad (2)$$

RESULTS

Spore germination induced by inosine and L-alanine was assessed on twelve *B. cereus* strains (Table 1) by measurement of the OD_{600} decrease of spore suspensions. A significant positive correlation ($r^2 = 0.95$, significant at $P < 0.001$) was obtained between the % OD_{600} decrease and the % phase-dark (germinated) spores, evaluated under a phase-contrast microscope at the end of the experiments. However, 100 % germinated spores under the microscope did not correspond to the same absolute OD_t value for all strains (Table 2). Preliminary germination assays were carried out with both heat-activated and untreated spores. We observed no significant difference (data not shown) and so subsequent tests were performed with non-activated spores.

For all the strains tested, maximum spore germination was obtained after 100 min and no additional germination was observed for longer incubation, up to 160-200 min. Spores of nine strains out of twelve showed a % OD_{600} decrease ranging from 40.8 to 63.6 in response to 1 mM inosine after 100 min., while spores of the three remaining strains, BmT, TZ415 and I11, exhibited a lower % OD_{600} decrease (from 18.8 to 28.8 %) (Fig 1A and Table 2). Similarly, these three strains together with I3 and BwT spores germinated poorly or not at all in response to 200 mM alanine (Fig 1B and Table 2). For the other strains, spore germination was between 36.1% and 65.6% of OD_{600} decrease (Table 2). A clear effect of inosine

concentration (0.05 to 1 mM) and alanine concentration (1 to 200 mM) on the kinetics of spore germination was observed, as shown with strain DSM 4282 (Figure 2) and for most of the other strains (Table 2). Significant germination was observed for concentrations equal to or higher than 0.2 mM inosine (Figure 2A) and 5 mM L-alanine (Figure 2B), and spore germination increased in step with germinant concentration. Interestingly, spores of INRA-5, I3, BmT, BwT, I11 and TZ415 showed a higher germination than others at 0.05 mM inosine, the lowest concentration tested. Increasing L-alanine concentration also greatly increased the final extent of germination for some strains, for example strain INRA-32, DSM 4282 or F4430, while it had no effect on other strains such as TL811 or TZ415 (Table 2). For strain TL811, L-alanine-triggered germination was greatest with the lowest concentration and higher than inosine-triggered germination. The concentration of germinant had an effect not only on the extent of spore germination but also on the germination rate defined as the maximum percentage in OD_{600} decrease per min. Different situations were observed according to the strains: in some cases, such as for BcT spores, the germination rate increased markedly with increasing germinant concentration, with both inosine and alanine (Fig 3 A and B). In other cases, for example I16, the germination rate increased to a lesser extent (Fig 3 A and B), despite a high overall rate of germination (Table 2). The germination rate of strain BwT was slightly affected by increasing germinant concentrations (Fig 3 A and B).

We investigated the effect of pH and NaCl concentration on spore germination triggered by either 1 mM inosine or 100 mM L-alanine. Spore germination in response to inosine decreased significantly with decreasing buffer pH for all the tested strains (Table 3), but the effect was more marked for some strains than for others, such as BwT or I11, for which no germination was observed at pH lower than 6.8. In contrast, for other strains such as TL811 or F4430, an OD_{600} decrease of 20% was still observed at pH 4.5. At pH 3.8, spore germination was very low or nil for all strains. L-alanine-triggered germination also decreased

with decreasing pH for most of the spores tested, and I11 and I3 spores showed no germination in this germinant at any pH (Table 3). For a given strain, the effect of pH on germination depended on the germinant. For instance, spores of BwT still germinated at pH 5.7 in the presence of L-alanine, whereas germination with inosine stopped at pH 6.8. With both germinants, lowering the pH decreased not only the extent of spore germination, but also the germination rate (data not shown). However, when *B. cereus* spores were inoculated in J-Broth medium at pH values ranging from 5.0 to 7.5, bacterial growth was always observed: time to turbidity at 30 °C was 3 days for strains INRA-32 and BwT and 1 day for the other strains. In J-Broth at pH 4.5, time to turbidity was 2-4 days for strains I11, I16, I3, BcT and 7 days for the remaining strains. At pH 3.8, no growth was detected after 7 days of incubation (data not shown).

For most of the strains tested except I11, the addition of up to 3.5 % NaCl only slightly modified spore germination in response to inosine (Table 4). Spores from strains BmT, BwT and TZ415 showed a 20 % OD_{600} decrease in the presence of 5% NaCl. For these spores, the germination rate was mainly affected for the highest NaCl concentrations tested, 3.5 % and 5 % (data not shown). In the presence of L-alanine, spores germinated less efficiently as the NaCl concentration increased except for BcT and F4430 spores. I3 and I11 spores did not germinate in response to this germinant at any NaCl concentration. The effect of salt was more pronounced on L-alanine germination than on inosine germination, for both extent (Table 4) and rate of spore germination (data not shown). We tested the effect on bacterial growth of increasing NaCl concentrations: cultures at 30°C from spore inocula in J-Broth medium were turbid after 1 day for all the NaCl concentrations, except with strains BmT and I3, which were turbid after 3 days with 3.5% and 5% NaCl, and with 5% NaCl respectively (data not shown).

DISCUSSION

Spore germination was investigated using twelve strains from the *B. cereus* group. These were laboratory strains, strains isolated from cooked foods or raw materials, and strains isolated from food-poisoning outbreaks, including both psychrotrophic and mesophilic strains. Spore germination was measured using an automated turbidometric system, Bioscreen, which had previously been successfully used to study spore germination of *B. subtilis* (Romick and Tharrington 1997) and *C. botulinum* (Plowman and Peck 2002; Alberto *et al.* 2003). As previously reported (Clements and Moir 1998), inosine and L-alanine were efficient germinants for most of the strains tested, particularly at the highest tested concentrations (1 mM and 200 mM respectively). For some strains, the extent of germination usually increased with increasing concentrations of germinants, as already reported (Collado *et al.* 2006). However, strains BmT, I11 and TZ415 germinated poorly, showing less than 30% decrease in OD_{600} at all concentrations of inosine, and (or) L-alanine (also BwT and I3). These results may be due to the number of germinant receptors present in the spore. The genome of *B. cereus* ATCC 14579, BcT in our study, contains seven putative *ger* operons termed *gerG, gerQ, gerI, gerL, gerK, gerR* and *gerS* (Hornstra *et al.* 2006b). These receptor-encoding genes are differentially expressed according to the sporulation medium and germination of the resulting spores in response to L-alanine or inosine differ significantly (Hornstra *et al*. 2006a). The respective roles of the receptors of the two germinants vary according to the strain: the *gerL* mutant in *B. cereus* ATCC 10876 exhibited no germination in response to alanine (Barlass *et al.* 2002), whereas no altered germination phenotype was attributed to loss of the homologous GerL receptor in the *B. cereus* ATCC 14579 (Hornstra *et al.* 2005). For I3 and I11, no germination was observed in L-alanine, suggesting the absence or non-expression of *gerL*, *gerR*, or of other genes possibly involved in this germination pathway, such as *sigB* encoding the stress response sigma factor. It has been shown that

spores of a *sigB* mutant show a severe defect in L-alanine-triggered germination (de Vries *et al.* 2005).

Interestingly, spores with a low extent of germination (around 30% OD_{600} decrease) at high germinant concentration, germinated nearly as well with a very low concentration of germinant (i.e. 0.05 mM). At low germinant concentration, spores of these strains exhibited a higher extent of germination than spores from other strains. We can assume that these mainly psychrotrophic spores would still germinate and lead to subsequent bacterial growth in food products or other environments containing only very low germinant concentrations.

The germination rate was usually correlated with the concentration of inosine or L-alanine. The highest germination rate was obtained with the highest concentration of germinant and consequently corresponded to the highest extent of germination, as previously described for strains BcT or ATCC 10876 (Clements and Moir 1998; Hornstra *et al*. 2005). However, the impact of inosine and L-alanine concentrations on the germination rate was different among strains and was not always correlated with extent of germination.

Many food preservation processes use acidification and (or) addition of NaCl to inhibit bacterial spore germination (Smoot and Pierson 1982). At pH 4.5 or 3.8, spores of the selected strains usually germinated poorly in response to 1 mM inosine or 100 mM L-alanine. The effect of low pH on germination was similar to what has been observed for *B. subtilis* spores (Ciarciaglini *et al.* 2000). Plowman and Peck (2002) showed no significant OD_{600} decrease, and therefore very low germination for *C. botulinum* spores at pH 5.0 or below. OD decreases of 20 % and 8.5 % were observed for BcT spores in L-alanine and inosine respectively at pH 3.8. These spores seemed to be less sensitive to this extreme pH than those of other strains. In contrast, spore germination of some strains was very pH-sensitive. Food acidification will therefore not affect spore germination of all *B. cereus* strains in the same way. As germination was affected differently by pH in the presence of L-alanine and inosine,

we can assume that the effect of pH was due to an action on germinant specific targets, *e.g.* the germinant receptors. Inhibition of spore germination at low pH could be due to inhibition of germinant binding by protonation of a functional group in or near the germinant receptor, as shown in both *B. cereus* and *C. botulinum* spores (Blocher and Busta 1985). However, this effect is reversible when spores were transferred at pH 7.0. Ciarciaglini *et al* (2000) showed that exposure of *B. subtilis* spores to low pH alone had little permanent effect on the L-alanine germination pathway; a combination of treatments (low pH, heat and lactic acid) was needed to cause permanent injury to the germination apparatus, mainly the spore's proteinaceous germinant receptors. In this work, growth was always observed in a nutrient broth at pH between 4.5 and 7.4, but with longer times to turbidity (from 48 h to 168 h according to the strains) as pH decreased. A small proportion of germinated spores may be sufficient to initiate bacterial growth. At pH lower than 4.5, growth of the tested strains was not observed within the 7 days of experimental incubation, in agreement with the limiting pH for growth reported for *B. cereus* (Lindsay *et al.* 2000).

Previous studies showed that sodium chloride at high concentration could inhibit the germination of spores of various species (Smoot and Pierson 1982). In the present study, spores of half of the *B. cereus* strains showed no inosine-triggered germination in the presence of 5% NaCl. In the same conditions, 8 out of the 12 tested strains still exhibited some germination in response to L-alanine. The effect of NaCl concentrations on spore germination was higher in the presence of inosine than of L-alanine, suggesting either that salt affected the specific germinant receptors differently, or that salt had an effect on the GerN protein, a germination Na^+/H^+-K^+ antiporter, that had a more marked influence on inosine than for L-alanine-initiated germination (Thackray *et al.* 2001). Growth of all *B. cereus* spores was observed one day after inoculation in a nutrient medium containing 0.06 % to 5% NaCl

and incubation at 30°C, indicating that high salt concentration can have an effect on spore germination but cannot prevent growth due to the development of a few germinated spores.

In conclusion, a wide variability of response to inosine or L-alanine was observed for spores of *B. cereus* within a set of strains covering some of the diversity of the *B. cereus* bacterial group. In real foods, populations of *B. cereus* are usually diverse, comprising several strains. The diversity in germination conditions of the various strains that may be present would presumably permit both outgrowth (from high germinating spores) and persistence of dormant and resistant spores (from low germinating strains) over a broad range of conditions, and we have shown that spores of some *B. cereus* strains can germinate at low germinant concentrations. These factors could influence the probability of *B. cereus* development, given that contamination of raw materials or food matrices by *B. cereus* is usually due to the presence of low numbers of spores (commonly <100 *B. cereus* spores/g) (Nguyen-the and Carlin 2005). However, efficient, fast germination may not mean shorter initial stages for growth: with non-proteolytic *C. botulinum* spores, there was no clear relation between rate of germination and time to first division or outgrowth (Stringer et al. 2005).

Assessing strain diversity may also be a promising and original approach to the exploration of the genetic germination process of *B. cereus*. We report diversity in response to germinants among the *B. cereus* strains that suggests different regulation pathways in the expression of germination genes during sporulation. The absence of some germination genes cannot be excluded for those of our strains that exhibited no germination with L-alanine.

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FIGURE LEGENDS

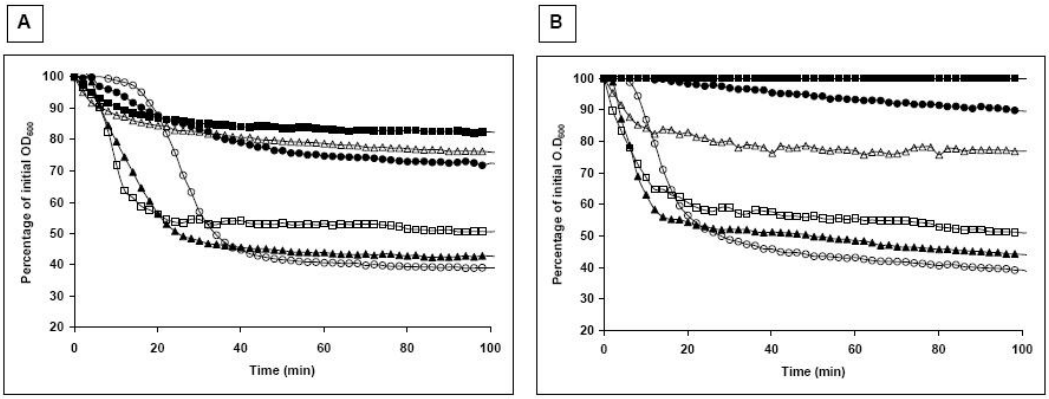
Figure 1: Decrease in OD_{600} (expressed as % initial OD_{600}) of spore suspensions during germination in 1 mM inosine (A) or 200 mM L-alanine (B) of *B. cereus* strain TZ415 (Δ), I11 (■), BmT (●), INRA-32 (○), DSM4282 (▲), and BcT (□).

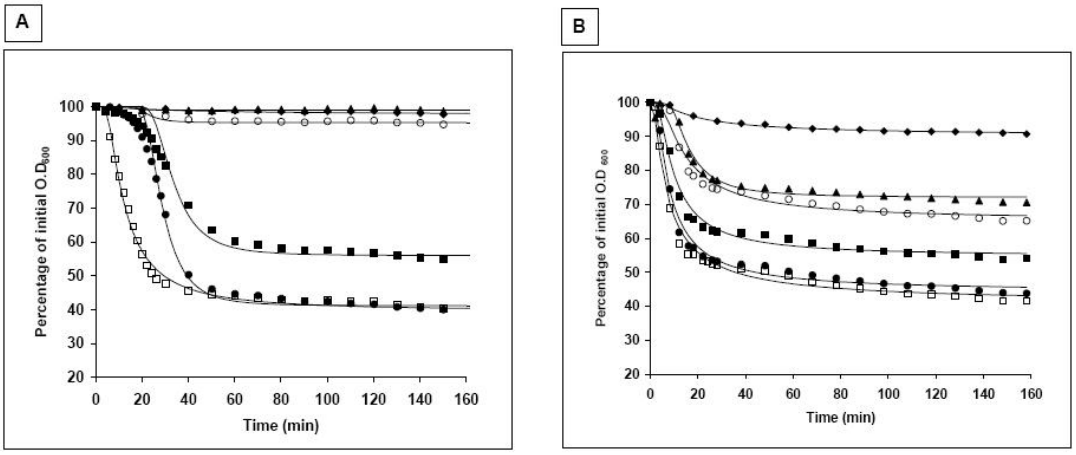
Figure 2: Effect of nutrient germinant concentrations on the decrease in OD_{600} (expressed as % initial OD_{600}) of spore suspensions of the *B. cereus* strain DSM 4282.

Panel A: symbols represent increasing concentrations of inosine, (◆, 0.05 mM), (▲, 0.1 mM), (○, 0.2 mM), (■, 0.3 mM), (●, 0.5 mM), (□, 1 mM)

Panel B: symbols represent increasing concentrations of L-alanine, (◆, 1 mM), (▲, 5 mM), (○, 10 mM), (■, 50 mM), (●, 100 mM), (□, 200 mM)

Figure 3: Effect of inosine (A) and L-alanine (B) concentrations on the germination rate (expressed as % initial $OD_{600}\,min^{-1}$) of *B. cereus* spores strain BcT (□), strain I16 (◇) and BwT (●). Bars represent standard deviation.





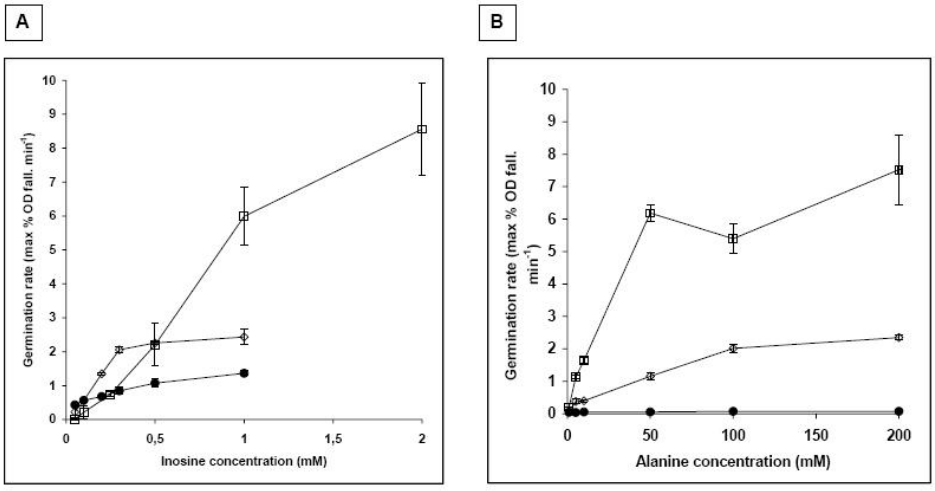


Table 1. Characteristics of the selected strains

Strain	Code in the study	Growth at 7°C *	Growth at 43°C *	Origin	Source †	Phylogenetic group ‡
B.cereus ATCC14579	BcT	-	+	type strain	ATCC	IV
B.mycoides CIP103472	BmT	+	-	type strain	CIP	VI
B.weihenstephanensis WSCB10204T	BwT	+	-	type strain	WSCB	VI
B.cereus TL811	TL811	-	+	isolated from vegetables	INRA	III
B.cereus TZ415	TZ415	+	-	isolated from vegetables	INRA	VI
B.cereus INRA-5	INRA-5	+	-	isolated from vegetable purees	INRA	VI
B.cereus I16	I16	-	+	isolated from cooked foods	ADRIA	IV
B.cereus I3	I3	+	-	isolated from cooked foods	ADRIA	VI
B.cereus INRA-32	INRA-32	-	+	isolated from vegetable purees	INRA	IV
B.cereus I11	I11	-	+	isolated from cooked foods	ADRIA	V
B.cereus F4430/73	F4430	-	+	diarrheic strain	NSVS	IV
B.cereus DSM4282	DSM4282	-	+	diarrheic strain	DSMZ	IV

*: test done according to Claus and Berkeley (1986)
†: see Materials and Methods
‡: Guinebretière *et al*, 2007

Table 2. Effect of inosine and L-alanine concentrations on the extent of germination (% decrease in initial OD_{600}) of *Bacillus cereus* spores

Strain	Inosine concentration						L-alanine concentration					
	0.05 mM	0.1 mM	0.2 mM	0.3 mM	0.5 mM	1 mM	1 mM	5 mM	10 mM	50 mM	100 mM	200 mM
BcT	8.1* a	12.1 ab	29.6† b	nt	**44.9** bc	**49.8** bc	35.3 a	**49.9** b	**53.4** b	**51.8** b	**56.2** b	**51.9** b
TL811	2.8 a	0.5 a	32.0 ac	**49.0** bc	**49.3** bc	**48.5** bc	**52.6** a	**52.4** a	**58.0** bc	**54.1** a	**54.9** ac	**58.9**b
DSM4282	2.6 a	1.3 a	5.7 a	47.1 b	**61.6** b	**61.9** b	9.3 a	29.4 b	34.8 b	45.9 c	**56.2** d	**58.5** d
INRA-5	27.9 a	38.3 b	47.1 c	**49.3** c	**52.5** c	**55.4** c	0.0 a	5.6 a	1.1 a	13.9 b	31.6 c	36.1 c
I16	4.7 a	12.3 b	39.4 c	**54.8** d	**59.9** e	**61.1** e	0.0 a	18.3 b	13.9 b	28.7 c	35.5 cd	42.3 d
I3	40.5 a	**41.6** abc	**44.2** bcd	**44.3** cd	**45.7** d	**40.8** ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
INRA-32	0.2 ab	0.0 a	22.7 ab	59.8 c	**63.6** c	**63.2** c	0.0 a	36.8 b	**48.5** c	**62.1** d	**65.4** d	**65.6** d
BmT	23.1 a	24.2 a	29.6 a	**28.9** a	**25.1** a	**28.8** a	3.3 a	12.6 b	23.3 c	18.6 c	20.9 c	21.0 c
BwT	34.4 a	39.4 b	43.9 c	**46.5** c	**46.8** c	**46.9** c	1.9 a	3.9 a	8.3 c	8.0 bc	9.2 c	14.0 d
F4430	12.6 a	6.0 a	12.6 ab	36.1 ab	**55.5** c	**63.6** c	26.8 a	42.6 a	**65.3** a	**41.6** a	**54.0** a	**61.6** a
I11	32.7 a	24.7 a	22.8 ab	**20.1** b	**22.6** b	**18.8** b	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
TZ415	37.6 a	**37.5** a	**34.3**† ab	nt	**31.0** bc	**27.4** cd	14.0 a	21.2 a	34.4 c	**25.3** d	**27.7** d	**28.2** d

* values are expressed as % decrease in initial OD_{600} of the spore suspension at the end of experiments. For each strain and germinant concentrations, values are the means of three replicates done simultaneously. For each strain and each germinant, values not followed by the same letter(s) are significantly different at $P < 0.05$ (Tukey's HSD). The incubation time ranged from 160 to 200 min; nt: concentration not tested; † 0.25 mM; % decrease in initial OD_{600} corresponding to a 100 % germination as observed under the microscope are indicated in bold type.

Table 3. Effect of pH on the extent of 1 mM inosine or 100 mM L-alanine-triggered germination (% decrease in initial OD_{600}) of *B. cereus* spores

Strain	Inosine							L-alanine						
	pH 7.5	pH 6.8	pH 6.2	pH 5.7	pH 5.0	pH 4.5	pH 3.8	pH 7.5	pH 6.8	pH 6.2	pH 5.7	pH 5.0	pH 4.5	pH 3.8
BcT	**73.7*** a	**47.4** b	42.4 b	25.7 c	17.5 cd	19.3 c	8.5 d	**64.7** a	**62.1** a	**60.9** a	**48.5** b	33.8 c	33.7 c	18.3 d
TL811	**58.1** a	**58.7** a	49.3 c	27.6 e	32.7 d	27.3 e	4.6 f	**61.1** a	**62.7** a	**54.8** b	**52.3** b	18.2 c	8.4 d	3.3 e
DSM 4282	**77.5** a	**62.1** a	22.3 b	20.9 bc	4.3 cd	0.0 d	6.1 bcd	**68.4** a	**62.4** ab	**56.6** b	40.4 c	26.8 d	12.4 e	8.8e
INRA-5	**56.0** a	50.7 a	34.0 b	10.1 c	1.9 cd	1.2 d	0.0 d	43.1 a	49.1 a	32.4 b	13.0 c	2.3 d	0.0 d	0.0 d
I16	**68.2** a	**64.5** a	47.6 b	36.2 b	20.0 c	2.9 cd	6.6 cd	**63.1** a	**68.7** a	**61.6** a	29.1 b	11.9 c	4.7 c	9.9 c
I3	**47.9** a	**37.2** ab	42.7 b	24.2 c	14.1 d	0.0 e	0.0 e	9.3 a	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
INRA-32	**71.0** a	**68.9** a	53.7 b	24.4c	9.6 d	0.9 e	1.5 e	**66.8** a	**70.9** a	**63.5** b	50.5 c	17.5 d	4.8 e	0.0 f
BmT	**34.4** a	**37.5** a	27.6 b	34.3a	0.0 c	0.0 c	1.1 c	**46.2** a	**44.9** a	**36.2** b	**27.2** c	19.5 d	8.0 e	7.9 e
BwT	**40.5** a	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	**50.9** a	**39.5** b	**28.4** c	20.1 d	1.0 e	1.5 e	0.0 e
F4430	**59.1** a	**59.4** a	33.9 b	29.9 b	14.1 bc	20.3 bc	6.4 c	**62.0** a	**65.0** a	**57.6** ab	41.8 b	14.5 c	12.7 c	6.8 c
I11	10.0 a	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
TZ415	**49.7** a	**54.6** a	**47.6** ab	41.3 b	22.4 c	18.2 c	2.9 d	**48.4** a	**46.8** a	38.9 a	38.0 a	18.5 b	0.0 c	0.0 c

* values are expressed as percentage decrease in initial OD_{600} of the spore suspension at the end of experiments. For each strain and each germinant, values not followed by the same letter(s) are significantly different at $P < 0.05$ (Tukey's HSD). For each strain and pH, values are the means of three replicates done simultaneously; the incubation time ranged from 160 to 200 min., % decrease in initial OD_{600} corresponding to a 100% germination as observed under the microscope are indicated in bold type

Table 4. Effect of NaCl concentrations on the extent of 1 mM inosine or 100 mM alanine-triggered germination (% decrease in initial OD_{600}) of *B. cereus* spores

Strains	Germination triggered by inosine							Germination triggered by L-alanine						
	NaCl concentrations (w/v)							NaCl concentrations (w/v)						
	0.06%	0.25%	0.5%	1%	2%	3.5%	5%	0.06%	0.25%	0.5%	1%	2%	3.5%	5%
BcT	**48.0** *a	**45.7** ab	**51.0** a	**46.0** ab	34.8 b	18.3 c	8.6 c	**53.0** a	**56.0** a	**49.5** a	**52.4** a	**49.6** a	**56.7** a	47.9 a
TL811	47.3† a	50.3† a	46.1† a	48.5† a	32.3† b	1.1† c	0.0† c	48.7† a	50.5† a	51.7† a	46.8† a	35.5† b	20.1† c	12.4† d
DSM 4282	**64.8** a	**71.6** a	**57.3** a	**61.3** a	6.3 b	0.0 b	0.0 b	**51.5** a	47.0 a	39.2 b	24.3 c	7.9 d	8.0 e	15.9 f
INRA-5	**53.6** ab	**53.5** ab	**62.0** ab	**57.9** ab	46.6 ab	36.4 b	0.0 c	**30.7** a	30.1 ab	20.4 b	8.0 c	0.0 d	6.5 c	3.3 cd
I16	59.3† a	53.8† ab	61.0† a	57.5† a	46.6† b	7.8† c	0.0† d	49.0† a	40.7† b	31.2† c	19.8† d	2.2† e	0.0† ef	0.0† ef
I3	**44.8** ab	**48.6** ac	**51.1** ac	**30.6** d	**51.4** c	43.3 ab	9.9 e	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
INRA-32	**70.6** ac	**64.7** abd	**74.4** ac	**71.7** abc	59.5bd	11.2 e	9.1 e	**66.9** a	**73.8** a	**72.8** a	52.3 b	29.3 c	10.0 d	7.0 d
Bmt	**25.9** a	**27.7** a	**30.6** a	**29.4** a	**30.0** a	28.1 a	27.9 a	42.1 a	32.4 ab	35.8 ab	35.8 ab	29.4 bc	21.2 c	8.1 d
Bwt	**46.1** a	**47.3** a	**50.0** ab	**51.3** ab	**49.0** a	40.6 ab	28.6 c	15.4 ab	21.0 ac	26.1 c	16.1 abc	10.2 ab	7.0 b	13.9 ab
F4430	64.0† a	64.0† a	44.6†ab	49.2† ab	23.0† bc	nt	1.7† c	**55.7** a	**53.6** a	**58.2** a	**52.9** a	**39.5** b	27.8 c	37.5 bc
I11	9.6 a	11.9 a	9.7 a	0.0 a	1.3 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
TZ415	39.0 ab	39.6 ab	42.8 abc	44.8 ac	46.3 c	37.9 b	22.2 d	16.5 abc	14.6 abcd	8.0 abc	7.3 abcd	0.0 bcd	9.0 abcd	0.0 bcd

* values are expressed as percentage decrease of the initial OD_{600} of the spore suspension at the end of experiments. For each strain, values not followed by the same letter(s) are significantly different at $P < 0.05$ (Tukey's HSD). For each strain and NaCl concentration, values are the means of three replicates done simultaneously. The incubation time ranged from 160 to 200 min; nt: concentration not tested; % decrease in initial OD_{600} corresponding to a 100 % germination as observed under the microscope are indicated in bold type; †: not examined under microscope.