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A transposon present in specific strains of *Bacillus subtilis* negatively affects nutrient- and dodecylamine-induced spore germination

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Summary

Spore germination shows a large inter-strain variability. Spores of certain *Bacillus subtilis* strains, including isolates from spoiled food products, exhibit different germination behavior from spores of the well-studied model organism *Bacillus subtilis* 168, often for unknown reasons. In this study, we analyzed spore germination efficiencies and kinetics of seventeen *B. subtilis* strains with previously sequenced genomes. A subsequent gene-trait matching analysis revealed a correlation between a slow germination phenotype and the presence of a mobile genetic element, i.e., a Tn*1546*-like transposon. A detailed investigation of the transposon elements showed an essential role of a specific operon (*spoVA*^{2mob}) in inhibiting spore germination with nutrients and with the cationic surfactant dodecylamine. Our results indicate that this operon negatively influences release of Ca-DPA by the SpoVA channel and may additionally alter earlier germination events, potentially by affecting proteins in the spore inner membrane. The *spoVA*^{2mob} operon is an important factor that contributes to inter-strain differences in spore germination. Screening for its genomic presence can be applied for identification of spores that exhibit specific properties that impede spore eradication by industrial processes.

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Introduction

In response to starvation, Gram-positive Bacilli and Clostridia can produce resistant and metabolically dormant (endo)spores through the process of sporulation (Higgins and Dworkin, 2012; Eijlander *et al.*, 2014; Tan and Ramamurthi, 2014). Protective layers, such as a proteinaceous coat, a peptidoglycan cortex and a dehydrated spore core, enable dormant spores to withstand external stresses such as extreme temperatures, radiation, desiccation or harmful chemicals (Setlow, 2006). This exceptional resistance of spores allows bacteria to survive adverse conditions and spread between environmental niches (Checinska *et al.*, 2015). When exposed to specific nutrient or non-nutrient germination triggers, spores can undergo the process of germination and the emerging cells can resume vegetative growth (Setlow, 2003; Moir, 2006; Paredes-Sabja *et al.*, 2011; Segev *et al.*, 2013; Setlow, 2014a; Sinai *et al.*, 2015). The germination process is characterized by a rapid spore rehydration, followed by the restoration of metabolic activity and the loss of stressor resistance (Setlow, 2014a).

Besides the ecological significance as transmission capsules that allow for bacterial survival, spores are highly relevant for industry and medicine. Some spore-formers, such as *Bacillus anthracis, Bacillus cereus, Clostridium perfringens* or *Clostridium difficile*, are human pathogens and their spores often facilitate spreading of disease (Mallozzi *et al.*, 2010). Spores are also a major concern to the food industry as heat regimes applied to ensure food safety are frequently insufficient for spore inactivation (Oomes *et al.*, 2007; Lima *et al.*, 2011; Lücking *et al.*, 2013; Berendsen *et al.*, 2015a). When spores of various *Bacillus* and *Clostridium* species survive such processes and germinate under conditions that support growth, these organisms can cause food spoilage, leading to significant economic losses (Rosenkvist and Hansen, 1995; Scheldeman *et al.*, 2005, 2006) and can in some cases be the cause of foodborne diseases (Lund, 1990). In contrast,

commercially produced *Bacillus* spore products are increasingly applied as natural insecticides for crop protection or as probiotics (Jensen *et al*., 2003; Cutting, 2011; Bader *et al*., 2012).

A thorough understanding of the spore germination processes is extremely important for both the eradication and the utilization of bacterial spores. On the one hand, spores need to resume vegetative growth via germination to exert their deleterious or beneficial effects. On the other hand, germination initiation is accompanied by the loss of spore (heat) resistance (Setlow, 2006, 2013) and therefore facilitates inactivation of spores (Nerandzic and Donskey, 2010, 2013).

Germination is commonly induced through a response to specific nutrients by germinant receptor proteins (Ger receptors, GRs) located in the inner membrane (IM) of the spore (Paidhungat and Setlow, 2000, 2001; Hudson *et al*., 2001). This process is positively influenced by a moderate heat treatment prior to exposure to nutrients (the so-called heat activation, HA), which is believed to act either directly on GR proteins or indirectly on the spore IM (Luu *et al*., 2015). In the gram-positive model organism *B. subtilis* 168, three functional GRs consisting of 3 or 4 subunits each (subunit A, B, C and D) have been characterized (Ross and Abel-Santos, 2010; Paredes-Sabja *et al*., 2011; Ramirez-Peralta *et al*., 2013). GerA responds specifically to L-alanine, while GerB and GerK cooperate in the germination response to a mixture of L-asparagine, glucose, fructose and potassium ions (AGFK) (Paidhungat and Setlow, 2000; Cabrera-Martinez *et al*., 2003). Various GRs form clusters (germinosomes) in the spore IM, together with another membrane protein GerD, which is required for full functionality of GRs (Hudson *et al*., 2001; Pelczar *et al*., 2007; Mongkolthanaruk *et al*., 2009; Griffiths *et al*., 2011; Korza and Setlow, 2013). Moreover, six coat proteins (GerPA-F) provide permeability of the spore coat to the nutrient germinants (Behravan *et al*., 2000; Butzin *et al*., 2012). After germination initiation, dipicolinic acid and Ca^{2+} ions (Ca-DPA) are released from the spore core in a process that involves SpoVA proteins, which presumably form a channel in the IM (Vepachedu and Setlow, 2004; 2007a; Wang *et al*., 2011; Velásquez *et al*., 2014). This initiates rehydration of the spore core, followed by degradation of the protective peptidoglycan cortex layer by two cortex lytic enzymes, SleB and CwlJ (Chirakkal *et al*., 2002). Various non-nutrient germinants, such as exogenous Ca-DPA, the cationic surfactant dodecylamine or high hydrostatic pressure can also trigger germination via slightly different mechanisms (Paredes-Sabja *et al*., 2011).

Strong variations in germination kinetics, germination efficiency, optimal heat-activation conditions and germination stimuli to which spores respond are often observed among spores of different species and strains (van der Voort *et al*., 2010; Alzahrani and Moir, 2014). This variability is currently attributed to differences in types, sequences and numbers of spore germination proteins and in general spore properties, such as core water content or the level of coat protein cross-linking (Ghosh *et al*., 2009; van der Voort *et al*., 2010; Eijlander *et al*., 2011; Abhyankar *et al*., 2015). The majority of our knowledge on spore germination and its variability is derived from studies using model spore-formers that are adapted to laboratory conditions. Importantly, spore germination behavior of such strains does not fully reflect that of strains associated with foods and other natural environments. Food processing can support selection of strains whose spores exhibit properties that increase their survival, such as elevated heat resistance or increased dormancy (Scheldeman *et al*., 2002; 2005; Postollec *et al*., 2012; Lücking *et al*., 2013).

Here, we applied gene-trait matching techniques to unravel the genetic basis of the variety in spore germination between recalcitrant food-spoilage isolates and non-food-related strains of *B. subtilis*. This approach revealed that the presence of a Tn*1546*-like transposon is responsible for slower spore germination with various triggers. We proved that specifically one operon of this transposon (named *spoVA*^{2mob}) plays a key role in the slow germination phenotype, in part by affecting the release of Ca-DPA through the spore IM. Moreover, as the same mobile genetic element has also been shown to elevate spore wet heat resistance (Berendsen *et al*., in press), we discuss the underlying genetic background of these two different spore properties.

Experimental Procedures

Strains, media and spore preparation

B. subtilis strains used in this study are listed in Table 1. Strains were cultured in Luria Bertani (LB) broth with the addition of chloramphenicol (Cm, 5 μg/ml) or spectinomycin (Spc, 10 μg/ml) when appropriate.

Spores were prepared on Schaeffer-agar plates at 37°C without the addition of antibiotics. Spores were harvested after seven days of incubation, washed with water and stored at 4°C as described previously (Berendsen *et al*., 2015a). All spores used in the same experiment were prepared simultaneously except for spores used in germination experiments for gene-trait matching analyses. Before each experiment, spores were washed with cold sterile Milli-Q water and checked for purity (>95% phase-bright spores) using phase-contrast microscopy. All experiments were performed at least twice using two independent spore crops.

Nutrient-induced spore germination

Nutrient-induced spore germination was routinely monitored by measuring the decrease in optical density at 600 nm (OD_{600}) over time. Release of Ca-DPA from spores and spore rehydration that occur during germination lead to a decrease in optical density of spore suspensions. A drop in OD_{600} of

Table 1. Strains used in this study

Strain	Genotype	Strain description	Reference
B4067		Food strain (peanut chicken soup); alternative name A163	(Cazemier *et al.*, 2001; Oomes and Brul, 2004; Kort *et al.*, 2005; Oomes *et al.*, 2007; Berendsen *et al.*, 2015a,; Berendsen *et al.*, 2016)
B4068		Food strain (curry cream soup); alternative name CC2	(Oomes *et al.*, 2007; Berendsen *et al.*, 2015a; Berendsen *et al.*, 2016)
B4069		Food strain (binding flour ingredient); alternative name IIC14	(Oomes *et al.*, 2007; Berendsen *et al.*, 2015a; Berendsen *et al.*, 2016)
B4070		Food strain (peanut chicken soup); alternative name A162	(Caspers *et al.*, 2011; Berendsen *et al.*, 2016)
B4071		Food strain (curry cream soup); alternative name CC16	(Oomes *et al.*, 2007; Berendsen *et al.*, 2015a, Berendsen *et al.*, 2016)
B4072		Food strain (red lasagna sauce); alternative name RL45	(Oomes *et al.*, 2007; Berendsen *et al.*, 2015a; Berendsen *et al.*, 2016)
B4073		Food strain (curry soup); alternative name MC85	(Oomes *et al.*, 2007; Berendsen *et al.*, 2015a, Berendsen *et al.*, 2016)
B4143		Food strain (surimi)	(Berendsen *et al.*, 2015a; Berendsen *et al.*, 2016)
B4145		Food strain (pasta)	(Berendsen *et al.*, 2015a; Berendsen *et al.*, 2016)
B4146		Food strain (curry sauce)	(Berendsen *et al.*, 2015a; Berendsen *et al.*, 2016)
B4055		Laboratory strain, derived from 168; alternative names JH642, BGSC1A96	(Brehm *et al.*, 1973; Smith *et al.*, 2014)
B4056		Laboratory strain, alternative names PY79, BGSC1A747	(Zeigler *et al.*, 2008; Schroeder and Simmons, 2013)
B4057		Laboratory strain; alternative names W23, BGSC2A9	(Earl *et al.*, 2007; Zeigler *et al.*, 2008; Zeigler, 2011)
B4058		Environmental isolate (Sahara Desert); alternative names TU-B-10T, BGSC2A11	(Nakamura *et al.*, 1999; Earl *et al.*, 2007)
B4060		Environmental isolate; alternative names NCIB3610T, BGSC3A1T	(Zeigler *et al.*, 2008; Berendsen *et al.*, 2015a)
B4061		Environmental isolate (Mojave Desert); alternative names RO-NN-1, BGSC3A27	(Nakamura *et al.*, 1999; Earl *et al.*, 2007)
168		Laboratory strain	(Brehm *et al.*, 1973; Kunst *et al.*, 1997)
168Δ*yitGF*	168 *yitGF*::*lox66-P32-cat-lox71*	Derivative of 168 with the *yitGF* operon replaced by *lox66-P32-cat-lox71*. Cm^r	(Berendsen *et al.*, in press)
168Δ*yitF*	168 *yitF*::*lox66-P32-cat-lox71*	Derivative of 168 with the *yitF* gene replaced by *lox66-P32-cat-lox71*. Cm^r	(Berendsen *et al.*, in press)
168*spoVA*^{2mob}	168 *amyE*::*spoVA*^{2mob}, Sp^r	*B. subtilis* 168 with operon 3 (*spoVA*^{2mob} operon) from the Tn*1546* transposon introduced in the *amyE* locus	(Berendsen *et al.*, in press)
B4417	168 *amyE*::*spec yitF*::Tn*1546*	168 *amyE*::Sp^r transduced with DNA fragment from *B. subtilis* B4067, ranging from *yitA* to *metC* including *Tn1546* transposon in *yitF*. Alternative name 168HR	(Berendsen *et al.*, in press)
B4417Δ*Tn1546*	B4417 *Tn1546*::*lox66-P32-cat-lox71*	Derivative of B4417 with entire Tn*1546* replaced with *lox66-P32-cat-lox71*. Alternative name 168HRΔ*Tn1546*	(Berendsen *et al.*, in press)
B4417Δop1	B4417 *operon1*::*lox66-P32-cat-lox71*	Derivative of B4417 with operon 1 of Tn*1546* replaced with *lox66-P32-cat-lox71*. Alternative name 168HRΔop1	(Berendsen *et al.*, in press)

Table 1. *cont.*

Strain	Genotype	Strain description	Reference
B4417Δop2	B4417 *operon2::lox66-P32-cat-lox71*	Derivative of B4417 with operon 2 of Tn*1546* replaced with a *lox66-P32-cat-lox71*. Alternative name 168HRΔop2	(Berendsen *et al.*, in press)
B4417Δ*spoVA*^{2mob}	B4417 *spoVA*^{2mob}*::lox66-P32-cat-lox71*	Derivative of B4417 with operon 3 (*spoVA*^{2mob}) of Tn*1546* replaced with *lox66-P32-cat-lox71*. Alternative name 168HRΔ*spoVA*^{2mob}	(Berendsen *et al.*, in press)
B4417Δop4	B4417 *operon4::lox66-P32-cat-lox71*	Derivative of B4417 with operon 4 of Tn*1546* replaced with *lox66-P32-cat-lox71*. Alternative name 168HRΔop4	(Berendsen *et al.*, in press)
B4417Δop5	B4417 *operon5::lox66-P32-cat-lox71*	Derivative of B4417 with operon 5 of Tn*1546* replaced with *lox66-P32-cat-lox71*. Alternative name 168HRΔop5	(Berendsen *et al.*, in press)
B4417Δ2DUF	B4417 *DUF421-DUF1657::lox66-P32-cat-lox71*	Derivative of B4417 with final gene of operon 3 (*spoVA*^{2mob} operon from Tn*1546*) encoding hypothetical membrane protein with DUF421 and DUF1657 domains replaced with *lox66-P32-cat-lox71*	(Berendsen *et al.*, in press)

approximately 60% (ΔOD∼60%) is generally accepted to correspond with complete germination (Atluri *et al.*, 2006; Nagler *et al.*, 2014). To increase the responsiveness of spores to nutrients (Luu *et al.*, 2015), spores of all strains (except for B4057 and B4143) were suspended in MQ water at an OD_{600} of 10 and were heat-activated for 30 minutes at 70°C, unless stated otherwise. After heat-activation, spores were cooled on ice for at least 15 minutes and examined for the occurrence of phase dark spores by phase-contrast microscopy (spores of strains B4057 and B4143 were not heat-activated due to their high sensitivity to heating). Then, spores were diluted to an OD_{600} of 1 in nutrient-containing solutions in a 96-microwell plate. Nutrient germinants (10 mM L-alanine; 10 mM or 1 mM mixture of L-asparagine, D-glucose, D-fructose and KCl) were diluted in 25 mM Tris-HCl (pH 7.4) with the addition of 0.01% Tween20 to avoid clumping and absorption of spores to the plate wells (Hornstra *et al.*, 2006). Alternatively, spores were resuspended in nutrient-rich LB medium with the addition of chloramphenicol (Cm, 5 μg/ml) or tetracycline (Tet, 6 μg/ml) to prevent outgrowth of germinated spores (Hu *et al.*, 2007; Smelt *et al.*, 2008). The 96-microwell plate was pre-warmed at 37°C and spore suspensions were incubated while shaking at 37°C in the TECAN Infinite® F200 microplate reader (Tecan Austria). OD_{600} measurements were performed at 2-3 minute intervals for at least 2 hours. OD_{600} values [$OD_{600}(t)$] were normalized in relation to the first measured value [OD_{600}(t0)] and expressed as percentages according to the formula:

$$OD_{600}(t)/OD_{600}(t0) * 100\%$$

Unless stated otherwise, typical graphs are shown when results were comparable in all experiments for each spore preparation.

Relative germination efficiencies [extents of spore germination, ΔOD_{600}(max)] per condition correspond to the maximum percentage of decrease in OD_{600} during 120 minutes of incubation of spores with germinants. The ΔOD_{600}(max) values were calculated using the following formula:

$$\Delta OD_{600}(\max) = 100\% - OD_{600}(\min)/OD_{600}(t0) * 100\%,$$

where

- OD_{600}(t0) constitutes the first measured value
- OD_{600}(min) constitutes the minimum OD_{600} achieved between the 110th and 120th minute.

Kinetics of spore germination were visualized by two parameters: (i) maximum rate of spore germination (max. rate); and (ii) time ($t_{90\%germ}$) needed for spore suspensions to reach 90% of ΔOD_{600}(max). Maximum germination rates were determined as an average of two largest decreases in OD_{600} on three consecutive time points. The $t_{90\%germ}$ values were distilled from the data as time-points when $OD_{600}(t_{90\%germ})$ is the closest to ΔOD_{600}(max)*90%.

Means and standard errors for the ΔOD_{600}(max), max. rate and $t_{90\%germ}$ parameters presented in Table 3 were calculated from experiments performed for three independent spore preparations and in at least two replicates.

After the plate reader assays, suspensions of germinated spores were also assessed by phase-contrast microscopy as described previously (Berendsen *et al.*, 2015b), to confirm the degree of spore germination.

Non-nutrient-induced germination

Non-heat-activated spores were germinated through the addition of Ca-DPA (60 mM $CaCl_2$ and 60 mM DPA, pH 7.4, adjusted with dry Tris base) at 30°C (Ramirez-Peralta *et al.*, 2012) or dodecylamine (1 mM in 10 mM Tris-HCl, pH=7.4) at 60°C. Ca-DPA-induced germination was monitored using phase-contrast microscopy as described previously (Berendsen *et al.*, 2015b). Dodecylamine-induced germination was determined by monitoring the release of DPA from spores at OD_{270} as described (Vepachedu and Setlow, 2007a). To determine the total amount of DPA in spores, samples of spore cultures were either boiled for 2 hours or autoclaved (boiling and autoclaving generated comparable results) and spun down in a microcentrifuge (12,000 rpm for 1.5 min) after which the OD_{270} of the supernatant was measured.

Spore coat removal

Spore suspensions with an OD_{600} of 15 were decoated using a mixture of 0.1 M NaOH, 0.1 M NaCl, 0.5% sodium dodecyl sulfate (SDS) and 0.1 M dithiothreitol (DTT). Suspensions were incubated for 30 minutes at 70°C as described (Paidhungat *et al.*, 2001; Bagyan and Setlow, 2002). Decoated spores were washed seven times with 0.15 M NaCl and resuspended in sterile demineralized water to a final OD_{600} of 10, checked by phase-contrast microscopy and stored at 4°C. Coat permeability was confirmed by a lysozyme sensitivity assay as described (Alzahrani and Moir, 2014). Afterwards, decoated spores were germinated with nutrients as described above.

Genome mining and gene-trait matching

For all predicted protein sequences encoded in the genomes of the 17 *B. subtilis* strains used in this study, an orthology prediction was performed using Ortho-MCL software (Li *et al.*, 2003). Based on the obtained orthology prediction, a presence and absence matrix of individual orthologous groups in every strain was created (Supporting Information dataset). Together with germination phenotypic data, i.e., fast germination or slow germination, the created matrix with presence of orthologous groups in the studied strains was used as an input for gene-trait matching analysis with Phenolink software under default settings [http://bamics2.cmbi.ru.nl/websoftware/phenolink/; (Bayjanov *et al.*, 2012)]. A list of orthologous groups important for either the slow or fast germination phenotype was obtained as an output of the automatic Phenolink analysis. The genes encoding listed orthologous groups in individual strains were further analyzed manually regarding i) their genomic context using Clone Manager software, ii) the predicted function of gene products using protein BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi; (Altschul *et al.*, 1990)] and iii) the predicted regulation of gene expression using the DBTBS Motif Location Search tool [http://dbtbs.hgc.jp/; (Sierro *et al.*, 2008)]. A schematic visualization of selected gene clusters was constructed with help of the draw context tool on the Genome2D server [http://genome2d.molgenrug.nl; (Baerends *et al.*, 2004)].

Construction of genetically modified strains

The construction of *B. subtilis* strain B4417 and derivatives has been described in detail in (Berendsen *et al.*, in press). In short, B4417 was obtained using natural transfer of DNA from food-borne donor strain B4067 to recipient strain *B. subtilis* 168 *amyE::spec trpC2*. Mitomycin C was added to induce a pro-phage in B4067 [which produces highly heat-resistant spores (Berendsen *et al.*, 2015a)] and phages of B4067 were mixed with cells of *B. subtilis* 168 *amyE::spec trpC2* on a filter. The transduced recipient strain was selected based on the increased heat resistance of its spores (survival at 100°C for 60 minutes), spectinomycin resistance, tryptophan deficiency and an altered colony morphology. The presence of the Tn*1546*-like transposon in the transduced strain was confirmed by PCR. The selected strain, from now on referred to as B4417, was sequenced by whole genome sequencing.

Subsequently, deletion mutants in strain B4417 were obtained for the entire Tn*1546*-like transposon, for the transposon individual operons as well as for the most downstream gene of its *spoVA*^{2mob} operon (DUF421-DUF1567) (Berendsen *et al.*, in press) with use of the *cre/lox* system (Lambert *et al.*, 2007; Yan *et al.*, 2008). The targeted elements of Tn*1546* were replaced with the *lox71-cat-lox66* cassette from pNZ5319. Correct deletion mutants were selected based on the chloramphenicol resistance and checked by PCR. The same method was used for the preparation of *yitF* and *yitGF* deletion mutants in *B. subtilis* 168.

For complementation purposes, the *spoVA*^{2mob} operon from Tn*1546* was amplified by PCR from *B. subtilis* B4067 and cloned into the pDG1730 vector (Guérout-Fleury *et al.*, 1996). The obtained construct was verified by sequencing and was subsequently integrated into the *amyE* locus of *B. subtilis* 168 (Berendsen *et al.*, in press).

Results

B. subtilis *strains can be divided into two distinct groups based on their spore germination rates*

To investigate the variety in germination of *B. subtilis* spores and to identify the responsible genetic components, we used ten *B. subtilis* isolates relevant to the food industry (Berendsen *et al.*, 2015a; Berendsen *et al.*, 2016) and seven non-food-related *B. subtilis* strains (Table 1). Spore germination was monitored by measuring the decrease in optical density over time after exposure to nutrient-rich LB medium. Strong differences were observed between spores of various strains in both germination efficiency (ranging from 5% germination to 100%, when assessed by phase-contrast microscopy) and germination rate (Fig. 1). The germination rate, in particular, allowed for a clear distinction of two separate phenotypic groups. Group 1 (slow germination, with an optical density drop between 0.1 and 1.1 OD_{600}/min) consisted of eight strains whose spores

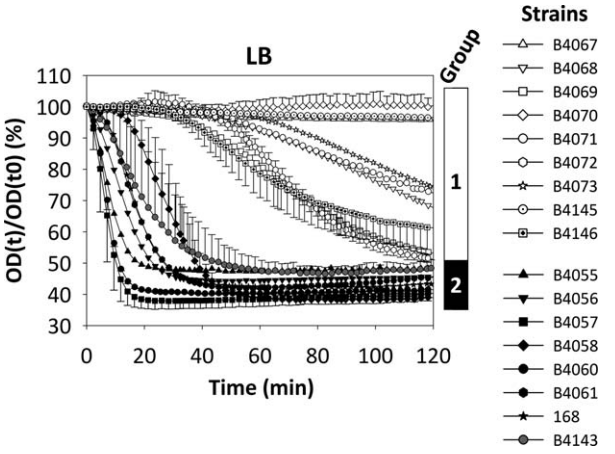

Fig. 1. Germination responses of spores of 17 *B. subtilis* strains in the LB medium. Spore germination is reflected by a decrease in optical density at 600 nm (*y*-axis) over time (*x*-axis).
Curves for individual strains were calculated as means from multiple experiments performed on multiple spore crops. Spores were divided into two groups based on the statistical significance of differing germination rates (Group 1, slow germination, open symbols and Group 2, fast germination, closed symbols). For clarity of the figure, standard errors are only shown for the most outer curves in each group.

required either more than 120 minutes to reach maximum germination or did not germinate at all (Fig. 1, open symbols). Group 2 (fast germination, with an optical density drop between 2.0 and 6.3 OD_{600}/min) contained spores of nine strains that reached maximum germination within 60 minutes after exposure to the medium (Fig. 1, closed symbols). With the exception of strain B4143, all strains that were isolated from food matrices (Table 1) belonged to group 1, whereas the non-food-related environmental and laboratory strains and food isolate B4143 clustered within group 2 (Fig. 1).

A transposon is responsible for the slow germination phenotype of B. subtilis *spores*

The remarkable finding that spores of the investigated food spoilage-associated strains germinated significantly less efficiently prompted us to study their genetic scaffolds by genome sequencing (Berendsen *et al*., 2016). Division of the strains into two phenotypic groups based on the observed spore germination rates was used as an input for the gene-trait matching analysis using Phenolink (Bayjanov *et al*., 2012).

In the analysis, the occurrence of each phenotype (slow/fast germination) was linked to the presence or absence of individual genes in the genomes of the 17 investigated strains. In-depth analysis of the candidate genes indicated by Phenolink led to the identification of two gene clusters that significantly co-occur with the slow spore germination phenotype, and are thus very likely to be involved in affected spore germination (Table 2 and Fig. 2). These two gene clusters were present in all strains of phenotypic group 1 (slow spore germination) and were absent from all strains of phenotypic group 2 (fast spore germination).

Cluster 1, which was previously identified in another context and from here on referred to as Tn*1546*-like transposon or Tn*1546* (Berendsen *et al*., in press), contains 15 genes divided into five operons (numbered 1 to 5) that are inserted into the *yitF* gene, thereby disrupting the *yitF* coding region (Table 2, Fig. 2). The five operons of this element are preceded by pseudogenes encoding a transposase and a resolvase (Table 2), which show significant similarity to the constituents of a similar transposon of *Enterococcus faecium* (Arthur *et al*., 1993; Simjee *et al*., 2002). This indicates that this gene cluster may have been transferred as a transposable element (Berendsen *et al*., in press). Analysis of predicted promoters upstream of the five operons in the Tn*1546*-like transposon revealed the existence of sporulation-specific σ factor binding sites (Fig. 2A) and the RNA sequencing data (data not shown) showed that transcription of these operons takes place during the process of spore formation.

The other cluster identified in this study, for clarity named cluster 2, contains five genes (six in strain B4146) that are distributed among four operons and are localized between the *bsn* and *yurJ* genes in strains B4067, B4068, B4069, B4070, B4071, B4072, B4073, B4145 and between the *groEL* and *ydjB* genes in strain B4146 (Fig. 2B). Moreover, a gene predicted to encode a transposase similar to the enzyme for the IS*Bma2* insertion sequence from *Burkholderia mallei* (Song *et al*., 2010) is also present in this region (Fig. 2B). Two of the operons of cluster 2 are also preceded by predicted sporulation-specific promoter sites (Fig. 2B).

To further investigate the role of the identified gene clusters in spore germination, genes in cluster 1 and cluster 2 were completely or partly introduced into the genetically accessible model strain *B. subtilis* 168. This laboratory strain belongs to the fast spore germination phenotypic group (Fig. 1, group 2) and does not naturally harbor these genes in its genome. No difference could be observed in the germination behavior between control spores and spores of *B. subtilis* 168 in which pairs of operons (operons 1 + 2 and 3 + 4) of cluster 2 (Fig. 2B) were introduced in the *amyE* locus (data not shown). This indicates that these operons encoded on cluster 2 are not solely responsible for poor germination of *B. subtilis* spores, at least not when they are inserted in the ectopic chromosomal locus *amyE*. In contrast, spores of *B. subtilis* 168 in which the Tn*1546*-like transposon was introduced via natural DNA transfer by an induced pro-phage (strain B4417, Berendsen *et al*., in press) showed the slow germination phenotype (Fig. 3 and Table 3), which we investigated further.

Table 2. Genes in two unique gene clusters present exclusively in slowly germinating *B. subtilis* strains

Operon	Gene	Predicted product (domains)	Gene locus tags in different strains of *B. subtilis*								
			B4067	B4068	B4069	B4070	B4071	B4072	B4073	B4145	B4146
Gene cluster 1 (transposon Tn*1546*)											
NA	*tnp*	Tn*1546* transposase (DDE-Tnp-Tn3)	B4067_4748 B4067_4749	B4068_4227	B4069_4135 B4069_4134	B4070_4487 B4070_4508	B4071_4409	B4072_4062 B4072_1114	B4073_4345	B4145_4623 B4145_4622	B4146_1165 B4146_1166
NA	*resolvase*	Tn*1546* resolvase				B4070_4509	B4071_4319				B4146_1167
1	*amidase*	N-acetylmuramoyl-L-alanine amidase (MurNAc-LAA)	B4067_4855	B4068_4218	B4069_4303	B4070_4542	B4071_4291	B4072_4326	B4073_4316	B4145_4711	B4146_1168
1	*ger(x)A*	GR subunit A	†	B4068_4206	B4069_4306	B4070_4543	B4071_4387	pseudogene	B4073_4328	†	B4146_1169
1	*ger(x)C*	GR subunit C	B4067_4717	B4068_4207	B4069_4305	B4070_4544	B4071_4388	B4072_4321	B4073_4327	B4145_4625	B4146_1170
2	*DUF2642*	hyp (DUF2642)	B4067_4718	B4068_4208	B4069_3291	†	B4071_3269	B4072_3297	B4073_4325	B4145_4626	B4146_1172
2	*Mn catalase*	manganese catalase (ferritin-like; CotJC)	B4067_4719	B4068_4209	B4069_4299	B4070_4457	B4071_4288	B4072_4330	B4073_4324	B4145_4627	B4146_1173
3	*DUF1657-1*	hyp (DUF1657)	B4067_4767	#	#	B4070_4475	#	#	#	#	B4146_1174
3	*yhcN/ylaJ*	lipoprotein (spore YhcN/YlaJ)	B4067_4768	B4068_4210	B4069_4296	B4070_4476	B4071_4287	B4072_4305	B4073_4323	B4145_4649	B4146_1175
3	*spoVAC*^{2mob}	stage V sporulation protein AC	B4067_4769	B4068_4211	B4069_4295	B4070_4477	B4071_4286	B4072_4306	B4073_4322	B4145_4648	B4146_1176
3	*spoVAD*^{2mob}	stage V sporulation protein AD	†	B4068_4212	B4069_4294	†	B4071_4285	B4072_4307	B4073_4321	†	B4146_1177
3	*spoVAEB* ^{2mob}	stage V sporulation protein AEB	B4067_4765	B4068_4213	B4069_4293	B4070_4540	B4071_4284	B4072_4308	†	B4145_4635	B4146_1178
3	*DUF1657-2*	hyp (DUF1657)	#	#	#	#	#	#	#	#	#
3	*DUF421-DUF1657 (2DUF)*	probable membrane protein (DUF421 and DUF1657)	†	B4068_4228	B4069_4292	†	B4071_4408	B4072_4309	B4073_4347	†	B4146_1179
4	*yetF N-term*	probable membrane YetF-like N-terminal (DUF421)	B4067_4745	B4068_4221	B4069_4291	B4070_4507	B4071_4407	B4072_4310	B4073_4333	B4145_4650	B4146_1180
4	*yetF C-term*	probable membrane YetF-like C-terminal (DUF421)	B4067_4746	B4068_4222	B4069_4290	pseudogene	B4071_4406	B4072_4311	B4073_4332	B4145_4651	B4146_1181
5	*Cls*	cardiolipin synthase (PLDc-CLS-1 and 2)	B4067_4747	B4068_4223	B4069_4289	†	B4071_4289	B4072_4312	B4073_4331	B4145_4652	B4146_1182
Gene cluster 2											
1	*azlC-like*	azaleucine resistance protein; branched-chain aa transporter permease (AzlC)	B4067_3677	B4068_3176	B4069_3216	B4070_3321	B4071_3344	B4072_3373	B4073_3264	B4145_3565	B4146_0648
2	*regulator*	GntR-family transcriptional regulator (AAT-like)	B4067_3676	B4068_3175	B4069_3215	B4070_3320	B4071_3345	B4072_3374	B4073_3265	B4145_3566	B4146_0649
NA	*tnp*	ISBma2-like transposase (DDE-Tnp-1-6)	B4067_3675	B4068_3174	B4069_3214	B4070_3319	B4071_3346	B4072_3375	B4073_3266	B4145_3567	
NA	*hyp*	hyp	B4067_3674	B4068_3173	B4069_3213	B4070_3318	B4071_3347	B4072_3376	B4073_3267	B4145_3568	
NA	*tnp*	ISBma2-like transposases (DUF772; DDE-Tnp-1-6)	B4067_3673 B4067_3672	B4068_3172 B4068_3171 B4068_3170	B4069_3212 B4069_3211 B4069_3210	B4070_3317 B4070_3316 B4070_3315	B4071_3348 B4071_3349 B4071_3350	B4072_3377 B4072_3378 B4072_3379	B4073_3268 B4073_3269 B4073_3270	B4145_3569 B4145_3570 B4145_3571	
3	*B3/B4(*)*	hyp (B3/4)	B4067_4757	B4068_3169	B4069_3209	B4070_3314	B4071_3351	B4072_3380	B4073_3271	B4145_3572	B4146_0650 B4146_0651(*)
3	*sulf-hydrylase*	O-acetylhomoserine sulfhydrylase; cystathionine β-lyase (CGS-like)	B4067_4756	B4068_3168	B4069_3208	B4070_3313	B4071_3352	B4072_3381	B4073_3272	B4145_3573	B4146_0652
4	*desaturase (**)*	fatty acid desaturase (membrane-FADS-like)	#	B4068_3167	B4069_3207	B4070_3312	B4071_3353	B4072_3382	B4073_3273	present	B4146_0653(**)

Operons are numbered 1-5 or 1-4 in clusters 1 and 2, respectively. Orthologous genes are indicated by locus tags assigned by automatic annotation.

#: gene is present, but no locus tag was assigned by automatic annotation.

†: gene is present, but is divided between multiple contigs of the non-closed genome sequences.

Abbreviations: NA—not applicable; hyp—hypothetical protein; DUF—domain of unknown function.

*: in strain #emi13386-bib-4146 the gene encoding the hypothetical protein with #emi13386-bib-0003/B4 domain is divided into two open reading frames due to the frameshift after 69 nt.

**: in strain #emi13386-bib-4146 the gene encoding the fatty acid desaturase is truncated and annotated as hypothetical.

Table 3. Extents of germination [OD_{600}(max)], maximum germination rates (max. rate) and time required for 90% germination ($t_{90\%germ}$)

	ΔOD_{600}(max) [%]				Max. rate (ΔOD/time) [%/min]				$t_{90\%germ}$ [min]			
Strain	LB nH		LB HA		LB nH		LB HA		LB nH		LB HA	
	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE
B4417	38.2	3.2	50.5	1.0	1.1	0.1	1.7	0.1	71.8	7.6	48.8	1.8
B4417ΔTn	46.3	4.0	53.7	0.7	1.8	0.3	3.1	0.1	38.2	6.0	29.1	2.9
B4417Δop3	48.4	2.9	54.3	1.3	1.7	0.2	3.0	0.2	42.9	8.3	33.5	5.0
B4417Δ2DUF	43.9	3.3	53.5	1.5	1.4	0.1	2.2	0.2	52.7	10.3	39.5	1.6
168	54.9	2.9	59.3	1.1	2.3	0.4	3.1	0.4	38.6	8.4	26.9	1.0
168*spoVA*^{2mob}	50.4	2.7	56.6	1.6	1.8	0.0	2.0	0.2	51.1	2.5	40.6	1.1

	AGFK nH		AGFK HA		AGFK*HA		AGFK nH		AGFK HA		AGFK* HA		AGFK nH		AGFK HA		AGFK* HA	
	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE
B4417	57.3	1.2	60.2	1.1	43.1	9.6	1.3	0.2	1.6	0.1	0.6	0.2	82.3	5.4	58.0	3.5	97.9	7.2
B4417ΔTn	61.2	0.5	60.1	1.1	58.1	2.7	2.2	0.3	2.4	0.4	1.3	0.3	37.5	4.1	35.8	4.6	73.2	13.4
B4417Δop3	60.3	0.8	59.5	1.2	54.5	4.4	2.0	0.3	2.2	0.2	1.1	0.2	42.6	6.5	38.9	4.1	84.5	9.4
B4417Δ2DUF	60.1	1.4	60.3	0.8	52.1	3.5	1.6	0.3	2.0	0.2	1.0	0.2	60.8	10.6	43.0	4.4	85.3	10.3
168	63.4	0.5	62.8	1.1	61.7	1.7	2.3	0.3	2.5	0.3	1.5	0.1	40.5	4.4	38.8	4.0	62.6	8.3
168*spoVA*^{2mob}	61.1	1.6	62.3	0.6	54.1	2.9	1.9	0.1	1.9	0.1	0.9	0.1	74.8	10.8	46.1	2.3	92.5	3.5

	Ala nH		Ala HA		Ala nH		Ala HA		Ala nH		Ala HA	
	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE	AVE	SE
B4417	38.4	1.5	44.3	3.6	1.1	0.2	1.2	0.1	72.9	4.1	58.0	2.3
B4417ΔTn	44.7	2.8	40.5	1.5	1.4	0.2	1.3	0.1	50.3	3.6	51.6	2.2
B4417Δop3	47.8	2.3	43.9	2.0	1.5	0.2	1.3	0.1	52.6	4.2	55.8	2.2
B4417Δ2DUF	44.8	2.5	41.1	2.3	1.3	0.2	1.1	0.0	63.1	5.5	59.9	3.3
168	54.6	2.4	52.0	2.5	1.6	0.2	1.8	0.2	49.1	3.2	47.7	3.9
168*spoVA*^{2mob}	46.3	0.9	46.9	0.2	1.3	0.2	1.5	0.1	63.1	5.1	53.3	1.1

Spores of six strains were either heat-activated at 70°C (HA) or not (nH) and germinated in LB (LB), 10 mM AGFK (AGFK), 1 mM AGFK (AGFK*) and 10 mM L-alanine (Ala) for 120 minutes. The extents of germination [ΔOD_{600}(max)] correspond to the maximum percentage of decrease in OD_{600} relative to the start OD_{600} within 120 minutes of germination. Means (AVE) and standard errors (SE) were calculated from experiments performed on three independent spore crops and for at least two technical replicates.

*Introduction of Tn*1546 *into* B. subtilis *168 slows down spore germination with nutrient germinants*

Strain B4417 contained an insertion of the Tn*1546*-like transposon into the *yitF* locus of *B. subtilis* 168. Genome sequencing analysis of this strain revealed multiple single nucleotide polymorphisms (SNPs) along the surrounding 100 kb long genomic fragment (Supporting Information Fig. S1). To discriminate between the influence of Tn*1546* and of the SNPs, the B4417Δ*Tn1546* strain (Berendsen *et al.*, in press), in which the Tn*1546*-like transposon was deleted, but the SNPs were preserved, was included as a control in all further germination experiments.

Spores of *B. subtilis* 168, B4417 and B4417ΔTn*1546* were (Fig. 3, right panel and Table 3) or were not (Fig. 3, left panel and Table 3) heat-activated at 70°C and germinated in LB medium (Fig. 3A and Table 3), AGFK (Fig. 3B and Table 3) or L-alanine (Fig. 3C and Table 3). Spores of *B. subtilis* 168 in which the transposon was introduced (B4417) germinated less rapidly (as demonstrated by lower max. germination rates and longer $t_{90\%germ}$ times in Table 3) with all tested nutrients compared with 168 spores. Additionally, in LB and L-alanine, the germination efficiency [reflected by ΔOD_{600}(max)] of B4417 spores was decreased compared to spores of *B. subtilis* 168. This effect was especially notable for non-heat-activated spores.

Deletion of Tn*1546* from strain B4417 (B4417ΔTn*1546*) restored the rate of nutrient-induced spore germination either completely (in AGFK and in LB for heat activated spores) or partially (in L-alanine and LB for non-activated spores) when compared to 168 spores (Fig. 3 and Table 3). Additionally, it enhanced the germination efficiency in LB and, for non-heat-activated spores, in L-alanine. In contrast, for heat-activated spores, the maximal rate and efficiency of germination with L-alanine were not significantly improved by the Tn*1546* deletion (Fig. 3C, right panel and Table 3).

The results obtained indicate that the Tn*1546*–like transposon has a negative effect on spore germination rates in

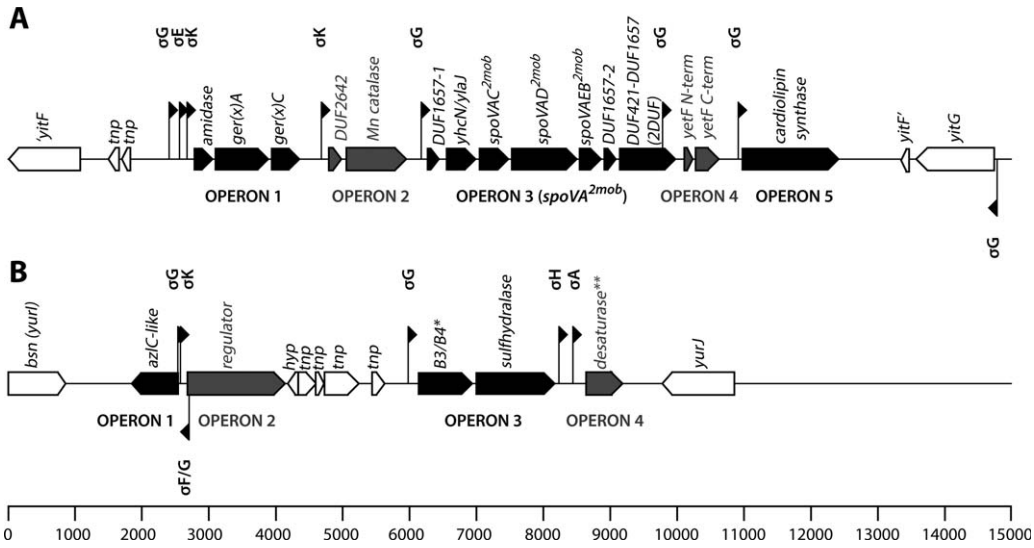

Fig. 2. Structures of gene cluster 1 (Tn*1546*) (A) and gene cluster 2 (B) that are associated with a slow germination phenotype.
A] Tn*1546* is inserted into the *yitF* gene, dividing it into a truncated N-terminal part (*yitF'*, 36 nt) and C-terminal part (*'yitF*, 1086 nt) B] Cluster 2 is inserted between the *bsn* and *yurJ* genes or the *groEL* and *ydjB* genes (in strain B4146). Operons 2 and 3 of cluster 2 are separated by genes encoding a hypothetical protein and transposases, which are both absent from strain B4146. The gene indicated as "B3/B4*" is divided into two open reading frames and the gene encoding desaturase** is truncated in strain B4146. Upstream of the individual operons, predicted binding sites for stationary-phase and sporulation σ factors (σ^H, σ^F, σ^E, σ^G and σ^K) and σ^A are marked by black arrows. Scales on the bottom of the figure show distances in nucleotide base pairs.

AGFK and on both rate and efficiency in LB and in L-alanine. Besides the Tn*1546*-like transposon, SNPs in the 100 kb genomic region distinguishing the 168 and B4417-derived strains (Supporting Information Fig. S1) also adversely influenced the response to L-alanine (Fig. 3C and Table 3), which is mediated via the GerA germinant receptor (Paidhungat and Setlow, 2000).

As B4417 spores are known to exhibit an increased heat-resistance phenotype (Berendsen *et al.*, in press), we speculated that the optimal heat-activation temperature to facilitate spore germination may also be higher for these spores. To verify this, B4417 spores were additionally heat-activated at 80°C and 87°C prior to germination. However, no differences in the germination behavior were observed after activation at 80°C and at 70°C whereas a 87°C heat treatment led to slightly less efficient germination of B4417 spores (Supporting Information Fig. S2). This indicates that the inhibitory effect of the Tn*1546* transposon on spore germination is not caused by an increase in spore heat-activation requirements.

*Operon 3 (*spoVA^{2mob}*) from Tn*1546 *has a major negative impact on nutrient-induced spore germination*

To elucidate which of the five operons of the Tn*1546* transposon negatively influence(s) spore germination, we studied the germination responses of individual operon deletion strains of *B. subtilis* B4417 (B4417Δop1, B4417Δop2, B4417Δ*spoVA*^{2mob}, B4417Δop4 and B4417Δop5 as described in Table 1). Additionally, *B. subtilis* 168 strains in which the *yitF* or the *yitF-yitG* genes were deleted (168Δ*yitF* and 168Δ*yitGF*) were included to investigate an effect of disruption of this locus by the Tn*1546*–like element. Spores of all B4417-derived deletion strains, except for B4417Δ*spoVA*^{2mob}, generated comparable germination curves as the B4417 control strain (data not shown). Similarly, no difference could be observed between the spore germination of *B. subtilis* 168, 168Δ*yitF* and 168Δ*yitGF* (data not shown). This indicates that genes belonging to these operons are not solely responsible for the slow germination phenotype observed for B4417.

In contrast, B4417Δ*spoVA*^{2mob} spores germinated significantly faster and in some conditions also more efficiently than spores of B4417 (Fig. 3 and Table 3). In fact, their germination behavior closely resembled the phenotype of B4417ΔTn*1546* spores (Fig. 3 and Table 3). Moreover, deletion of only the last gene of this operon (referred to as *DUF421-DUF1657* or *2DUF*), which encodes a putative membrane protein, improved nutrient-induced spore germination when compared with the control (Fig. 3 and Table 3), yet to a slightly lower extent than observed for B4417Δ*spoVA*^{2mob} spores (Fig. 3 and Table 3). Thus, the *spoVA*^{2mob} operon, with a possible involvement of its last gene, seems to be a major player in the inhibition of spore germination in *B. subtilis*. This hypothesis was further supported by introduction of the *spoVA*^{2mob} operon into the *amyE* locus of *B. subtilis* 168 (Table 1, 168*spoVA*^{2mob}), which significantly decreased the spore

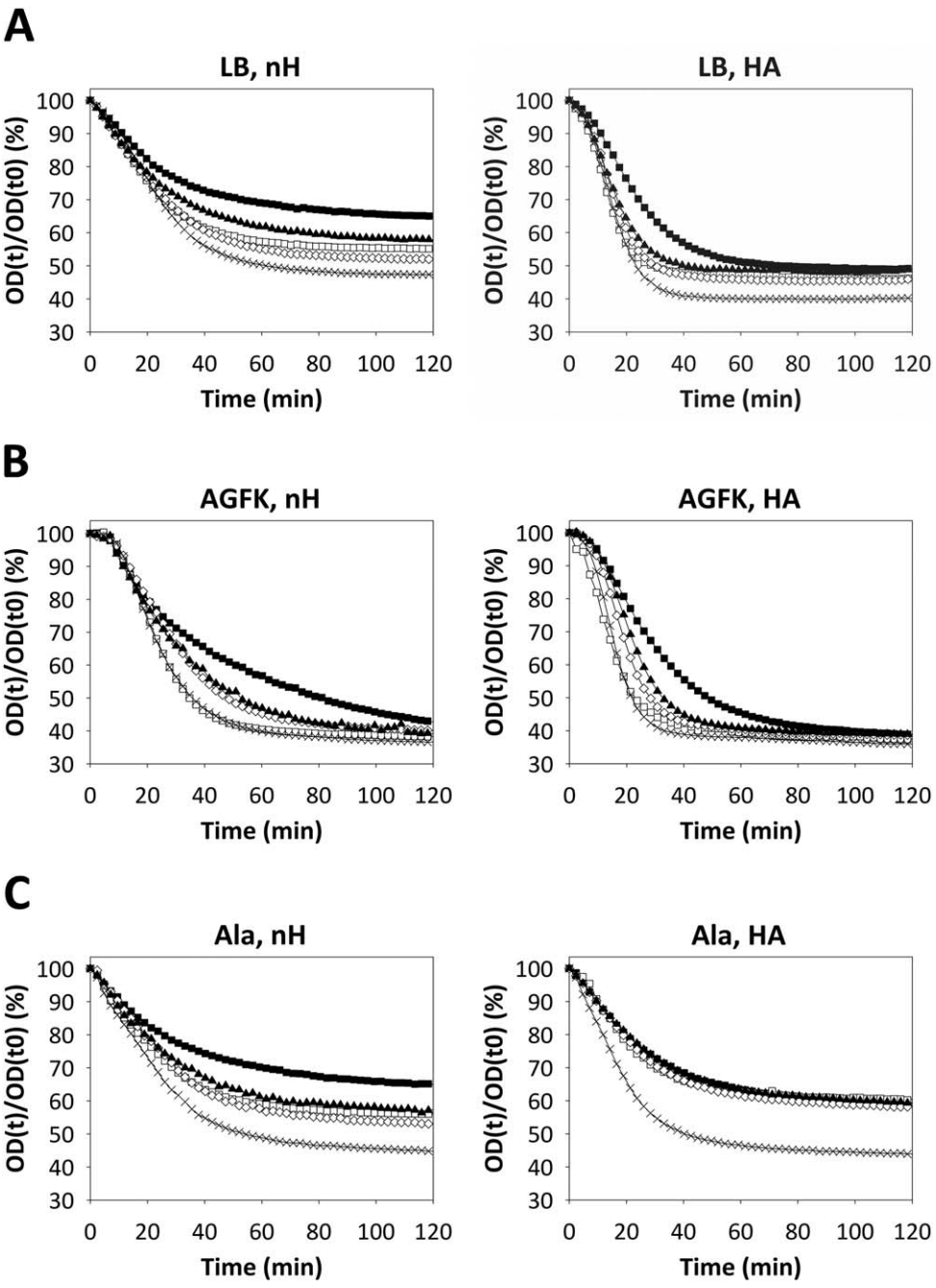

Fig. 3. Effects of deletion of Tn*1546* components on nutrient-induced germination of B4417 spores. Representative graphs of decrease in OD_{600} in time in response to LB (A), AGFK (B) and L-alanine (C) are shown for non-heat-activated (nH; left panel) and heat-activated (HA; right panel) spores of strains: B4417 (■), B4417Δ*Tn1546* (□), B4417Δ*spoVA*^{2mob}(¤), B4417Δ*2DUF* (▲) and 168 (X).

germination rate in LB and 1 mM AGFK (Table 3 and Supporting Information Fig. S3A).

The spoVA^{2mob} *operon inhibits release of Ca-DPA during dodecylamine-triggered germination*

To investigate at which stage the germination process is inhibited by the presence of the Tn*1546*–like transposon (and in particular the *spoVA*^{2mob} operon), we germinated spores under various conditions. First, spores of strains 168, B4417 and B4417Δ*Tn1546* were subjected to the decoating procedure at 70°C, which improves the access of nutrients to GRs as it removes a barrier of the spore coat (Behravan *et al.*, 2000; Butzin *et al.*, 2012). Upon exposure to AGFK and LB, similar germination trends were observed for the decoated spores as previously for the spores with intact coats (Supporting Information Fig. S4). B4417 spores with the removed spore coat still showed poorer germination responses to nutrients than spores of B4417Δ*Tn1546* and 168 strains. Therefore, the spore coat does not seem to play a significant role in slowing down nutrient-induced germination by genes of

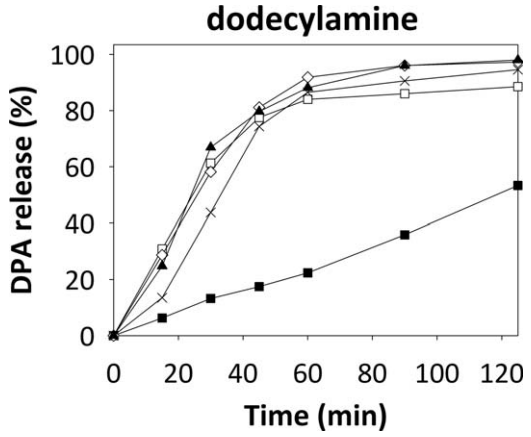

Fig. 4. Percentage of Ca-DPA release in time during exposure to dodecylamine from spores of B4417 (■), B4417Δ*Tn1546* (□), B4417Δ*spoVA*^{2mob} (◇), B4417Δ*2DUF* (▲) and 168 (X).

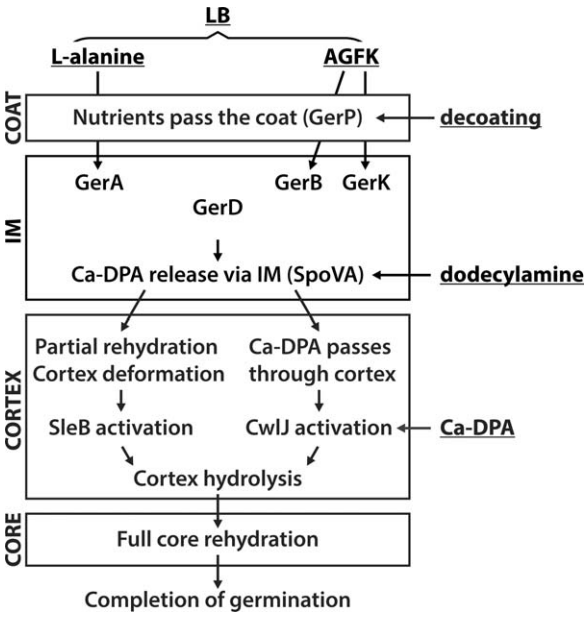

Fig. 5. Schematic representation of pathways and proteins involved in spore germination [adapted from (Paredes-Sabja *et al.*, 2011)]. Methods for investigating specific stages of germination are underlined. Germination pathways, proteins and spore structures possibly affected by *spoVA*^{2mob} are indicated in black, whereas probable unaffected ones are indicated in gray.

Tn*1546*. Secondly, 168, B4417 and B4417Δ*Tn1546* spores were incubated with exogenous Ca-DPA, which causes germination via direct activation of the cortex lytic enzyme CwlJ (Paidhungat *et al.*, 2001), thus skipping all the preceding events that occur during germination initiated by nutrient germinants (Fig. 5). Spores of the three strains germinated alike with Ca-DPA (Supporting Information Fig. S5), suggesting that the final stages of spore germination, which involve cortex hydrolysis, are also unaffected by the Tn*1546*–like element.

Finally, the cationic surfactant dodecylamine, which acts on the SpoVA channel, directly triggering Ca-DPA release and thereby bypassing the requirement for GRs (Vepachedu and Setlow, 2007a; Velásquez *et al.*, 2014), was used as a germinant. When 168, B4417 and B4417Δ*Tn1546* spores were exposed to dodecylamine, a significant decline in Ca-DPA release was detected for B4417 spores (Fig. 4). Removal of either the entire *spoVA*^{2mob} operon or only the *2DUF* gene resulted in similar levels of Ca-DPA release as observed for B4417Δ*Tn1546* and 168 spores (Fig. 4). A role of *spoVA*^{2mob} in decreasing the amounts of Ca-DPA released during dodecylamine-induced germination was further confirmed by insertion of *spoVA*^{2mob} into the *amyE* locus of *B. subtilis* 168 (Supporting Information Fig. S3B). Altogether, the data show that the *spoVA*^{2mob} operon of Tn*1546* at least negatively influences the release of Ca-DPA through the SpoVA channel and may also affect earlier germination events that involve the GRs and GerD in the IM.

Discussion

By employing a gene-trait matching approach with a subsequent phenotypic analysis of genetically modified strains, we show that the presence of a Tn*1546*-like transposon slows down nutrient- and dodecylamine-induced spore germination in *B. subtilis* strains derived from food environments. Our study reveals that one of the operons of Tn*1546*, namely the *spoVA*^{2mob}, is mainly responsible for the inhibited spore germination (Table 3, Figs. 3 and 4 and Supporting Information Fig. S3). The same operon has been shown independently to cause an increase in spore wet heat resistance (Berendsen *et al.*, in press), indicating a common genetic basis for a reduced germination capacity and elevated spore heat resistance. The co-occurrence of these two spore features has been reported previously after certain modifications of sporulation conditions (Rose *et al.*, 2007; van der Voort and Abee, 2012) and for superdormant *Bacillus* spores that concurrently exhibit elevated wet heat resistance (Ghosh *et al.*, 2009). Nevertheless, this work in combination with the study by Berendsen *et al.* (in press) demonstrates the first clear genetic link between increased spore heat resistance and poorer nutrient- and dodecylamine-induced germination.

Our findings suggest two potential mechanisms via which the products of *spoVA*^{2mob} could simultaneously cause both of these spore phenotypes: (i) increased DPA spore content and (ii) altered spore IM properties. Firstly, the presence of the *spoVA*^{2mob} operon has been shown to result in elevated DPA concentrations in spores (Berendsen *et al.*, in press). As spores with low DPA content are prone to spontaneous germination (Paidhungat *et al.*, 2000; Magge *et al.*, 2008), it is possible that higher DPA

concentrations lead to an increase in spore stability and dormancy. Furthermore, accumulation of DPA in the spore core is associated with a lower spore water content, which is essential for wet heat resistance (Setlow, 2014b) and has been related to spore superdormancy (Ghosh *et al*., 2009). Secondly, the spore IM is crucial for both efficient spore germination and spore resistance to the wet heat (Rose *et al*., 2007; Griffiths and Setlow, 2009; Setlow, 2014b) and four out of seven products of the *spoVA*^{2mob} operon are predicted to localize within the IM [the YhcN/YlaJ-like lipoprotein, SpoVAC^{2mob}, SpoVAEB^{2mob} and 2DUF (Table 2, Fig. 2)]. Indeed, the deletion of the last gene on the *spoVA*^{2mob} operon, encoding the putative membrane protein, 2DUF, from strain B4117 reduces spore heat resistance (Berendsen *et al*., in press) and improves spore germination (Table 3, Figs. 3 and 4). Although an effect of this deletion might be indirect, the membrane-localized products of *spoVA*^{2mob} could conceivably influence the IM properties.

In fact, the types of spore germination pathways (nutrient and dodecylamine) affected by *spoVA*^{2mob} (Table 3, Figs. 3 and 4, Supporting Information Fig. S3) indicate that this operon alters the early stages of germination that depend on proteins localized in or adjacent to the spore IM (Fig. 5), namely GRs with the GerD protein (Hudson *et al*., 2001; Mongkolthanaruk *et al*., 2009; Griffiths *et al*., 2011; Korza and Setlow, 2013) and the core component of the IM channel for Ca-DPA transport, SpoVAC (Vepachedu and Setlow, 2005; 2007a; Korza and Setlow, 2013; Velásquez *et al*., 2014). The products of *spoVA*^{2mob} could delay germination after exposure to both dodecylamine and to nutrients through inhibition of the Ca-DPA release across the IM. However, one cannot exclude that the products of *spoVA*^{2mob} also influence earlier IM-dependent germination events, such as sensing of nutrients by GRs and signal transduction between the GR and SpoVA proteins (Paredes-Sabja *et al*., 2011; Setlow, 2014a) (Fig. 5). In turn, permeability of the coat to germinants (Supporting Information Fig. S4) and later stages in germination (Supporting Information Fig. S5), which include the degradation of the peptidoglycan cortex and full rehydration of the spore core (Paredes-Sabja *et al*., 2011; Setlow, 2014a), seem unaffected by the Tn*1546*–like element (Fig. 5).

Even though we established a clear role of the *spoVA*^{2mob} operon in the important germination events, the exact contribution of the seven genes encoded on this operon (Table 2, Fig. 2) to spore germination remains unclear. Four genes encoding hypothetical proteins with domains of unknown function (DUF1657, DUF421, and DUF1657) and a putative lipoprotein bear no similarity to described proteins, which complicates a function prediction for these four components. In contrast, the remaining three gene products, SpoVAC^{2mob}, SpoVAD^{2mob} and SpoVAEB^{2mob}, share significant amino-acid sequence identities with the SpoVAC, SpoVAD and SpoVAEB proteins (55%, 49%, and 59%, respectively) encoded in the heptacistronic *spoVAA-AF* operon of *B. subtilis* 168. Proteins encoded by this operon are responsible for the transport of Ca-DPA across the IM into the spore core during sporulation and from the spore core upon germination (Fort and Errington, 1985; Vepachedu and Setlow, 2004; 2007a; Paredes-Sabja *et al*., 2011; Wang *et al*., 2011; Li *et al*., 2012; Setlow, 2014a). In *B. subtilis* 168, SpoVAC is an integral IM protein that presumably functions as a mechanosensitive channel for this Ca-DPA transfer (Velásquez *et al*., 2014). SpoVAD is located on the outer surface of the IM and has the ability to bind Ca-DPA (Vepachedu and Setlow, 2005; Li *et al*., 2012), while SpoVAEB is a predicted IM protein that is essential for Ca-DPA uptake during sporulation (Li *et al*., 2012). Although the SpoVA^{2mob} proteins have been hypothesized to increase uptake of Ca-DPA into the spore core during sporulation (Berendsen *et al*., in press), their exact role in germination is unknown. In *B. subtilis* 168, overexpression of the native *spoVAA-AF* operon has resulted in faster nutrient-induced germination (Wang *et al*., 2011). However, this is not the case for B4417 spores, in which the presence of the *spoVA*^{2mob} operon decreases spore germination. We therefore speculate that the three SpoVA^{2mob} proteins could interfere with the action of the existing SpoVA channel or compete for Ca-DPA binding, leading to the observed inhibition of Ca-DPA release. Additionally, they might disturb the interactions between the "regular" SpoVA proteins and germinant receptors' subunits (Atluri *et al*., 2006; Vepachedu and Setlow, 2007b), which are present in spores in limited numbers (Stewart and Setlow, 2013). Furthermore, as mentioned above, the IM-associated products of the *spoVA*^{2mob} operon, including DUF421-DUF1657, could alter general properties of the spore IM. More in-depth studies are required to elucidate the exact role of the *spoVAC*^{2mob}, *spoVAD*^{2mob}, *spoVAEB*^{2mob} and four other hypothetical genes encoded on Tn*1546*.

An important observation in this study is that the insertion of the *spoVA*^{2mob} operon in *amyE* in *B. subtilis* 168 affects germination (Table 3 and Supporting Information Fig. S3) and spore heat resistance (Berendsen *et al*., in press) to a lesser extent than its presence within the native locus on Tn*1546* in B4417. This suggests that the genomic context of *spoVA*^{2mob} might play a role in the development of the altered phenotypes, for instance by affecting expression of the *spoVA*^{2mob} operon during sporulation (Higgins and Dworkin, 2012). Alternatively, other operons of the transposon or the SNPs outside of Tn*1546* that distinguish B4417 and 168 strains (Supporting Information Fig. S1) may have some secondary effects on the properties of B4417 spores. Indeed, next to *spoVA*^{2mob}, the SNPs introduced to B4417 along with the Tn*1546* transposon during transduction of DNA from the isolate B4067 (Supporting

Information Fig. S1) are partially responsible for a decrease in the rate and efficiency of germination of B4417 spores in L-alanine (Table 3 and Fig. 3C). Additionally, they seem to prevent an enhancement of L-alanine-induced germination upon heat-activation for B4417Δ*Tn1546*, B4417Δ*spoVA*^{2mob} and B4417Δ2DUF spores (Table 3 and Fig. 3C). Although reasons for this phenomenon are unclear, the influence of the SNPs appears to be limited to germination via the GerA germinant receptor, which mediates the response to L-alanine (Paidhungat and Setlow, 2000; Atluri *et al*., 2006). In contrast, germination with AGFK, which requires cooperative action of the GerB and GerK receptors (Paidhungat and Setlow, 2000; Atluri *et al*., 2006), is unaffected by differences in genomic sequences of 168 and B4417 outside the Tn*1546* transposon.

Interestingly, operon 1 of Tn*1546* contains genes that are orthologous to known germination genes (Table 2 and Fig. 2). This operon encodes the putative GR subunits A and C with a length of 337 and 132 amino-acids, respectively, but misses the gene for subunit B (Table 2 and Fig. 2). Within the tested conditions, no effect of operon 1 on spore germination was observed (data not shown). This could be caused by the lack of a corresponding gene encoding the GR subunit B, which in known GRs is thought to be involved in the recognition of specific nutrient(s) (Paredes-Sabja *et al*., 2011) or by the truncation of the *ger(x)A* and *ger(x)C* genes (data not shown). Thus, it is likely that the encoded subunit A and C do not build a functional germinant receptor. However, the two GR subunits encoded on the first operon of Tn*1546* could potentially interact with subunits of the GerA, GerB and GerK GRs within the germinosome cluster in the spore IM, thereby influencing the spore germination properties (Atluri *et al*., 2006; Griffiths *et al*., 2011).

The *spoVA*^{2mob} operon contributes to inter-strain variability in spore germination and heat resistance properties not only in *B. subtilis* but also in the other *Bacillus* species. The operon has been proposed to stem from the pXOI-like plasmid of *B. cereus*, from where it has spread to multiple strains of *B. subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus thermoamylovorans* and *Bacillus sporothermodurans*, presumably via horizontal gene transfer (Berendsen *et al*, in press). Similar to what has been observed for spores of *B. subtilis*, the correlation between the presence of *spoVA*^{2mob} and elevated wet heat resistance has been shown for various *B. amyloliquefaciens* and *B. licheniformis* strains (Berendsen *et al*., in press). Additionally, four *Bacillus thermoamylovorans* strains that harbor the *spoVA*^{2mob} operon in their genomes produce spores that are characterized by extreme wet heat resistance and a very poor germination capacity after exposure to nutrients (Berendsen *et al*., 2015b). Thus, the effect of the *spoVA*^{2mob} operon on such spore properties seems universal amongst different *Bacillus* species and partly explains the strong variations observed therein.

To conclude, our findings also have various practical implications for the food industry. We provide significant evidence that the *spoVA*^{2mob} operon plays a major role in causing the two spore properties, slow germination and high heat resistance, which both complicate spore inactivation and control in industrial settings. The operon can thus function as a genetic marker for the identification of the problematic spores, thereby improving risk assessments. This is especially important as food processing treatments impose selective pressures that favor spores bearing these features (Scheldeman *et al*., 2002; 2005; Postollec *et al*., 2012; Lücking *et al*., 2013), likely leading to the observed over-representation of the strains containing *spoVA*^{2mob} amongst the studied food isolates. Secondly, fractional germination of spores containing *spoVA*^{2mob} needs to be taken into consideration when quantifying spores by the traditional plating techniques that depend on spore germination and outgrowth. Finally, the presence of the Tn*1546*-like transposon in the investigated food isolates and its absence in common laboratory strains underline the importance of thoroughly studying the genetic basis of the diversity in spore germination and clearly show that studies focusing on domesticated laboratory strain often fail to uncover the effects of gene products that occur in nature or in industrial settings.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supporting Information dataset. Presence and absence matrix of orthologous groups in 17 *B. subtilis* strains based on the orthology prediction by Ortho-MCL. Orthologous groups present in individual strains are indicated by locus tags of the corresponding genes.

Fig. S1. Schematic overview of construction of B4417 and B4417Δ*Tn1546* strains. Strain B4417 was obtained via transfer of a 100 kb long chromosomal fragment, encompassing Tn*1546*, from the donor strain B4067 into the recipient strain *B. subtilis* 168 *amyE*::*spec* by an induced pro-phage. As a result, the region between the *yitA* (BSU10920) and *metC* (BSU11880) genes in *B. subtilis* 168 *amyE*::*spec* was replaced by the homologous area from B4067 (indicated in gray), which included the Tn*1546* transposon (indicated by black and white stripes). Subsequently, the control strain B4417Δ*Tn1546* was derived from B4417 by replacement of Tn*1546* with the CmR cassette (*cat*, indicated in white).

Fig. S2. Effects of more severe heat-activation conditions on germination of B4417 spores. Representative graphs of decrease in OD_{600} over time due to germination with AGFK are shown for spores of strains: B4417

(squares), B4417Δ*Tn1546* (circles) and 168 (triangles) heat-activated at 70°C (white symbols), 80°C (gray symbols) and for B4417 also 87°C (black symbols).

Fig. S3. Effects of insertion of the *spoVA*^{2mob} operon into the *amyE* locus of *B. subtilis* 168 on spore germination with 1 mM AGFK (A) and dodecylamine (B). Representative graphs of decrease in OD_{600} (A) and Ca-DPA release (B) over time are shown for spores of strains 168*spoVA*^{2mob} (▼), B4417 (■) and 168 (X).

Fig. S4. Effects of coat removal on germination with AGFK (A) and LB (B) of B4417 spores. Graphs of decrease in OD_{600} in time are shown for chemically decoated (at 70°C) spores of the strains: B4417 (■), B4417Δ*Tn1546* (□) and 168 (X).

Fig. S5. **Effect of Tn*1546* on spore germination with Ca-DPA.** Percentages of germinated (phase-dark) spores were quantified for strains B4417 (black), B4417Δ*Tn1546* (light gray) and 168 (dark gray) based on phase-contrast microscopy images made after 10, 25, 40, 55 and 70 minutes of incubation with 60 mM Ca-DPA. Mean values of two independent experiments are shown, including error bars based on standard errors.