Germinant receptor diversity and germination responses of four strains of the *Bacillus cereus* group

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Running title: Diversity in germination of four *B. cereus* strains

Abstract

Four strains of the *Bacillus cereus* group were compared for their germinant receptor composition and spore germination capacity. Phylogenetic analysis of the germinant receptor encoding operons of the enterotoxic strains *B. cereus* ATCC 14579 and ATCC 10987, the emetic strain AH187, and the psychrotolerant strain *Bacillus weihenstephanensis* KBAB4, indicated a core group of five germinant receptor operons to be present in the four strains, with each strain containing one to three additional receptors. Using quantitative PCR, induction of expression during sporulation was confirmed for all identified germinant receptor operons in these strains. Despite the large overlap in receptors, diversity in amino-acid-induced germination capacity was observed, with six out of 20 amino-acids, serving as germinants for spores of all four strains. Each strain showed unique features: efficient germination of strain KBAB4 spores required non-inducing amounts of inosine as the co-germinant, strain ATCC 10987 spores germinated only efficiently after heat-activation. Furthermore, strain ATCC 14579 and AH187 spores germinated without heat activation or inosine, with strain ATCC 14579 spores being triggered by all amino acids except phenylalanine and strain AH187 spores being specifically triggered efficiently only by phenylalanine. Analysis of all germination data did not reveal strict linkages between specific germinants and germinant receptors. Finally, the diversity in nutrient-induced germination capacity was also reflected in the diverse germination responses of heat-activated spores of the four *B. cereus* strains in food matrices, such as milk, rice water and meat bouillon, indicating that amino acid composition and/or availability of inosine are important germination determinants in foods.

Key words: *ger* operon, food preservation, *B. weihenstephanensis*, sporulation

Introduction

Bacilli constitute a diverse group of Gram-positive bacteria that produce spores as survival vehicles, and it is through these highly resistant spores that foodborne pathogenic species such as *Bacillus cereus* can survive a wide range of stress conditions such as encountered in certain foods and during gastric passage (Gould, 2006). Notably, in recent years, the development and implementation of consumer driven mild preservation techniques have become more popular (Gould, 2001), and this may further enhance the spore survival, and thus the persistence of sporeformers in food production environments. Germination of spores can be triggered upon exposure to nutrients such as present in foods, where after they can grow out and multiply, and produce toxins (Gould, 2001).

Food poisoning by *B. cereus* can either be caused by an infection or an intoxication, which lead to a diarrhoeal or an emetic type of illness, respectively (Stenfors Arnesen et al., 2008). The diarrhoeal type of illness is caused by the production of enterotoxins by *B. cereus*, including the hemolytic enterotoxin (Hbl), the non-hemolytic enterotoxin (Nhe) and cytotoxin K (CytK), in the human small intestine after consumption of contaminated food (Granum and Lund, 1997; McKillip, 2000; Schoeni and Wong, 2005). Foods that are often related to food poisoning by enterotoxin producing *B. cereus* strains are meat products, soups, vegetables, sauces and dairy products (Schoeni and Wong, 2005). The emetic type of illness is caused by production of the emetic toxin Cereulide by *B. cereus* in foods before consumption and causes nausea and vomiting (Ehling-Schulz et al., 2004). Foods associated with *B. cereus* strains causing the emetic type of food poisoning are mainly rice and pasta (Schoeni and Wong, 2005). Generally, symptoms caused by *B. cereus* food poisoning

are regarded as mild, and therefore *B. cereus* food poisoning is probably under reported (Griffiths and Schraft, 2002).

In addition, food spoilage by *B. cereus* is often caused by psychrotolerant strains of the *B. cereus* group, as these strains can grow at refrigerator temperatures. Amongst the psychrotolerant strains of the *B. cereus* group are the *B. weihenstephanensis* strains, which are distinguished from *B. cereus* strains because of their growth at low temperatures, and no growth at a temperature of 43°C (Lechner et al., 1998; Stenfors and Granum, 2001). These psychrotolerant strains of the *B. cereus* group have only been reported to be involved in food poisoning events sporadically (van Netten et al., 1990).

Germination of *Bacillus* spores has been shown to be induced by nutrients, the so-called germinants. This nutrient-induced germination is mediated by the interaction of the nutrients and the so-called germinant receptors. However, the activation mechanism of germination by interaction of nutrients and its receptors is still poorly understood (Moir et al., 2002). Germinant receptors are generally encoded by tricistronic operons (Moir et al., 2002), although tetracistronic operons have recently been reported (Christie et al., 2008). Transcription of the germinant receptor encoding operons in *B. subtilis* has been shown to occur during sporulation and under the control of σ^G (Paidhungat and Setlow, 2002; Wang et al., 2006). Recently, a selection of germinant receptors and their inducing nutrients have been studied in members of the *B. cereus* group, as described below. The GerR receptor encoded by the *gerR* operon was found to play an important role in the germination of *B. cereus* strain ATCC 14579 (Hornstra et al., 2005; Hornstra et al., 2006), whereas no such role was found in *B. cereus* strain 569 and in *B. anthracis* (Barlass et al., 2002). However, as a commonality for these three strains, the germinant receptor encoded by the *gerI*

operon (or the orthologous *gerH* operon) was shown to be involved in inosine related germination. Interestingly, for both *B. anthracis* and *B. cereus* ATCC 14579 the germinant receptor encoded by *gerI* was shown to be involved in germination in response human cells, i.e. macrophages and differentiated Caco-2 cells, respectively (Hornstra et al., 2009; Weiner and Hanna, 2003). In addition, spores derived from *B. cereus* group members were shown to germinate efficiently when exposed to high concentrations of a combination of L-alanine and inosine (Broussolle et al., 2008). For *B. cereus* strain ATCC 10876 a germination response could also be triggered by a combination of L-histidine and inosine (Senior and Moir, 2008), whereas no such response could be identified for strain ATCC 14579 (Hornstra et al., 2006). This shows diversity in germinants and germinant receptors for the different members of the *B. cereus* group, but a direct comparative analysis including a wide range of germinants is still lacking, whereas such knowledge may contribute significantly to efficient control of germination and outgrowth of spore-forming bacteria in food.

Therefore, this study assesses germinant receptors and germination characteristics of four representatives of the *B. cereus* group, the enterotoxic strains *B. cereus* ATCC 14579 and ATCC 10987, the emetic toxin producing strain AH187, and the psychrotolerant strain *B. weihenstephanensis* KBAB4, in defined conditions and in selected model foods. The role of germinants as wake-up calls in a range of environments is discussed, including their potential linkage to specific receptors.

Materials and Methods

Strains and culture and sporulation conditions

The four sequenced strains used in this study were the enterotoxin producing *Bacillus cereus* type strain ATCC 14579 (Frankland and Frankland, 1887; Ivanova et al., 2003), the enterotoxin producing *B. cereus* ATCC 10987 (Rasko et al., 2004), the emetic toxin producing *B. cereus* AH187 (Ehling-Schulz et al., 2005), NCBI genome project 17715) and the psychrotolerant *B. weihenstephanensis* KBAB4 (Lapidus et al., 2008). Overnight cultures for inoculation of sporulation medium were obtained in tubes with 5ml Luria Broth (Difco) at 30°C and 200rpm rotary shaking. The sporulation medium was based on the Difco sporulation medium (Schaeffer et al., 1965), fortified with sporulation elements of the defined medium for *B. cereus* (de Vries et al., 2004) and maltose to increase the yield of cells (data not shown). The medium contained Nutrient Broth (8g/l, Difco), maltose (10mM), $(NH_4)_2SO_4$ (5 mM), $MgCl_2$ (1 mM), $Ca(NO_3)_2$ (1 mM), $FeSO_4$ (1 mM), $MnSO_4$ (66 µM), $ZnCl_2$ (12.5 µM), $CuCl_2$ (2.5 µM), Na_2MoO_4 (2.5 µM) and $CoCl_2$ (2.5 µM), and will be referred to as Maltose Sporulation Medium (MSM). Sporulation was performed in 50ml MSM in 250ml Erlenmeyer flasks, at 30°C and 200 rpm rotary shaking. Sporulation efficiency was determined by microscopic observation and droplet plating before and after heating of sporulating cultures. In order to obtain spore batches containing only spores (>95%), spores were washed in 10mM potassium phosphate buffer (pH 7.4), at least 10 times during the first 3 weeks, before starting any further experiments.

Determination of spore properties

144 To determine the spore sizes, forward scatter parameters of the different spore
145 batches were analysed by flow cytometry using a FACSCalibur flow-cytometer
146 (Becton Dickinson, San Jose, Calif.). The Flow Cytometry Size Calibration Kit
147 (Molecular Probes BV) that contains collections of beads with different diameters was
148 used to correlate the forward scatter parameters with size values. By measuring the
149 forward scatter, the length of the spores was analysed and this resulted in a
150 distribution of the size of the oval-shaped spores that was a normal distribution and
151 therefore the average size values were determined by fitting a normal distribution of
152 the forward scatter with Excel Solver (Frontline Systems, Inc.). Hydrophobicity of the
153 spores was determined as described previously (Rosenberg et al., 1980). In short,
154 spores were suspended in water and measured for their OD_{600} (OD_{before}, values of 0.4
155 to 0.5). Next, 50µl of n-hexadecane (Sigma Aldrich) was added to 1ml of spore
156 suspension in a plastic cuvette. This mixture was vortexed for 1 min, after which the
157 phases were allowed to separate for 15 min. Subsequently, the OD_{600} of the aqueous
158 phase was determined (OD_{after}), and the transfer percentage to the n-hexadecane
159 phase was determined by calculation ($100 - [(OD_{after}/OD_{before}) \times 100]$).
160 Heat resistance of the spores was determined at 95°C, by heating a 20µl spore
161 suspension of an OD_{600} of 0.1 in a 200µl thin-walled tube (Biorad). Appropriate
162 dilutions were pour-plated after 1, 5, 10, 15 and 20 minutes of heating, and colony
163 forming units, resulting from surviving spores, were determined after 48 hours using
164 BHI agar plates incubated at 30 °C. The weibull-model, with the beta-parameter
165 between 1.0 and 2.5, was used to determine the reported d1(delta)-value
166 corresponding to the first decimal reduction of viable spores, as used previously
167 (Couvert et al., 2005; den Besten et al., 2006). Observed differences between the four

strains for all properties were considered significant when the $p<0.05$, as determined by use of a student's t-test (MS Excel).

Germination assays

For germination of non-heat activated spores, the spores were washed and resuspended in germination buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl). For germination of heat-activated spores, the spores were heat-activated by heating at 70°C for 15 min in the washing buffer (Hornstra et al., 2005), and subsequently washed and resuspended in germination buffer. Spore germination experiments were performed at a spore density of OD_{600} 0.4-0.6 and at a temperature of 30°C. Spore germination was measured by monitoring the reduction of the OD_{600} during the transformation of phase bright spores into phase dark spores. Germination assays were performed in 96-wells plates and OD_{600} was measured by use of the Spectramax plus^{384} (Molecular Devices). Experiments with duration of 90 min were measured every min during incubation in the Spectramax plus^{384} at 30°C with 30 sec shaking between measurements, whereas 24 h experiments were measured after shaking in the Spectramax plus^{384} at 0, 1, and 24 h, with incubation in a 30°C incubator. Nutrients were applied by adding 50 µl of 5 times concentrated solutions to 200µl of spore suspensions. Final concentration used for amino acid germination were 20mM L-alanine (Ala), 10mM L-phenyl-alanine (Phe), 20mM L-glycine (Gly), 20mM L-valine (Val), 20mM L-leucine (Leu), 20mM L-iso-leucine (Ile), 20mM L-cysteine (Cys), 20mM L-methionine (Met), 20mM L-serine (Ser), 20mM L-threonine (Thr), 20mM L-glutamine (Gln), 20mM L-arginine (Arg), 20mM L-asparagine (Asn), 20mM L-aspartic acid (Asp), 20mM L-glutamic acid (Glu), 20mM L-histidine (His), 20mM L-lysine (Lys), 20mM L-proline (Pro), 5mM L-tryptophan (Trp), and 10mM L-tyrosine

(Tyr). The concentration of inosine used to aid amino acid germination was 0.1mM (Hornstra et al., 2006). Heat-activated spores were used for germination assays in the previously described model foods rice water (Lassi), meat bouillon (Maggi) (Hornstra et al., 2005) and pasteurized semi-skimmed cow milk (Frieslandfoods). Germination efficiencies in meat bouillon were assessed by OD_{600} measurements, as described above, and verified by microscopic analysis. Germination efficiencies in rice water were based on microscopic analysis. For germination in pasteurized semi-skimmed cow milk, spores were heat-activated, washed and resuspended in germination buffer. Subsequently, 200µl of milk was added to 50µl of spore suspension. After 90 min incubation in 96 wells plates at 30°C samples were diluted in physiological salt solution and plated before and after heat killing (70°C for 15 min) of germinated spores and vegetative cells, in order to determine the percentage of non-germinated spores. All germination experiments were performed in biological duplicates.

RNA isolations and quantitative PCR

During growth and sporulation in MSM 2ml samples were taken at different time points of the four strains and snap frozen in liquid N_2 for RNA isolation. After thawing on ice, cell cultures were centrifuged at 13.000 rpm and resuspended in TriReagent (Ambion). Subsequently, after bead-beating by use of a mini-bead-beater 8 (Biospec) and Lysing Matrix B (qBiogene), RNA was isolated according to the TriReagent protocol. After RNA isolation a DNAse treatment was performed by use of Turbo DNAse free (Ambion) according to the manufacturer's protocol. Making of cDNA for quantitative PCR (qPCR) was performed as described previously (van Schaik et al., 2007), by use of 500ng of total RNA and a mix of reverse primers relevant for the specific strain. Subsequently, qPCRs were performed as described

before, primers used are indicated in Table S1. Design of primers was done by use of Primer3 (Rozen and Skaletsky, 2000), and as much as possible, primer pairs were designed for multiple strains per orthologous gene, allowing one mismatch per primer (Table S1). Primer efficiency was checked by PCR on genomic DNA.

In silico genome analysis

The amino acid sequence of gerRA and gerLA of strain ATCC 14579 were used to identify the A components of the different germinant receptors, gerRB and gerLB of strain ATCC 14579 were used for B-components, and gerRC and gerLC of strain ATCC 14579 for the C-components, for all three components BLAST was used for identification (Altschul et al., 1990). For each set of components (gerA-components and gerB-components, data not shown, and gerC-components, Figure 2), an alignment was performed by use of Muscle 3.6 (Edgar, 2004). A boot-strapped phylogenetic tree of the alignments was constructed using ClustalW (Thompson et al., 2002), the subsequent tree was rooted using LOFT (van der Heijden et al., 2007), and visualized by use of Treeview (Page, 2002).

Results

Growth and sporulation

Growth and sporulation of the four *B. cereus* strains were studied in MSM. The four strains displayed similar growth performances, although ATCC 10987 reached a slightly lower end OD_{600} (Figure 1). Upon entering the stationary phase of growth, all four strains sporulated efficiently (over 85%) in MSM, however at different time frames. For strain AH187 heat resistant spores were identified already within two hours into the stationary phase, whereas for ATCC 10987 and KBAB4 the first heat resistant spores were identified after four and five hours into the stationary phase, respectively. For strain ATCC 14579 heat resistant spores could only be detected after eight hours into the stationary phase. A similar difference was observed in the time needed to complete sporulation, as for ATCC 10987 and AH187 this took two hours, whereas for ATCC 14579 and KBAB4 sporulation was completed in seven and eight hours, respectively (Figure 1).

Subsequent analysis of spore properties showed clear differences in size and heat resistance (Table 1). Spores derived from strain ATCC 14579 were the smallest (0.77µM), and spores of strain AH187 were the largest (1.84µM). Spore heat resistance at 95ºC showed strains ATCC 10987 and AH187 to be significantly more resistant than the KBAB4 and ATCC 14579 strains, with KBAB4 spores being the least resistant. Spores derived from the four strains displayed similar hydrophobic properties.

Germinant receptors

Phylogenetic analyses of amino acid compositions of the separate GerA, GerB, and GerC components of the three gene germinant receptor encoding operons

present in the four *B. cereus* strains showed that there are ten distinct groups of germinant receptors present within the *B. cereus* group members. In the phylogenetic tree of the GerA and GerB components the different GerX receptors do not cluster in one group (data not shown), however, phylogenetic analysis of the amino-acid sequences of the GerC components suggests the GerX receptors of the different strains to be evolutionarily related and part of one group, resulting in the ten distinct groups of germinant receptors (Figure 2). A core set of five of these ten germinant receptors, GerR, GerL, GerI (with GerH as ortholog), GerS and GerK, are present in the four studied strains. The presence of these germinant receptors in these four strains indicates this group to be important. In addition to the core set of five germinant receptors, *B. weihenstephanensis* KBAB4 contains an operon situated on a plasmid that encodes a germinant receptor related to GerS, and therefore, this germinant receptor is named GerS2. Also in *B. cereus* ATCC 10987 one additional germinant receptor encoding operon is present, GerX, that is related to the GerX receptor described previously for *B. anthracis* (Guidi-Rontani et al., 1999). For *B. cereus* ATCC 14579 two additional operons encoding germinant receptors are present on the genome, encoding the receptors GerG (Hornstra et al., 2006) and GerQ (Barlass et al., 2002; Hornstra et al., 2006). Moreover, *B. cereus* AH187 contains three additional operons encoding germinant receptors, of which GerG (Hornstra et al., 2006) and GerX (Guidi-Rontani et al., 1999) have been described previously, with the *gerX* operon being present on a plasmid (NCBI genome project 17715). Furthermore, the third additional germinant receptor belongs to a phylogenetic group that has not been previously described; we suggest naming the encoded germinant receptor GerT (Figure 2). Finally, next to the core set of five germinant receptors, the five remaining germinant receptors are present divided over the four strains.

In order to find out whether the annotated germinant receptors were actively transcribed, expression of the gene encoding the GerA-components was analysed in the four strains (Figure 3). In general, for all four strains expression of all operons encoding germinant receptors was observed, and in each strain, the different *gerA* genes representing the different germinant receptors, showed similar expression levels. Furthermore, induction of expression of the germinant receptor encoding operons was observed to be at the highest two hours before the first heat resistant spores were observed (Figure 3). The trend of the expression was similar to the trend of spore formation, as the highest expression levels for *B. cereus* ATCC 10987 and AH187 was observed already two hours after the first induction (Figure 3), whereas for *B. cereus* ATCC 14579 and *B. weihenstephanensis* KBAB4 the highest expression levels were reached five hours after the first induction (Figure 3). The highest relative expression of germinant receptor encoding operons was observed for ATCC 14579. For the three other studied strains the relative expression level was shown to be similar (Figure 3). As the expression of germinant receptor encoding operons in *B. subtilis* is regulated by σ^G (Wang et al., 2006), the expression of *sigG*, encoding σ^G, was also monitored for the four *B. cereus* group strains studied here. In the 150bp up-stream regions, for all operons encoding Ger receptors, a putative σ^G binding site similar to the *B. subtilis* binding site could be identified (data not shown). Expression of *sigG* for all four strains was shown to be induced approximately one hour before induction of the germinant receptor encoding operons, also reaching the highest point of induction two hours before the first heat resistant spores were observed (Figure 3). Remarkably, for *B. cereus* AH187 this signifies *sigG* expression to be already induced during the growth phase (Figure 3d).

Germination responses

Germination in response to an array of nutrients showed that heat-treated spores of all four strains germinated in response to high concentrations of inosine plus L-alanine with strain AH187 showing a somewhat lower response, i.e. microscopy revealed approximately 50% of the spores had turned phase dark, whereas the other strains reached 100% phase-bright phase-dark transitions (Figure 4). No germination was observed in response to a combination of glucose, fructose, asparagine and potassium phosphate (data not shown), although it is the strongest germinant combination for *B. subtilis* spores (Wax and Freese, 1968). Testing all 20 standard L-amino acids showed a great diversity in germination activation with eleven amino acids, whereas nine amino acids (Arg, Asn, Asp, Glu, His, Lys, Pro, Trp and Tyr) did not trigger germination at all. Diversity in germination response is illustrated by the data obtained with L-alanine (Figure 5A) and L-phenylalanine (Figure 5B). With L-alanine, spores of strain KBAB4 could only be induced to germinate efficiently when also inosine was present, whereas heat activation has no added effect. In contrast, strain ATCC 10987 spores germinated only after heat activation of the spores, with no added effect of inosine. Germination of strain ATCC 14579 and AH187 was shown to be assisted by both heat activation and addition of inosine. Data obtained with phenylalanine revealed similar, but reduced responses for all strains, except the emetic strain AH187 strain that showed an efficient germination response to this amino acid that was not further affected by heat activation and/or inosine (Figure 5B).

An overview of the germination responses for the other nine germination triggering amino acids is shown in Table 2. This revealed similar trends as described above, i.e., inosine is essential for strain KBAB4 spore germination, strain ATCC 10987 spores require heat activation, strain ATCC 14579 spore germination is

335 stimulated by inosine and heat activation, and strain AH187 spores show a very
336 limited response, with L-cysteine and L-serine as notable exceptions, acting as
337 efficient germinants for this emetic *B. cereus* strain. Prolonged incubation up to 24 h
338 reveals similar results as observed after 1 h incubation. Summarizing the 24 h data
339 reveals heat activated spores of strain KBAB4, ATCC 10987 and ATCC 14579 to
340 have germinated with eleven amino acids in the presence of inosine, whereas AH187
341 spores failed to germinate with L-glycine, L-valine, L-isoleucine, L-methionine and
342 L-glutamine (data not shown).
343 Germination responses of the different spores were finally assessed in selected
344 food matrices. Incubation in pasteurized milk led to efficient spore germination of
345 strain ATCC 10987 and ATCC 14579, to only marginal spore germination of strain
346 KBAB4 and to no spore germination for strain AH187 (Figure 6). In contrast, meat
347 bouillon efficiently induced germination of all strains, albeit with a slightly reduced
348 response of strain KBAB4 spores, i.e., 40% germination for KBAB4 versus 100% for
349 the other three strains (data not shown). Finally, spores of all four strains showed poor
350 germination in rice water, with less than 10% germination for ATCC 10987 and
351 ATCC 14579, and less than 1% germination for KBAB4 and AH187 spores (data not
352 shown).
353

Discussion

Germination of spores of the food spoiling and food poisoning *Bacillus cereus* strains in food products cause many problems to the food industry. To control germination of *B. cereus* it is important to identify spore features involved in germination and triggers of germination. Therefore, four *B. cereus* strains were studied for their spore and germination properties.

First of all, by use of *in silico* analysis a core set of five germinant receptors was identified to be present on the genome of the four strains of the *B.* cereus group studied. Furthermore, all germinant receptors present in these strains were shown to be expressed by qPCR. Despite the presence and expression of the core set of germinant receptors, specific germination properties were identified for each strain. Strain KBAB4 only germinated in response to amino acids in co-presence of inosine, strain ATCC 10987 only germinated efficiently after heat activation, strain ATCC 14579 was able to germinate without any additional activation and the emetic strain AH187 germinated in response to only six amino acids, whereas the strains KBAB4, ATCC 10987 and ATCC 14579 germinated in response to 11 amino acids (Table 2). Correlation analysis did not reveal direct linkages between specific amino acid germinants and the expressed germinant receptors in the selected strains. It is conceivable that nutrient permeability of the outer layers of the spore (exosporium, coat and cortex) also affects the triggering efficiency of germinants. The strongly enhanced germination of strain ATCC 10987 after heat treatment is possibly linked to the fact that a heat treatment of spores enhances the permeability of these outer spore layers. In addition, it has been reported that enzymes present and active in the cortex of the spore hamper germination by neutralizing the germination inducing nutrient, such as the purine-specific nucleoside hydrolase IunH of *B. thuringiensis*, which is

involved in decreasing the inosine and adenosine induced germination rate (Liang et al., 2008), and the spore specific alanine racemase of *B. anthracis* (Chesnokova et al., 2009). Both nucleoside hydrolase and alanine racemase encoding genes are present in all four *B. cereus* strains tested, but their contribution, if any, to the germination efficiency of different strains remains to be established.

Interestingly, strain AH187 was the only strain that germinated efficiently in response to L-phenyl-alanine as a single nutrient in all conditions tested. L-phenyl-alanine is a highly hydrophobic amino-acid at a neutral pH. The capacity of this specific amino acid to specifically induce efficient germination of strain AH187 spores could be influenced by diversity between the different strains in spore surface charge and hydrophobicity. However, only minor differences were identified in hydrophobicity of the spores of the four strains tested (Table 1). Still diversity in surface charge could influence germination (Brahmbhatt et al., 2007). Furthermore, the efficient induction of spore germination of strain AH187 by L-phenyl-alanine is intriguing and future studies may reveal whether the strain-specific GerT receptor (Figure 2) is involved in the L-phenyl-alanine-induced germination response.

In foods spores can encounter many different nutrients and many different conditions, such as varying temperatures and pH values. The four strains studied all germinated in response to L-alanine, L-phenyl-alanine, L-Leucine, L-Cysteine, L-Serine and L-Threonine, with varying efficiencies. With the selected strains in our study, L-histidine did not induce germination at a pH of 7.4. Notably, a study by Senior and Moir (2008) showed germination of *B. cereus* strain ATCC 10876 spores in response to L-histidine in a basic environment with pH 8.9. Furthermore, an acidic environment has been shown to reduce germination of *B. cereus* strains in response to the combination of L-alanine and inosine (Broussolle et al., 2008). It is conceivable

that part of the differences observed in the various studies on nutrient-induced germination of *Bacillus* spores have their origin in the various experimental conditions, such as neutral versus alkaline pH values used.

In order to link the observed differences in germination capacity to practice, induction of spore germination was also tested in response to food matrices associated with *B. cereus* related problems, including rice (Fricker et al., 2007; Wijnands et al., 2006), milk (Bartoszewicz et al., 2008) and meat (Anderson Borge et al., 2001; Midura et al., 1970). Interestingly, highly efficient germination of heat activated spores for all four strains tested, was observed in meat bouillon, that is known to be rich in amino acids and ribonucleosides. Furthermore, spores of the strains KBAB4, ATCC 10987 and ATCC 14579 were found to germinate in milk, whereas spores of the emetic toxin producing strain AH187 only displayed a poor germination response. It remains to be studied in general whether emetic toxin strains germinate poorly in response to milk. Moreover, for all four strains tested only a small fraction of the spores, ranging from 1-10%, was able to germinate in rice water. In spite of the low germination frequencies observed in our study with the selected emetic strain used, and the low presence of emetic toxin producing *B. cereus* strains in the environment (Bartoszewicz et al., 2008) and in rice (Ankolekar et al., 2009), the emetic type of illness is often caused by *B. cereus* in association with starchy foods, such as rice (Fricker et al., 2007; Wijnands et al., 2006). Studies including a larger collection of emetic strains may reveal whether the poor germination is rice is a general feature of (emetic) *B. cereus* strains. Controlled activation of spore germination in food and food processing equipment could be of assistance to prevent the presence of spore formers in food. In contrast to highly resistant spores, the germinated spores are vulnerable for cleaning procedures and more importantly for mild processing steps. In this study it is

shown that diversity in spore germination of four strains complicates the activation of efficient germination of multiple strains in the same conditions. However, a combination of high concentrations of L-alanine and inosine induced efficient germination of heat-activated spores of the four strains (Figure 4). Insight in the diversity in germination of spore formers may aid in the establishment of a combined germination-inactivation procedure allowing for the eradication of spores from food.

In conclusion, specific germination properties have been identified for each of the four strains studied. Furthermore, these specific properties could not be directly linked to the strain specific, expressed germinant receptor operons, pointing to a contribution of additional factors, such as spore outer layers and their enzymes, and the transduction of germination signals, to germination characteristics. Further characterisation of these features may provide important links for the control of spores in food and food processing.

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606 **Figure legends**

607 Figure 1:

608

609 Growth and sporulation of the four *B. cereus* strains in MSM. Growth is measured by
610 increase in OD_{600} for KBAB4 (lightest gray, triangles), ATCC10987 (light gray,
611 circles), ATCC14579 (dark gray, squares), and AH187 (black, diamonds). The bars
612 indicate sporulation of the cells, starting at the end of growth (start of the striped
613 bars), followed by detection of the first heat resistant spore (start of solid bar) and up
614 to the completion of the production of heat resistant spores (end of solid bar).

615

616 Figure 2:

617

618 Rooted neighbour-joining phylogenetic tree of the GerC-component of the three gene
619 *ger*-operons present in the four studied species. K4 is strain KBAB4, A10 is strain
620 ATCC 10987, A14 is strain ATCC 14579 and AH187 is strain AH187. * indicates a
621 gene that was not annotated in the ERGO database.

622

623 Figure 3:

624

625 Expression of *sigG* and the *gerA* components of the *ger*-operons. Relative expression
626 for the four strains of *sigG* (gray triangles) and the average of the *gerA*-components of
627 the ger-operons (black squares). For the average expression of the *gerA*-components
628 the standard deviation is presented. The end of growth (1) and the first heat resistant
629 spore detected (2) are indicated by striped vertical lines in the graphs.

630

Figure 4:

Germination response of heat-activated spores upon exposure to the combination of the nutrients L-alanine (5mM) and inosine (2.5mM) for KBAB4 (lightest gray, triangles), ATCC10987 (light gray, circles), ATCC14579 (dark gray, squares), and AH187 (black, diamonds).

Figure 5

Spore germination of the four strains, in L-Alanine (A) and L-phenyl-alanine (B) after one hour incubation at 30°C as measured by OD_{600}. Spore batches were non heat activated (black and white bars) or heat activated (grey scale bars), and germination was without (black and darkest grey bars) or with (white and lightest grey bars) inosine. Germination percentages were determined by regarding a drop in OD_{600} of 65% for strain KBAB4 (K4), 55% for strain ATCC 10987 (A10), 60% for strain ATCC 14579 (A14) and 40% for strain AH187 after 1 hour of incubation as 100% germination. Germination of 100% at these percentages was verified by microscopic observations. The error bars indicate the standard deviations of biological duplicate experiments.

Figure 6: Germination of heat activated spores in food.

Germination of heat activated spores in milk. For spore germination in milk the percentage of germinated spores was determined from the remaining heat resistant spores after 90 min incubation in milk at 30°C.

Figure 1

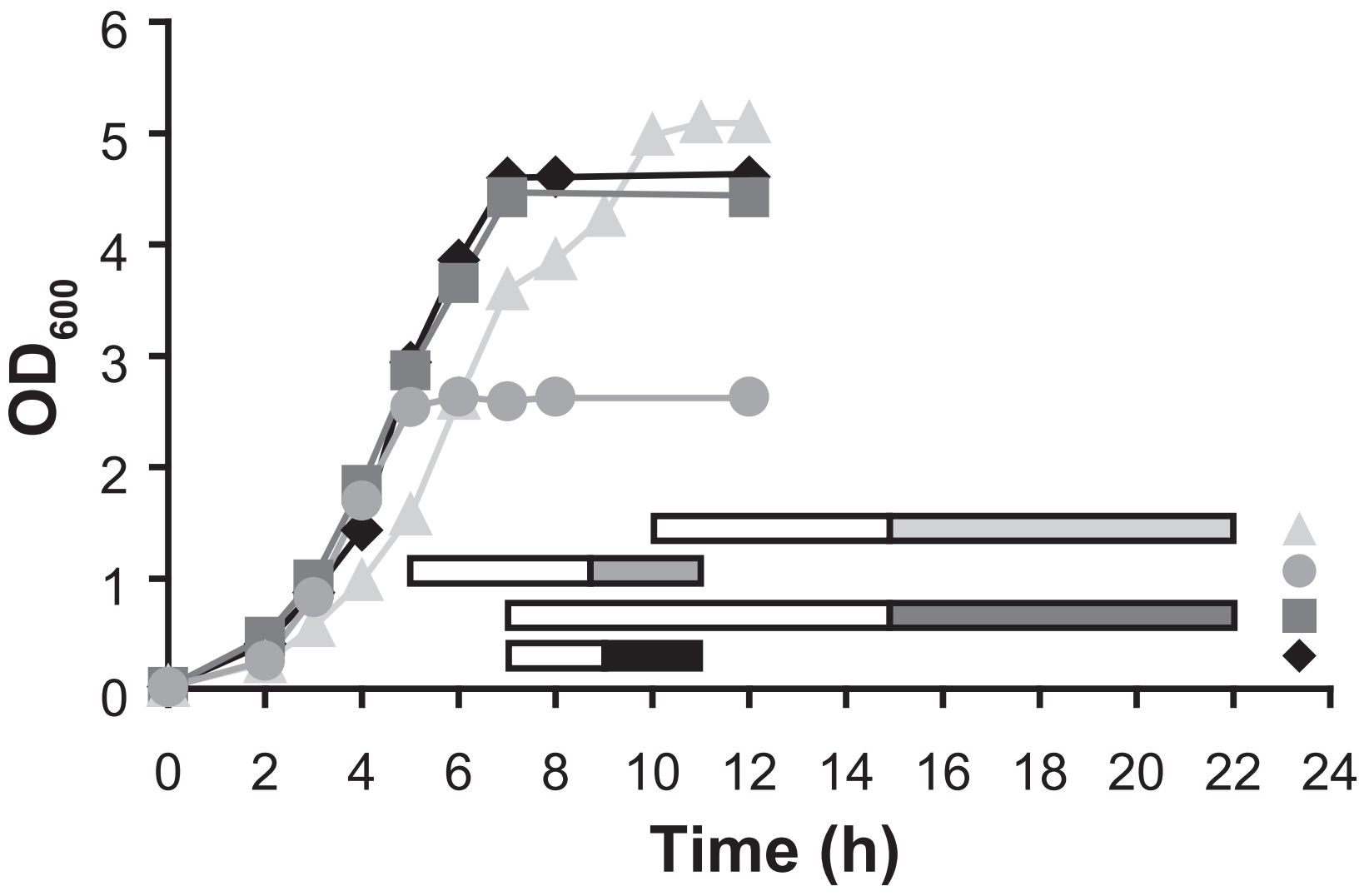

Figure 2

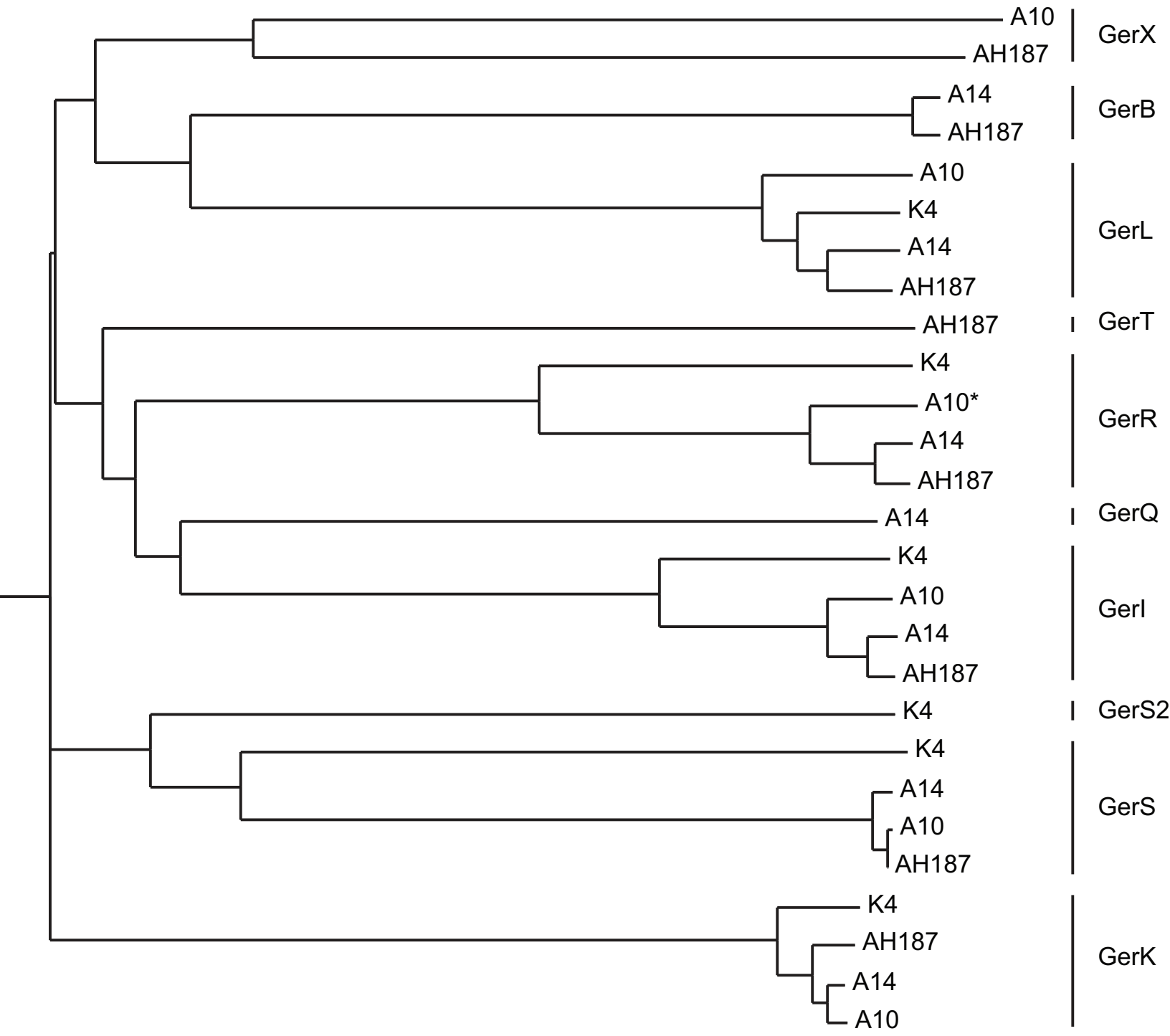
A10
AH187
GerX
A14
AH187
GerB
A10
K4
A14
AH187
GerL
AH187
GerT
K4
A10*
A14
AH187
GerR
A14
GerQ
K4
A10
A14
AH187
GerI
K4
GerS2
K4
A14
A10
AH187
GerS
K4
AH187
A14
A10
GerK

Figure 3

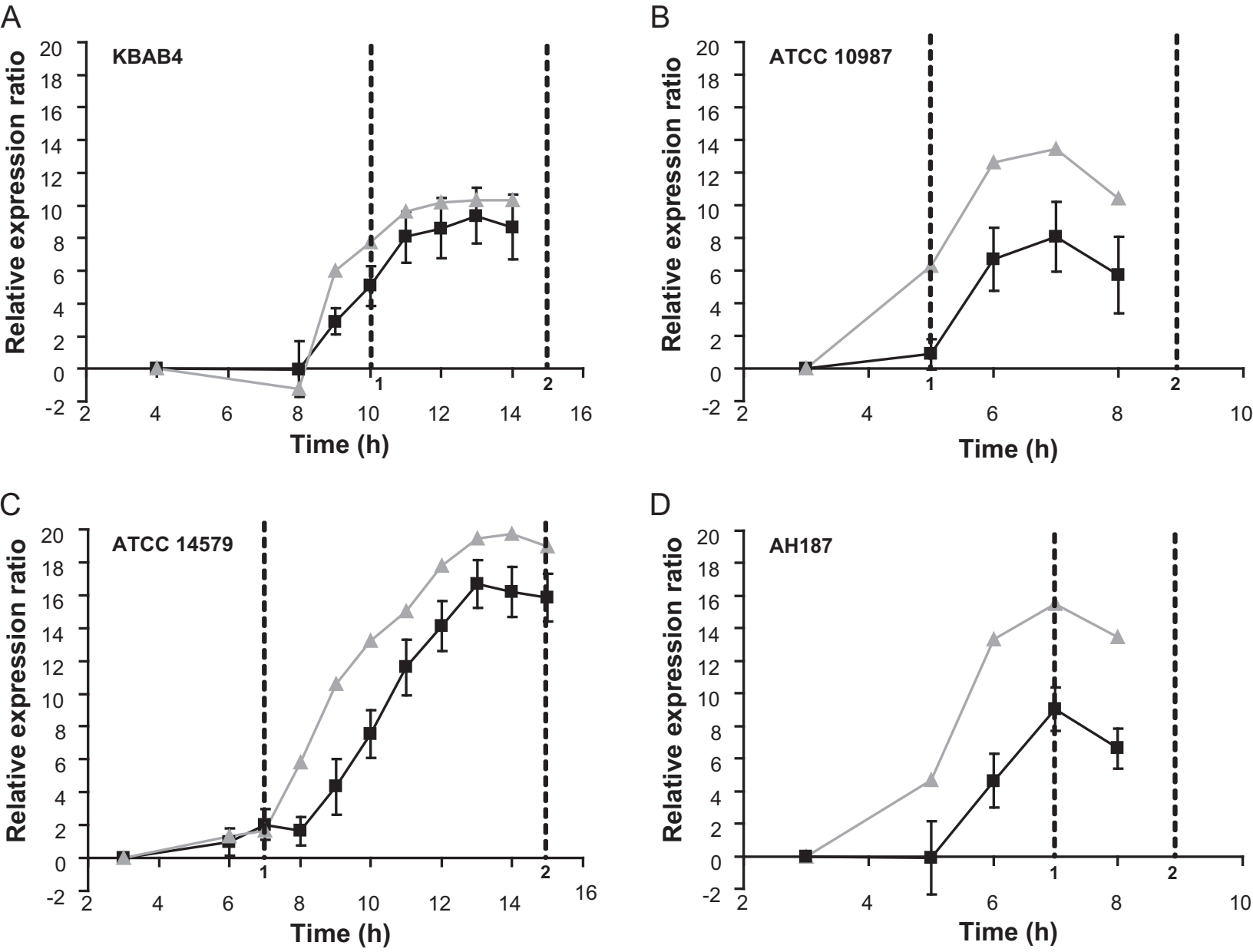
A
KBAB4
Relative expression ratio
Time (h)
B
ATCC 10987
Relative expression ratio
Time (h)
C
ATCC 14579
Relative expression ratio
Time (h)
D
AH187
Relative expression ratio
Time (h)

Figure 4

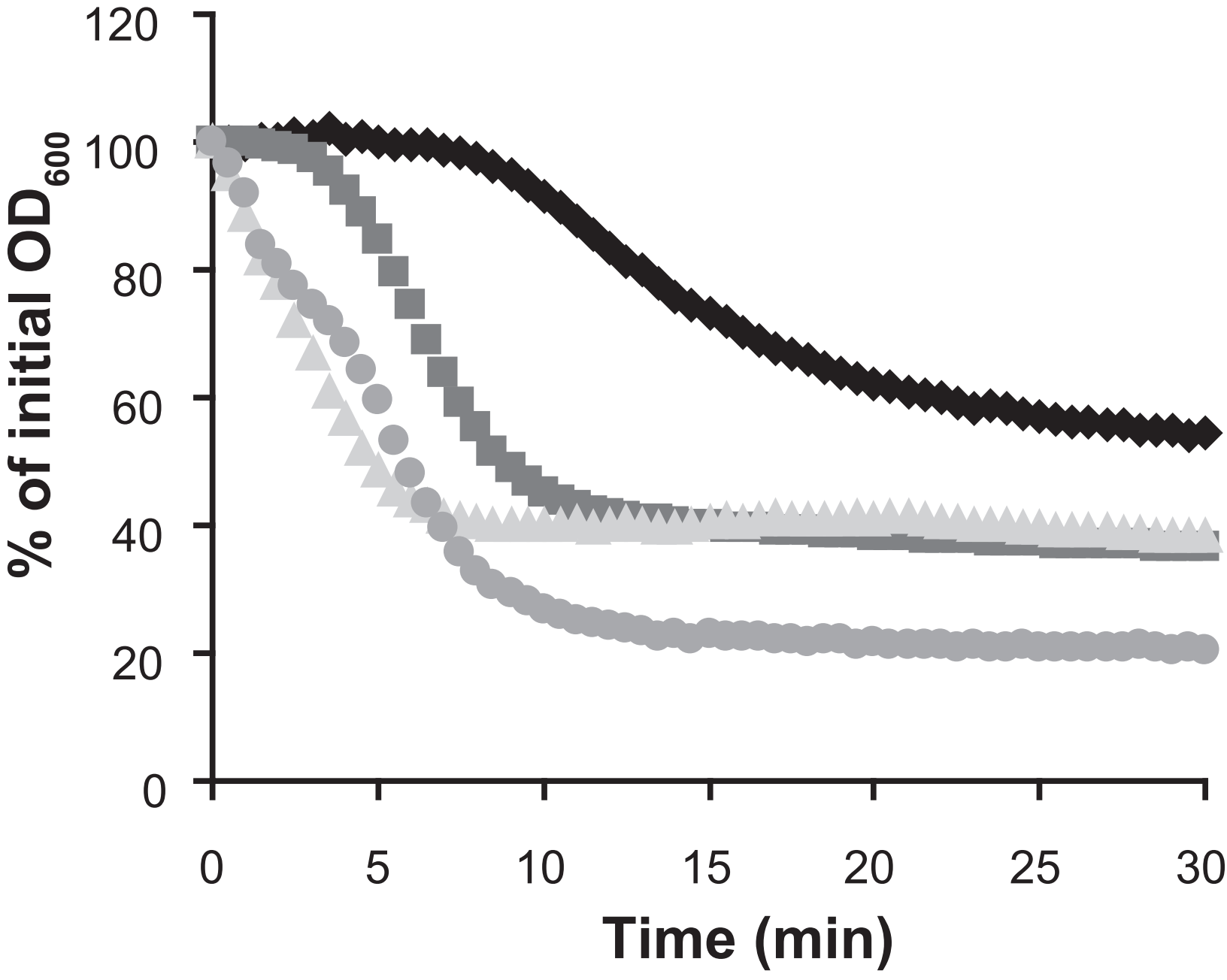

Figure 5

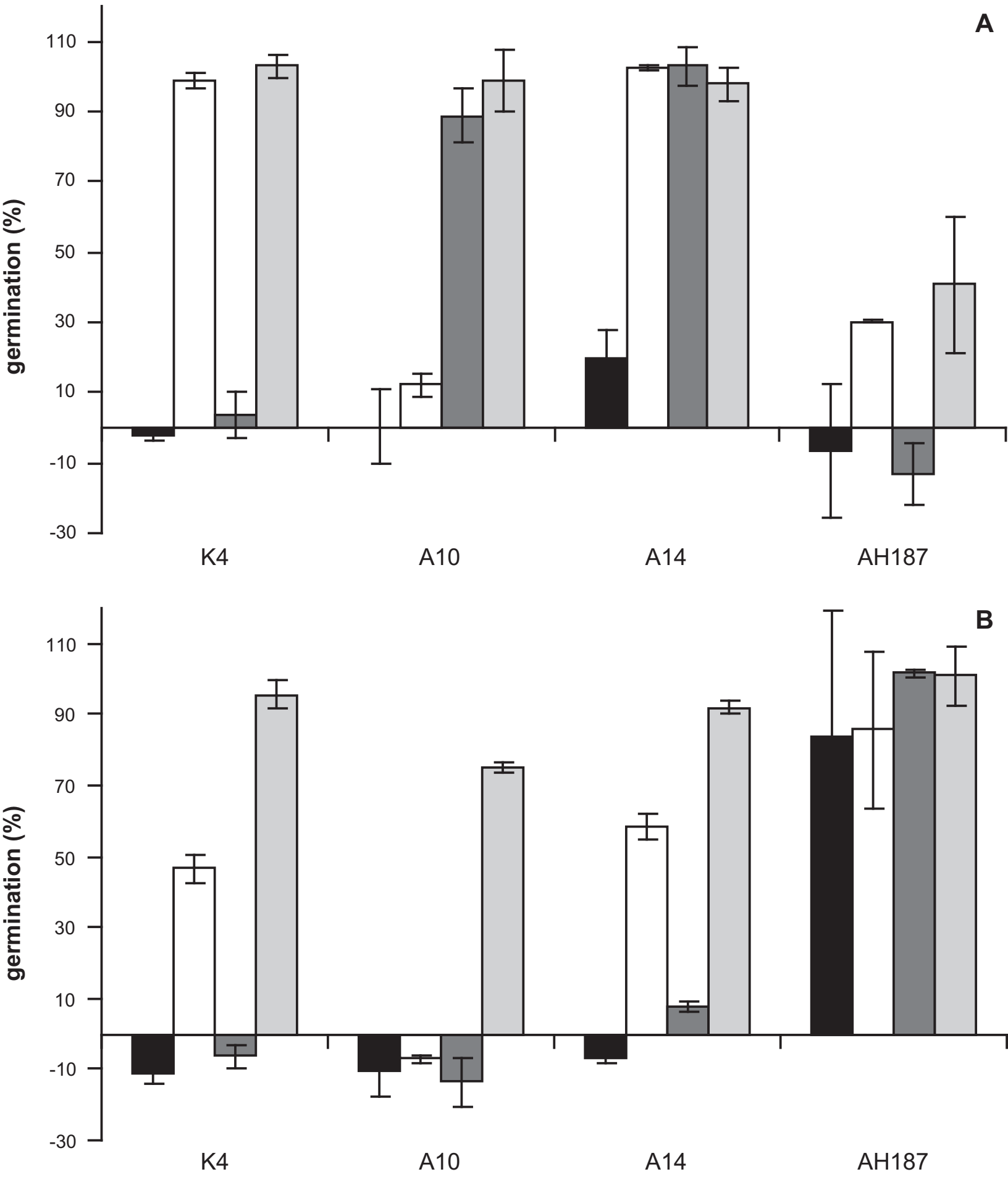
A
110
90
70
50
30
10
-10
-30
germination (%)
K4
A10
A14
AH187
B
110
90
70
50
30
10
-10
-30
germination (%)
K4
A10
A14
AH187

Figure 6

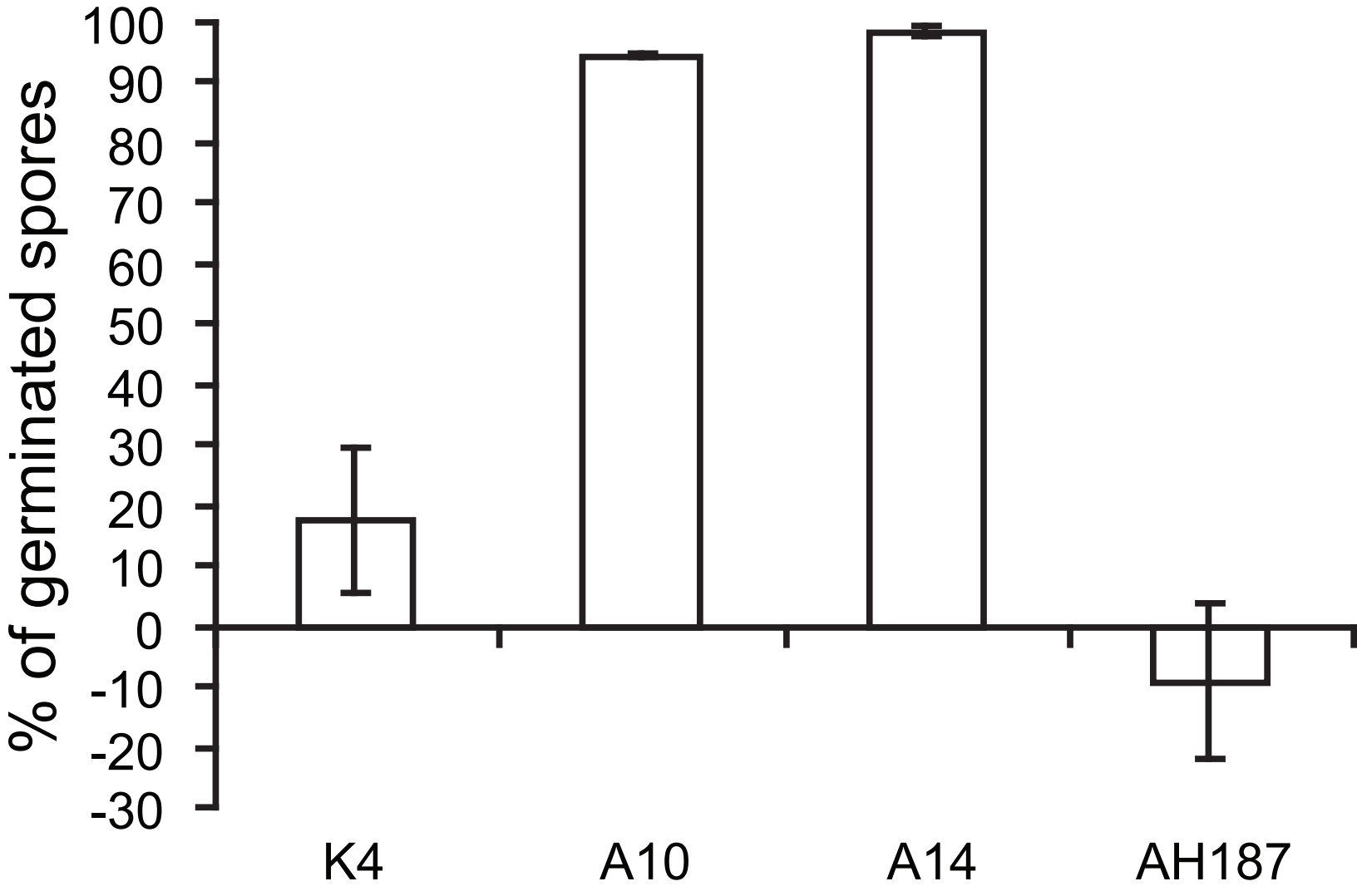
% of germinated spores
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
K4
A10
A14
AH187

Table 1

Table 1: General spore properties of the four strains

	Size (µm)	Hydrophobicity (%)	Heat resistance (d1 in min)	Sporulation efficiency (%)
KBAB4	1.12 (0.003)	92.36 (0.84)	5.66 (2.27)	90-100
ATCC 10987	1.11 (0.036)	92.84 (1.56)	34.94 (7.19)	80-90
ATCC 14579	0.77 (0.037)	87.74 (1.65)	12.40 (0.49)	90-100
AH187	1.84 (0.072)	90.70 (2.48)	23.36 (3.33)	90-100

Standard deviations are presented between brackets.

Table 2

Table 2: Spore germination as measured by OD_{600} after 1h.

	KBAB4				ATCC 10987				ATCC 14579				AH187			
	No Heat		Heat		No Heat		Heat		No Heat		Heat		No Heat		Heat	
	-ino	+ino	-ino	+ino	-ino	+ino	-ino	+ino	-ino	+ino	-ino	+ino	-ino	+ino	-ino	+ino
Gly	-	+/-	-	++++	-	-	-	++	-	++++	-	++++	-	-	-	-
Val	-	-	-	+	-	+/-	++++	++++	-	+	++++	++++	-	-	-	-
Leu	-	+	-	++++	-	-	++	++++	-	-	+	++++	-	-	-	+/-
Ile	-	-	-	+	-	+/-	++++	++++	-	++	++	++++	-	-	-	-
Cys	-	++	-	++++	-	+/-	++++	++++	+	++++	++++	++++	+/-	-	++	++
Met	-	-	-	++	-	+/-	++	++++	-	+++	+++	++++	-	-	-	-
Ser	-	++++	-	++++	-	-	+	+++	-	++++	+++	++++	+	++++	+	+++
Thr	-	-	-	+	-	+/-	+++	++++	-	-	++++	++++	+/-	+	-	-
Gln	-	+/-	-	++	-	-	-	+++	-	++++	+++	++++	-	-	-	-

- = no germination, +/- = up to 20% germination, + = 20 to 40% germination, ++ = 40 to 60% germination, +++ = 60 to 80% germination, ++++ 80 to 100% germination.

Table S1: Primers used in this study

Primer	Sequence
gerB_forw_A14_AH187	TGCTGCGATATTTGGCTTGT
gerB_rev_A14_AH187	ATGGAACAGCAGGGCTGGTA
gerI_forw_A10_A14_AH187	TATCGTCCTTCGCTTGATGG
gerI_rev_A10_A14_AH187	AATGCCGCTCATTGGAATATG
gerI_forw_K4	TCAATTGCACCAGCAAGATCA
gerI_rev_K4	TGCGGACCGATAACTGTTGA
gerK_forw_K4_A14_AH187	CCTCCTATTTTTGAAGCTCTCTT
gerK_rev_K4_A14_AH187	ACCGATTGTTTGTCCAACTC
gerK_forw_A10	TTGCACCAATGCGGATTAAA
gerK_rev_A10	CTCGTTACGTGTTGGCGTTG
gerL_forw_A10_AH187	GATCGGCGTCTTCAATCCAT
gerL_rev_A10_AH187	CGGAAACGGCGACAAATTAT
gerL_forw_K4	AGAGCCGCCAGTGACAGAAG
gerL_rev_K4	TCACGTAAACCACGGCGAAT
gerL_forw_A14	GGGAAGGTGTTCCGTTTCCT
gerL_rev_A14	TCGTTTGTCCCATCGGTTTC
gerQ_forw_A14	TGGCTCACCTTATGCGATCA
gerQ_rev_A14	GGCAATAATCCAAGGCATGGT
gerR_forw_A14_AH187	ACAGATGGCTCTCCATATGCTT
gerR_rev_A14_AH187	CGATCCAATCATCCACGGTAA
gerR_forw_A10	AGTTGGGCAAACGATTGGAA
gerR_rev_A10	TGCAAGGGCAGATAAAGCAA
gerR_forw_K4	CGAGGCTGGAGCTCGTTTAC
gerR_rev_K4	ACAGATGCCTGGCCAATGAC
gerS_forw_A10_A14_AH187	GCTCCATAAAAAGCTCACACGA
gerS_rev_A10_A14_AH187	TCGCGATTTGGAATATATCGAC
gerS_forw_K4	TGCACCATTTCGTCTCCAAG
gerS_rev_K4	TTTGTTCCGGCTGTCCTGTA
gerS2_forw_K4	GACTCCCTCTTAGGCGCTGA
gerS2_rev_K4	ATTTGAAACCGGCGAATCAC
gerT_forw_AH187	GGGGTGTGTTGGAGTCA
gerT_rev_AH187	GTCTGGTCGGTATTTGCTT
gerX_forw_A10	AATTTGGTCCTTTCGCAGCA
gerX_rev_A10	CAAGCCACTGATACACGGAGA
gerX_forw_AH187	TGGGCAATCACGTTCACAAG
gerX_rev_AH187	CGTGCCCCAGCCTCTCTAA