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The *Bacillus subtilis* germinant receptor GerA triggers premature germination in response to morphological defects during sporulation

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Summary

During sporulation in *Bacillus subtilis*, germinant receptors assemble in the inner membrane of the developing spore. In response to specific nutrients, these receptors trigger germination and outgrowth. In a transposon-sequencing screen, we serendipitously discovered that loss of function mutations in the *gerA* receptor partially suppress the phenotypes of >25 sporulation mutants. Most of these mutants have modest defects in the assembly of the spore protective layers that are exacerbated in the presence of a functional GerA receptor. Several lines of evidence indicate that these mutants inappropriately trigger the activation of GerA during sporulation resulting in premature germination. These findings led us to discover that up to 8% of wild-type sporulating cells trigger premature germination during differentiation in a GerA-dependent manner. This phenomenon was observed in domesticated and undomesticated wild-type strains sporulating in liquid and on solid media. Our data indicate that the GerA receptor is poised on a knife's edge during spore development. We propose that this sensitized state ensures a rapid response to nutrient availability and also elicits premature germination of spores with improperly assembled protective layers resulting in the elimination of even mildly defective individuals from the population.

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Introduction

Mutant phenotypes are often the direct consequence of the absence of a gene product. However, in many cases, a phenotype is largely indirect resulting from the inappropriate activation or inhibition of downstream events. In these instances, a small perturbation can be amplified leading to large phenotypic consequences. Here, we report a striking example of the latter in which a large set of mutants that have relatively modest defects in spore maturation inappropriately trigger germination resulting in loss of resistance properties and inviability. These findings led us to discover that a sizeable proportion of wild-type sporulating cells inappropriately trigger germination during the process of sporulation. Our data suggest that the requirement for dormant spores to sensitively monitor and rapidly respond to nutrients comes at a high cost, in which errors in morphogenesis lead to a significant loss of viable spores from the population.

In response to starvation, *Bacillus subtilis* enters the sporulation pathway in which one cell type (a nutrient deprived cell) differentiates into two: a mother cell (that ultimately lyses) and a dormant spore (reviewed in Piggot and Hilbert, 2004; Higgins and Dworkin, 2012; Tan and Ramamurthi, 2014). These two cells follow different programs of gene expression controlled by a cascade of alternative sigma factors that are activated in a stage and cell-type-specific manner. The first landmark event in this morphological process is the formation of polar septum that divides the cell into a large mother cell and smaller forespore compartment. Shortly after polar division, the mother cell membranes migrate around the forespore generating a cell within a cell surrounded by a membrane derived from the forespore (the inner forespore membrane), a thin layer of peptidoglycan (the germ cell wall) and a membrane derived from the mother cell (the outer forespore membrane). At this

stage, the spore prepares for dormancy, which includes the production of small acid soluble DNA-binding proteins (SASPs) that protect the spore chromosome from radiation, heat and genotoxic chemicals (Setlow, 2014b). Concomitantly the mother cell packages the spore in a series of protective layers including a thick and loosely cross-linked layer of specialized peptidoglycan (the cortex) in the space between the outer forespore membranes and the germ cell wall (Meador-Parton and Popham, 2000). The cortex is composed of a modified peptidoglycan in which ~ 50% of the *N*-acetyl muramic acid (MurNAc) sugars in the heteropolymeric glycan strands are converted to muramic delta lactam (Gilmore *et al*., 2004). The mother cell also assembles a multi-layered coat composed of > 70 proteins on the cytoplasmic face of the outer forespore membrane (McKenney *et al*., 2013) that protects the spore from predation and degradative enzymes (Klobutcher *et al*., 2006). Finally, the mother cell produces the small molecule dipicolinic acid (DPA) that is transported into the spore as a Ca^{2+} chelate. Ca^{2+}-DPA replaces much of the water in the spore core, contributing to heat resistance and maintenance of spore dormancy (Paidhungat *et al*., 2000). Once the spore is mature the mother cell lyses releasing it into the environment.

Spores can remain dormant for years but can rapidly germinate and resume vegetative growth in the presence of nutrients (reviewed in Moir, 2006; Setlow, 2014a; Moir and Cooper, 2015). *B. subtilis* encodes five paralogous germinant receptors that are produced in the forespore after engulfment is complete. Each receptor is composed of three subunits (A, B and C) that are thought to form a membrane complex in the inner forespore membrane (reviewed in Ross and Abel-Santos, 2010). The A subunits are polytopic membrane proteins that are only homologous to other A subunits from endospore formers. The B subunits represent a branch of the APC (Amino acid-Polyamine-organoCation) superfamily of membrane transporters (Wong *et al*., 2012). The C subunits are lipoproteins. These receptors are required to respond to specific nutrients (called germinants) in the environment. It is not known how the receptors sense or transduce this information but germinants trigger exit from dormancy. Germination is thought to begin by the release of monovalent ions from the core, followed by the release of Ca^{2+}-DPA through a putative channel complex in the inner spore membrane. These steps are followed by the degradation of the spore cortex by two partially redundant spore cortex lytic enzymes that specifically target the muramic delta lactam, leaving the germ cell wall intact (Popham and Bernhards, 2015). Ca^{2+}-DPA release and cortex degradation allow an influx of water and the transition from a desiccated phase-bright spore to a swollen phase-dark one. Spore re-hydration restores metabolism and the capacity for efficient macromolecular synthesis. Finally, the coat breaks open, resulting in outgrowth of the germinated spore into a vegetative cell. The exit from dormancy is a decision not to be taken lightly but also requires that dormant cells not miss opportunities to take advantage of scarce and limited nutrients.

Here, we performed a genetic screen to identify additional factors involved in the signal transduction pathways that lead to spore germination. Instead, we serendipitously discovered that > 25 sporulation mutants that are impaired in the synthesis of the spore protective layers or core dehydration trigger premature activation of the GerA germinant receptor during development. These observations led us to discover that a surprisingly large percentage of wild-type sporulating cells inappropriately trigger germination in a GerA-dependent fashion. We further show that the majority of these prematurely germinated spores are inviable. Thus, our data suggest that the GerA receptor is highly sensitized to sense and respond to nutrients and the cost of this sensitivity is the loss of up to 8% of the sporulating population as a result of errors in morphogenesis.

Results

Many sporulation mutants are partially suppressed by the absence of the GerA receptor

In an attempt to identify novel factors that function in the signaling pathways that trigger spore germination, we used transposon-sequencing (Tn-seq) (van Opijnen and Camilli, 2013) to screen for genes that become critical for germination when sporulating cells rely on a single germinant receptor. Cells lacking the three principal germinant receptors GerA, GerB and GerK have a > 1000-fold reduction in spore germination on LB agar plates (Paidhungat *et al*., 2000). However, as long as either the GerA or GerK receptor is present, germination is ~ 80% of wild-type levels. Accordingly, to identify factors that act specifically in the GerA signaling pathway, we screened for transposon insertions that had strong germination defects in a strain in which GerA was the only functional receptor. Although the two minor germinant receptors encoded in the *ynd* and *yfk* operons are not known to be critical for germination (Paidhungat *et al*., 2000), our 'GerA-only' strain had loss of function mutations in the *gerB*, *gerK*, *ynd* and *yfk* operons (referred to as Δ4 *gerA*$^+$). Similarly, we screened for factors specific to the GerK signaling pathway using a strain in which GerK was the only functional receptor (Δ4 *gerK*$^+$). In both strains, the genes encoding the components that resemble permeases (the 'B' subunits) (Cooper and Moir, 2011) of these receptors were deleted (specifically,

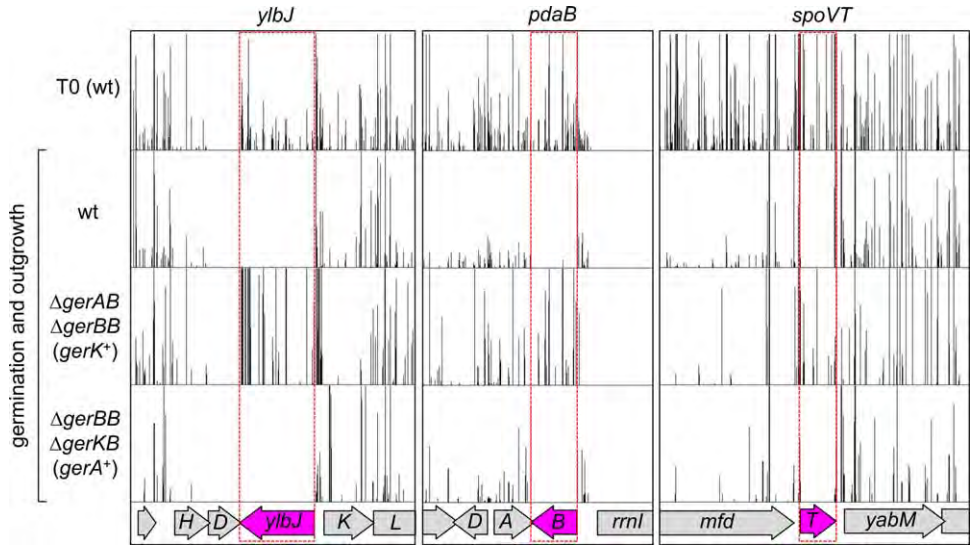

Fig. 1. Transposon insertions were over-represented in genes required for sporulation in the absence of a functional GerA receptor. Transposon insertion profiles from three different regions of the genome are depicted. Mariner-based transposon libraries from the indicated strains were grown in Difco sporulation medium (DSM) until nutrient exhaustion. A sample was saved from the wild-type (wt) library at the onset of starvation (T0). The cultures were sporulated for 24 h. Vegetative cells and mutants defective in spore formation were killed by heat treatment at 80°C for 20 min. The spores were germinated and outgrown on LB agar and pooled. The transposon insertion sites were identified by deep sequencing and mapped to the *B. subtilis* 168 reference genome. Boxes highlight *ylbJ*, *pdaB* (*B*) and *spoVT* (*T*) (purple) that are significantly enriched ($P < 0.005$) for transposon insertions in the Δ*gerAB* Δ*gerBB* (*gerK*$^+$) compared to WT and to Δ*gerBB* Δ*gerKB* (*gerA*$^+$). Both strains lacking germinant receptors also harbor mutations in *yndB* and *yfkB* encoding subunits of minor germinant receptors. The height of each line represents the number of the sequencing reads at this position. The maximum number of reads depicted was 200 for the *ylbJ* and *spoVT* genomic regions and 300 for the *pdaB* region.

Δ*gerAB*, Δ*gerKB*, Δ*gerBB*, Δ*yndB* and Δ*yfkB*). Saturating transposon libraries were constructed in wild-type and in the two quadruple mutants. At the onset of starvation (T0), a sample was removed from the wild-type library and the three cultures were allowed to exhaust their nutrients and sporulate over the next 24 h (T24). The cultures were then incubated at 80°C for 20 min to kill vegetative and sporulation-defective cells and plated on LB agar. More than 500,000 colonies from germination proficient spores were pooled from each library and the transposon insertions were mapped by deep sequencing (see 'Experimental procedures' section). The transposon insertion profiles from the wild-type library and the Δ4 *gerA*$^+$ and Δ4 *gerK*$^+$ libraries were compared to each other and to the wild-type library harvested at T0.

As expected, transposon insertions in all three genes in the *gerA* operon were significantly under-represented in the Δ4 *gerA*$^+$ library (Supporting Information Fig. S1). Similarly, insertions in the *gerK* operon were largely absent in the Δ4 *gerK*$^+$ library. Consistent with the idea that the germinant receptors do not work with pathway-specific signaling proteins that are critical for germination (Li *et al*., 2014), there were no additional genes in which transposons were specifically under-represented in one but not the other library. However, examination of the transposon insertion profiles identified an unanticipated set of genes in which insertions were over-represented in the Δ4 *gerK*$^+$ library compared to the wild-type and Δ4 *gerA*$^+$ libraries (Fig. 1 and Supporting Information Fig. S2). Three particularly clear examples are shown in Fig. 1. The *ylbJ* gene encodes a polytopic membrane protein of unknown function that is induced during sporulation under the control of the mother cell transcription factor SigE (Eichenberger *et al*., 2003). PdaB (also known as SpoVIE) is a peptidoglycan deacetylase involved in spore cortex synthesis and is also produced in the mother cell under SigE control (Fukushima *et al*., 2004; Silvaggi *et al*., 2004). SpoVT is a late-acting forespore transcription factor (Bagyan *et al*., 1996). Loss-of-function mutations in all three genes have been reported to reduce sporulation efficiency. Consistent with these data, in the wild-type library, transposon insertions in all three genes were present at the time of starvation (T0) but were virtually absent after sporulation, heat treatment, germination and outgrowth (Fig. 1). By contrast, in the Δ4 *gerK*$^+$ strain, insertions in these genes appeared to have no impact on sporulation and/or germination. In other words, the absence of one or several of the germinant receptors appeared to suppress the sporulation/germination defects of Δ*ylbJ*, Δ*pdaB* and Δ*spoVT*. Moreover, transposon insertions in *ylbJ* and several other hits in the screen appeared to be over-represented compared to the wild-type library at T0 (Fig. 1 and Supporting Information Fig. S2).

In total, we identified 37 genes in which transposon insertions were over-represented in the Δ4 *gerK*$^+$ library compared to the wild-type and Δ4 *gerA*$^+$ libraries at T24 (Table 1 and Supporting Information Fig. S2). Mutations in 23 of them (including *ylbJ*, *pdaB* and *spoVT*) have been reported to result in sporulation defects. Mutations in 9 have recently been found to delay but not block spore maturation (Meeske *et al*., 2016). Finally, five of the genes were not previously reported to impact sporulation. As expected, the transposon insertion profiles in the wild-type library for these last two groups of genes were not significantly different between T0 and T24. However, insertions in these genes were over-represented in the Δ4 *gerK*$^+$ library, similar to the pattern observed for *ylbJ* (Supporting Information Fig. S2). Among the 37 genes identified, 28 have been demonstrated or are predicted to be expressed under sporulation control (Eichenberger *et al*., 2004; Nicolas *et al*., 2012; Arrieta-Ortiz *et al*., 2015), of which more than a third are in the SigE regulon. Most of those that have been characterized have been implicated in the synthesis of the spore envelope layers (the coat and cortex) or are involved in spore core dehydration.

Interestingly, previous work from Setlow and colleagues has shown that cells lacking the three principal germinant receptors (GerA, GerB and GerK) suppress the sporulation defect of a Δ*spoVF* mutant (Paidhungat *et al*., 2000). The *spoVF* locus is a bi-cistronic operon that is expressed in the mother cell under the control of SigK (Daniel and Errington, 2003). The two genes in this operon encode the enzymes responsible for the synthesis of dipicolinic acid (DPA) that contributes to spore core dehydration and wet heat resistance (Paidhungat *et al*., 2000). In our Tn-seq screen, insertions in the *spoVF* operon were significantly over-represented in the strain in which the GerA, GerB, Ynd and Yfk receptors were inactivated (Δ4 *gerK*$^+$) (Supporting Information Fig. S2). However, cells with a functional GerA receptor but lacking the other four (Δ4 *gerA*$^+$) did not display this suppression. In fact, the Δ4 *gerA*$^+$ (GerA-only) strain did not appear to suppress insertions at any genomic locus.

The GerA receptor is necessary and sufficient to enhance the sporulation defect of many sporulation mutants

To validate the suppression identified by Tn-seq, we began our analysis with the *ylbJ* gene. We combined a *ylbJ* null mutation with the Δ4 *gerK*$^+$ and Δ4 *gerA*$^+$ strains. Using the production of heat-resistant spores as our assay for sporulation efficiency, we compared these strains to wild-type and the Δ*ylbJ* mutant. As reported previously (Eichenberger *et al*., 2003; Meeske *et al*., 2016), cells lacking *ylbJ* were $>$ 1,000,000-fold reduced in sporulation efficiency compared to wild-type. Furthermore, a Δ*ylbJ* mutant with only a functional GerA receptor (Δ4 *gerA*$^+$) was similarly impaired (Supporting Information Table S1). However, and consistent with our Tn-seq data, cells with nonfunctional GerA, GerB, Ynd and Yfk receptors (Δ4 *gerK*$^+$) suppressed the Δ*ylbJ* sporulation defect by $>$ 15,000-fold sporulating at 0.6% efficiency (Supporting Information Table S1). It is unclear why the suppression was not complete as might have been anticipated from the over-representation of insertions in the *ylbJ* gene in the Δ4 *gerK*$^+$ library compared to wild-type at T0 (Fig. 1). One possible explanation is that germination is delayed in cells lacking four of the five germinant receptors and in the absence of *ylbJ*, germination occurred more quickly resulting in the over-representation of transposon insertions in it relative to other insertions in the library. However, our attempts to reconstitute this effect in mixing experiments have been unsuccessful. Nevertheless, the $>$ 15,000-fold suppression of Δ*ylbJ* mutant validated the screen and prompted further analysis.

Based on the data described above, we hypothesized that the presence of a functional GerA receptor enhances the sporulation defect of Δ*ylbJ*. To test this, we generated a series of strains with different combinations of receptor mutations and analyzed their sporulation efficiency in the presence and absence of *ylbJ*. As can be seen in Supporting Information Table S1, cells lacking the *B* gene from the *gerA* operon (Δ*gerAB*) or the entire *gerA* (Δ*gerA*) locus largely phenocopied the Δ4 *gerK*$^+$ strain, suppressing the Δ*ylbJ* mutation $>$ 15,000-fold. By contrast, the Δ*gerKB* mutation suppressed $\sim$ 200-fold, while the Δ*gerBB* mutation only 15-fold. These data and the absence of significant suppression in a strain in which GerA is the only functional receptor (Supporting Information Table S1) are consistent with the central role played by the *gerAB* mutation in suppressing the sporulation defects of the hits in our Tn-seq screen. To more rigorously test this idea, we generated in-frame deletions for four additional genes identified in our screen (*pdaB*, *spoVT*, *spoVFA* and *uppP*) and analyzed their sporulation efficiencies in the Δ4 *gerK*$^+$ strain (Supporting Information Table S2); in the absence of the GerA receptor (Δ*gerAB*); and in the strain in which GerA was the only functional receptor (Δ4 *gerA*$^+$). The strain lacking four of the germinant receptors including GerA (Δ4 *gerK*$^+$) and the one that lacked GerA (Δ*gerAB*) partially suppressed the sporulation defects of all four mutants (Supporting Information Table S2). The suppression ranged from 2.7- to 279-fold and was similar in the two backgrounds. Furthermore, in cells in which GerA was the only functional receptor, the sporulation efficiencies of the mutations were similar to those obtained in a wild-type background in which all five

Table 1. Genes with more transposon insertions in Δ4 (*gerK*+) compared to wild-type

Gene	*P*-value[a]	Fold change[b]	Description	% spo (*gerA*$^+$)[c]	% spo (Δ*gerA*)[c]	Fold suppression	Cytological suppression	Promoter(s)	Spo$^-$[e]	Delay[f]
araR	3.5×10^{-4}	12.3	Transcriptional repressor of arabinose utilization	37.5	38.8	1.03	No	SigA	Yes	Yes
asnO	1.9×10^{-9}	1.05×10^{3}	Asparagine synthase	0	0	–	No	SigE	Yes	Yes
ccdA	4.7×10^{-6}	265.2	Thiol-disulfide oxidoreductase (SpoVD activity)	2.3	10.6	4.61	Yes	SigH[d]	Yes	Yes
dacB	7.7×10^{-6}	99.8	D-Alanyl-D-alanine carboxypeptidase	3.6	7.1	1.97	Yes	Spo0A, SigE	Yes	Yes
gerPC	0.18	3.4	Spore coat assembly and/or permeability	11.1	34.7	3.13	Yes	SigK	No	No
pdaB (spoVIE)	6.0×10^{-4}	21.1	Polysaccharide deacetylase (spore cortex formation)	3.8	9.8	2.58	Yes	SigE	Yes	Yes
prkA	1.4×10^{-4}	14.5	Putative serine protein kinase	8.2	15.7	1.91	Yes	SigE	Yes	Yes
prkC	0.24	7.4	Serine protein kinase	44.3	52.9	1.19	Yes	SigA	No	No
prpC	0.015	13.0	Protein phosphatase (antagonist of PrkC)	35.7	47.8	1.34	Yes	SigA	No	Yes
qcrA	0.041	46.1	Menaquinol:cytochrome *c* oxidoreductase	ND	ND	ND	ND	AbrB, Spo0A, SigH[d]	Yes	Yes
rho	1.9	3.8	Transcription termination	78.9	93.6	1.19	Yes	SigA, SigH[d]	No	Yes
skfB	4.6×10^{-3}	5.3	Synthesis of spore killing factor	ND	ND	ND	ND	AbrB, Spo0A	No	No
skfC	1.7×10^{-3}	3.8	Synthesis of spore killing factor	44.5	60.2	1.35	No	AbrB, Spo0A	No	No
spmA	1.4×10^{-3}	32.1	Spore maturation protein (spore core dehydration)	15.6	23.7	1.52	Yes	Spo0A, SigE	No	Yes
spmB	0.021	13.6	Spore maturation protein (spore core dehydration)	ND	ND	ND	ND	Spo0A, SigE	No	Yes
spoVAA	1.9×10^{-4}	1.89×10^{3}	Uptake and release of Ca^{2+}:DPA	39.9	54.7	1.37	Yes	SigG	Yes	Yes
spoVAB	2.3×10^{-3}	1.55×10^{3}	Uptake and release of Ca^{2+}:DPA	ND	ND	ND	ND	SigG	Yes	Yes
spoVFA	0.49	15.4	Dipicolinate synthase (subunit A)	0.002	0.207	134.1	Yes	SigK	Yes	Yes
spoVFB	0.38	18.3	Dipicolinate synthase (subunit B)	ND	ND	ND	ND	SigK	Yes	Yes
spoVG	0.013	72.9	Pleitropic sporulation factor	16.3	33.4	2.05	Yes	SigH	Yes	Yes
spoVR	5.6×10^{-3}	7.3	Spore cortex synthesis	7.6	30.1	3.96	Yes	SigE	Yes	Yes
spoVS	0.039	101.0	Spore coat assembly/spore core dehydration	0.3	0.9	3.00	Yes	SigH, SigE	Yes	Yes
spoVT	3.6×10^{-3}	2.9	Transcriptional regulator of SigG-dependent genes	0.02	0.073	3.65	Yes	SigG	Yes	Yes
spoVIGA (*ytrH*)	0.017	79.1	Spore cortex/coat synthesis	11.7	24	2.05	Yes	SigE	Yes	Yes
spoVIGB (*ytrI*)	0.040	2.0	Spore cortex/coat synthesis	10.5	25.2	2.40	Yes	SigE	Yes	Yes
uppP	4.0×10^{-6}	27.4	Minor undecaprenyl pyrophosphate phosphatase	0.6	7.8	13.00	Yes	SigA	Yes	Yes
yabQ	0.075	4.4	Spore cortex synthesis	0.14	0.27	1.93	Yes	SigE	Yes	Yes
ydzQ	0.031	25.6	Unknown	ND	ND	ND	ND	GlnL, SknR, YdfI	No	Yes
yerC	0.043	56.0	Unknown	ND	ND	ND	ND	SigA	Yes	Yes
yfmI	5.9×10^{-8}	2.8	Similar to macrolide efflux transporters	62.3	67.4	1.08	Yes	AbrB, Spo0A	No	No
yhbH	0.054	9.0	Unknown; sporulation or germination	12	27.5	2.29	Yes	SigE	Yes	Yes
ylbJ	3.3×10^{-6}	2.89×10^{3}	Unknown	2×10^{-5}	0.33	1.66×10^{4}	yes	SigE	Yes	Yes
ypbH	0.031	3.5	Putative adaptor protein for ClpC–ClpP	18.9	39.1	2.07	Yes	SigK	No	Yes
ypmB (*tseB*)	0.040	88.0	Unknown	ND	ND	ND	ND	SigA	No	Yes
yqzK	0.030	35.8	Unknown	34.5	52	1.51	Yes	SigF	No	Yes
yrbG	0.44	5.0	Unknown	50.9	61.2	1.20	Yes	SigG	No	Yes
ytaF	0.47	8.8	Similar to Ca^{2+}-binding membrane transporters	32.9	48	1.46	Yes	SigK	Yes	Yes

a. Based on Mann–Whitney *U* test.
b. Fold-change in transposon insertions compared to the wild-type after sporulation, 80°C for 20 min, germination and outgrowth.
c. Sporulation efficiency (spo) was determined 30h after starvation and refers to heat-resistant (80°C for 20 min) colony forming units (CFU) of the null mutant compared to wild-type.
d. Predicted promoter(s).
e. Previously reported to have a sporulation defect.
f. Previously reported to be delayed in spore maturation.
ND, no determined.

receptors were intact. Taken together, these results indicate that the GerA receptor is both necessary and sufficient to enhance the sporulation defects of these mutations. Although the suppression was highly reproducible (Supporting Information Table S2), similar to our analysis for *ylbJ*, it was more modest than predicted from the Tn-seq screen (see 'Discussion' section).

To survey a larger number of hits from our screen, we tested whether the Δ*gerAB* mutation could suppress 22 additional mutants. In-frame deletions were generated from the *B. subtilis* null mutant collection and analyzed for sporulation efficiency in the presence and absence of a functional GerA receptor (Table 1). Eight of these mutants were suppressed by greater than twofold in the Δ*gerAB* background. Nine were suppressed between 1.3- and 2-fold. The remaining six mutants were suppressed by less than 1.3-fold. We conclude that the sporulation defects of a large set of genes are partially suppressed by the absence of the GerA receptor.

ΔgerAB *suppresses the cytological defects of the* ΔylbJ *mutant*

To further characterize the suppression mediated by Δ*gerAB*, we analyzed the Δ*ylbJ* mutant and the Δ*gerAB* Δ*ylbJ* double mutant by phase contrast microscopy. Wild-type, Δ*gerA* and Δ*ylbJ* single mutants and the Δ*gerAB* Δ*ylbJ* double mutant were induced to sporulate by nutrient exhaustion in liquid Difco sporulation medium (DSM) and analyzed in a 20-h time course. Representative images from four time points [hours 8, 12, 16 and 20 (T8–T20)] are shown in Fig. 2. The complete time course can be found in Supporting Information Fig. S3A. By hour 8, wild-type and the Δ*gerAB* mutant had phase-grey and phase-bright forespores inside mother cells. By hour 16, many phase-bright spores had been released through mother cell lysis. By contrast and as reported previously (Eichenberger *et al*., 2003; Meeske *et al*., 2016), sporulating cells lacking *ylbJ* appeared to be stalled in development, forming phase-grey forespores. By hour 16, the cultures appeared heterogeneous (Fig. 2) with phase-grey and larger phase-dark spores. By hour 20, a few spores remained phase-grey while most were phase-dark, had a dull grey appearance that we refer to as dull phase-grey, or looked hollow or empty (Fig. 2 and Supporting Information Fig. S3A). Strikingly, in the Δ*gerAB* Δ*ylbJ* mutant, the spores differentiated more uniformly to phase-grey and then maintained this state through hour 20. Based on sporulation efficiency (Table 1 and Supporting Information Table S1), only a small subset of the Δ*gerAB* Δ*ylbJ* spores in these fields are heat resistant; however, the absence of GerA largely suppressed the formation of phase-dark, dull-grey and hollow spores observed in the Δ*ylbJ* single mutant. This cytological suppression was even more pronounced on DSM agar plates (Supporting Information Fig. S3B). Furthermore, addition of nutrients to the two spore populations revealed that the Δ*gerAB* Δ*ylbJ* phase grey spores were capable of germination and outgrowth while the Δ*ylbJ* mutant spores were largely nonviable, even when the spores were not heat-treated (Supporting Information Fig. S4). Finally, analysis of a series of strains lacking one or several germinant receptors revealed that a functional GerA receptor was both necessary and sufficient to enhance the cytological defects and the viability of the Δ*ylbJ* mutant (Supporting Information Fig. S5 and Table S1).

ΔgerAB *suppresses the cytological defects of many sporulation mutants*

The dramatic cytological suppression of Δ*ylbJ* in cells lacking a functional GerA receptor prompted us to investigate whether Δ*gerAB* could suppress the cytological phenotypes of other mutants identified in our Tn-seq screen. We examined 28 mutants in the presence and absence of *gerAB* by phase-contrast microscopy at hour 24 of sporulation. With the exception of Δ*asnO*, Δ*skfC* and Δ*araR*, the remaining 25 mutants were appreciably suppressed in the absence of the GerA receptor (Table 1). Figure 3 shows representative images of four mutants (Δ*pdaB*, Δ*spoVFA*, Δ*spoVR* and Δ*spoVT*), and Supporting Information Fig. S6 shows larger fields of these 4 mutants and the other 22 mutants that were suppressed by Δ*gerAB*. In all cases, the single mutants were heterogeneous, with phase-dark, dull phase-grey and empty spores, while the Δ*gerAB* mutation suppressed these phenotypes with a larger population of phase-grey and/or phase-bright spores. As in the case of Δ*ylbJ*, a functional GerA receptor was both necessary and sufficient to enhance the cytological defects (Supporting Information Fig. S7). Notably, the suppression by Δ*gerAB* was incomplete indicating that the mutants do indeed impair spore maturation. However, the data clearly indicate that the morphological defects are more extreme when the GerA receptor is present.

Among the 29 mutants we analyzed by phase-contrast microscopy, six delayed spore maturation (*prpC*, *rho*, *spmA*, *yhbH*, *ypbH* and *yqzK*) (Meeske *et al*., 2016) and four were not previously reported to have a sporulation phenotype (*gerPC*, *prkC*, *skfC* and *yfmI*). Phase-contrast microscopy revealed that all but Δ*skfC* had clearly detectable cytological phenotypes. In all cases, these mutants had many phase-bright spores, as would be expected from their modest sporulation defects. However, an appreciable number of the spores in these cultures were phase-dark or appeared hollow

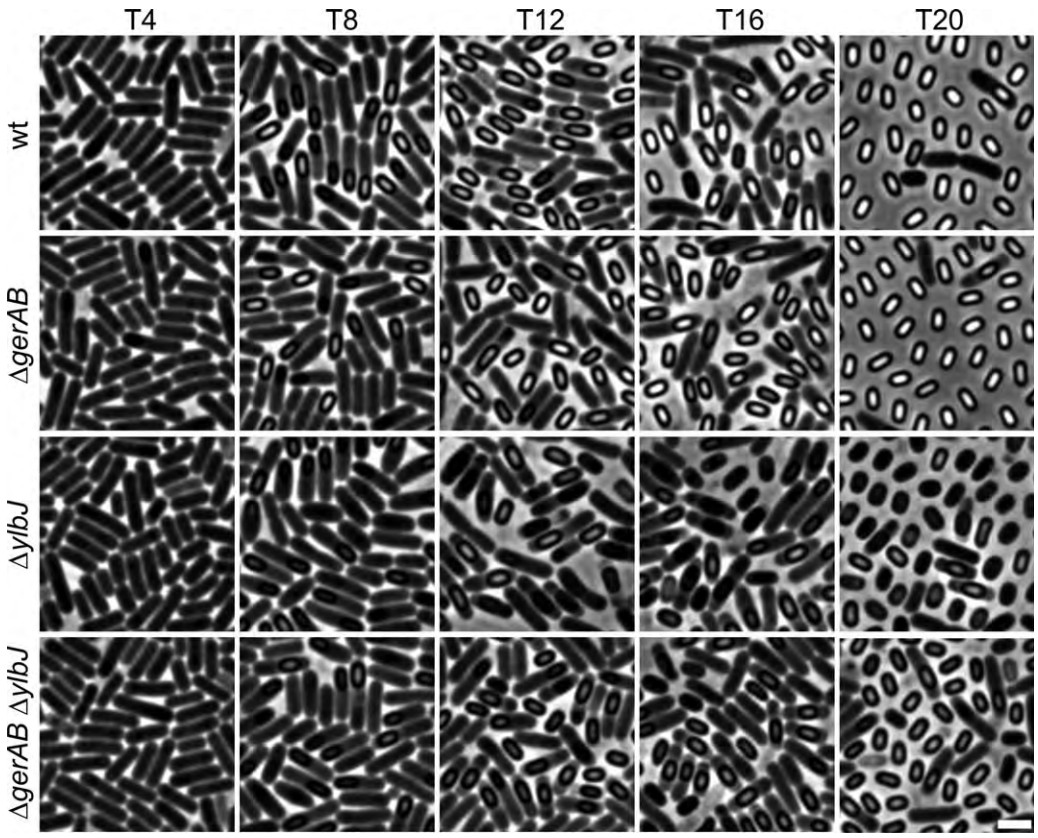

Fig. 2. Cytological suppression of Δ*ylbJ* in the absence of a functional GerA receptor.
Representative phase-contrast images of sporulating cells during a sporulation time course. Wild-type (wt), Δ*gerAB*, Δ*ylbJ* and the Δ*gerAB* Δ*ylbJ* double mutant were induced to sporulate by nutrient exhaustion at 37°C in liquid DSM. Images from 4, 8, 12, 16 and 20 h after the initiation of sporulation are shown. The complete time course can be found in Supporting Information Fig. S3A. Scale bar indicates 2 μm.

(Supporting Information Fig. S6). Importantly, the Δ*gerAB* mutation largely suppressed these phenotypes. Finally, we note that four of the mutants (Δ*prkC*, Δ*rho*, Δ*yfmI* and Δ*yrbG*) that displayed cytological suppression in the absence of *gerAB* were suppressed by 1.2-fold or less in sporulation efficiency (Supporting Information Fig. S6; Table 1) arguing that this small increase in sporulation efficiency reflects true suppression.

The mutant phenotypes described here and their suppression by Δ*gerAB* were not unique to the 168 background. We detected similar phenotypes and Δ*gerAB*-dependent suppression for Δ*ylbJ* and Δ*pdaB* in the PY79 background and in the undomesticated strain 3610 (Supporting Information Fig. S8). Collectively, these data indicate that the GerA receptor enhances the morphological defects of a large collection of sporulation mutants.

Inactivation of the spore cortex lytic enzymes suppresses the cytological defects of the mutants

A hallmark of spore germination is the transition from phase-bright to phase-dark spores. The presence of phase-dark spores in the mutants analyzed above and the reduction in this class of spores in the cells lacking a functional GerA receptor raised the possibility that morphological defects in spore development inappropriately trigger the GerA receptor leading to premature germination and the more severe phenotypes observed. Two effectors in the germination pathway that act downstream of the germinant receptors that are critical for the transition from phase-bright to phase-dark spores are the spore cortex lytic enzymes CwlJ and SleB (reviewed in Popham and Bernhards, 2015). Both proteins are synthesized during sporulation and packaged in the dormant spore. These cell wall degrading enzymes specifically target the muramic delta-lactam of the spore cortex peptidoglycan. Degradation of this protective layer allows the spore to take up more water leading to swollen phase-dark spores and ultimately exit from dormancy. If defects in spore maturation inappropriately trigger the GerA receptor and premature activation of the cortex lytic enzymes, then cells lacking CwlJ and SleB might similarly suppress the phenotypes of the mutants described above. Unlike Δ*gerAB*, Δ*cwlJ* Δ*sleB* double mutant spores are incapable of germinating (Ishikawa *et al.*, 1998), so we could not use

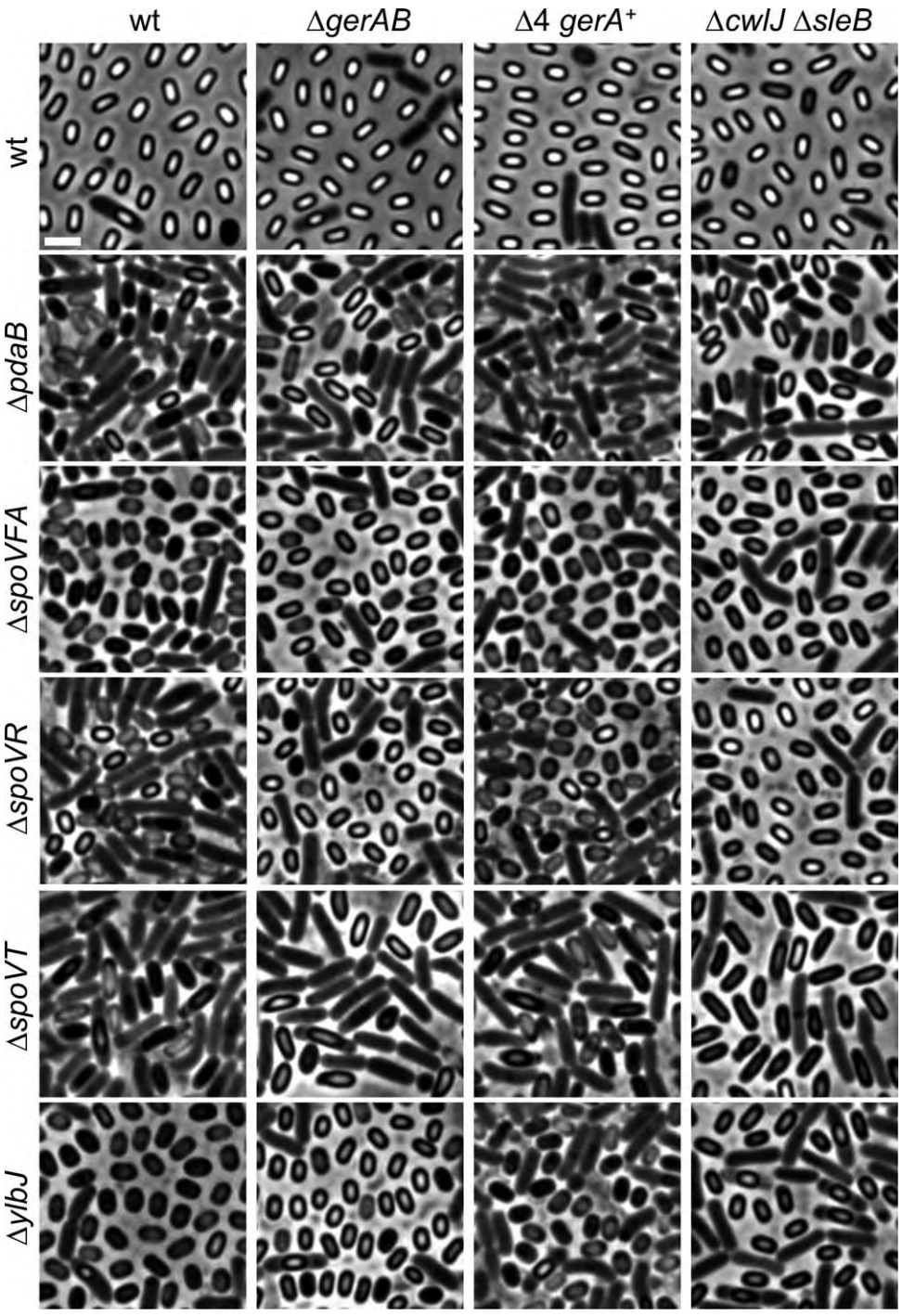

Fig. 3. The GerA receptor enhances the cytological defects of many sporulation mutants.
Representative phase-contrast images of cells sporulated by nutrient exhaustion at 37°C in liquid DSM for 24 h. The set of sporulation mutants (left column) and wild-type (wt) were analyzed in a strain lacking a functional GerA receptor (Δ*gerAB*), a strain in which GerA was the only functional receptor (Δ4 *gerA*$^{+}$) and a strain in which the two spore cortex lytic enzymes were absent (Δ*cwlJ* Δ*sleB*). Larger images of the strains in which the GerA receptor is absent (Δ*gerAB*) can be found in Supporting Information Fig. S6. Scale bar indicates 2 μm.

sporulation efficiency as our assay for suppression. Instead, we investigated whether the double mutant could suppress the cytological phenotypes of Δ*pdaB*, Δ*spoVFA*, Δ*spoVR*, Δ*spoVT* and Δ*ylbJ*. Strikingly, the Δ*cwlJ* Δ*sleB* double mutant phenocopied the Δ*gerAB* mutant, largely suppressing the cytological defects of the five mutants tested (Fig. 3). These results support the idea that defects in spore envelope and core maturation inappropriately trigger the GerA receptor leading to degradation of the cortex.

L-Alanine addition to sporulating cells triggers premature germination

To directly test whether GerA activation during sporulation contributes to the mutant phenotypes reported here

we sought to inappropriately activate GerA in wild-type sporulating cells during the stage when the protective layers are being assembled. To do so, we sporulated wild-type and the Δ*gerAB* mutant by nutrient exhaustion and at hour 4.5 of sporulation we split the cultures and added the germinant L-alanine or an equivalent amount of water to each and followed spore differentiation by phase-contrast microscopy. In the absence of germinant, wild-type cells differentiated into phase-bright spores over the next 3–5 h (Fig. 4). However, in the presence of L-alanine, the developing spores remained phase-grey for a longer period of time (Fig. 4). Approximately half of the spore population ultimately transitioned to phase-bright while the other half became phase-dark or appeared hollow. By contrast, the Δ*gerAB* mutant differentiated into phase-bright spores in the presence and absence of L-alanine, in a manner similar to wild-type cells in the absence of germinant. The sporulation efficiencies of the cultures as assayed by heat-resistant colony-forming units at hour 30 were consistent with the cytological analysis (Fig. 4). These data provide further support for the idea that defects in spore maturation inappropriately activate GerA leading to premature germination and spore lysis.

The GerA receptor triggers premature germination in wild-type sporulating cells

Our data suggest that defects in the assembly of the spore envelope layers and spore core dehydration trigger the GerA germinant receptor causing inappropriate germination. We wondered whether premature germination might be triggered in wild-type sporulating cells. To investigate this, we systematically compared fields of wild-type, Δ*gerA* and Δ*cwlJ* Δ*sleB* sporulating cells at hour 24 of sporulation. Consistent with the idea that the GerA receptor is triggered during sporulation of wild-type cells, we found that 8.4% of wild-type spores were phase-dark, dull phase-grey, or appeared empty or hollow (Fig. 5A). By contrast, 2% of the Δ*gerAB* spores had these phenotypes. None of the spores from the Δ*cwlJ* Δ*sleB* mutant appeared phase-dark or hollow, however, 5.9% had a grayish appearance with a dark halo suggesting that these spores may have triggered germination but were unable to complete the process. These results suggest that the GerA receptor prematurely triggers germination in a substantial proportion of the sporulating population of wild-type cells.

The GerA receptor triggers germination in different wild-type strains under different sporulation regiments

We wondered whether the premature germination phenotypes were unique to wild-type 168 cells sporulated in liquid medium. Accordingly, we analyzed spore-formation on sporulation agar plates. Wild-type 168 cells and the Δ*gerAB* mutant were spotted on DSM agar plates and allowed to sporulate at 37°C. To avoid edge effects, the cells were surrounded by additional spots of cells that competed for nearby nutrients (Supporting Information Fig. S9A). At hour 96, the entire spot was scraped and thoroughly mixed, and six fields from wild-type and Δ*gerAB* were visualized by phase-contrast microscopy. As was observed in liquid medium, 8.2% of the wild-type spores were phase-dark, dull phase-gray or appeared hollow, while $< 0.1\%$ of the Δ*gerAB* mutant had these phenotypes (Supporting Information Fig. S9B and Table S3). Similar results were obtained using the wild-type strain PY79 sporulated in liquid and on solid medium (Fig. 5B and Supporting Information Table S3) and the undomesticated wild-type strain 3610 sporulated on DSM agar and minimal medium (MSgg) agar that supports biofilm formation (Branda *et al.*, 2001) (Fig. 5C and Supporting Information Fig. S9B and Table S3). Although we cannot rule out the possibility that some of the spores germinated after release from the mother cell, visualization of wild-type cells in a sporulation time course on DSM agar plates and in liquid medium indicates that a significant percentage of the premature germination occurred during the process of differentiation (Supporting Information Figs S10 and S11). Recent studies in the bacterium *Bacillus cereus* suggest that this spore former might also trigger premature germination in the absence of the regulator SpoVT (Eijlander *et al.*, 2016) (see 'Discussion' section). Consistent with this idea, we found that 1.6% of a *B. cereus* wild-type spore population was dull phase-grey, phase-dark or hollow (Supporting Information Fig. S12 and Table S3). Collectively, these data suggest that errors in spore morphogenesis trigger GerA-dependent premature germination during sporulation and that inappropriate germination is likely to be a general feature of wild-type sporulating cells and not an idiosyncrasy of domestication.

Prematurely germinated spores are inviable or lack resistance properties

To investigate whether the phase-dark, dull phase-grey and hollow spores resulting from premature germination in wild-type cells retain their resistance properties, we monitored germination and outgrowth by time-lapse microscopy. Wild-type 168 spore preparations were treated at 80°C for 20 min and then incubated on an LB agar pad at 37°C and monitored using a Nikon TiE microscope. Within 30 min, virtually all of the phase-bright spores became swollen and phase-dark (Fig. 6A).

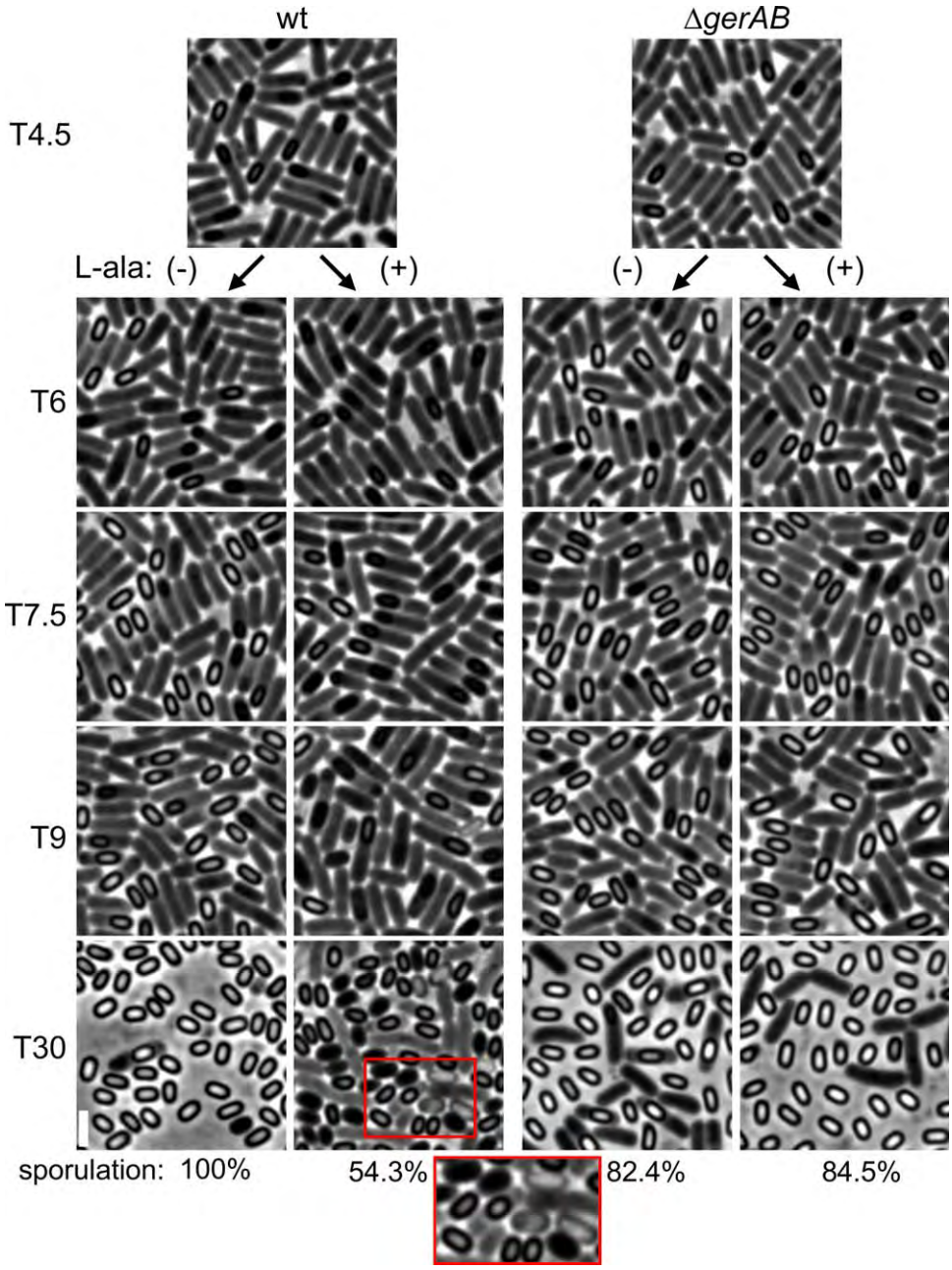

Fig. 4. Cytological phenotypes resulting from premature germination induced by L-alanine in sporulating cells. Representative phase-contrast images from a sporulation time course. Wild-type (wt) and cells lacking a functional GerA receptor (Δ*gerAB*) were induced to sporulate by nutrient exhaustion at 37°C in liquid DSM. 4.5 h (T4.5) after the initiation of sporulation, the cultures were divided in two. One set (+) was treated with L-alanine (L-ala; 30 mM, final concentration). An equal volume of ddH_2O was added to the other set (–). At the indicated time points (in hours), the four cultures were analyzed by phase-contrast microscopy. The sporulation efficiencies after 30 h are indicated below the images. A larger image (boxed in red) highlighting the phase-dark and phase-grey/hollow spores resulting from premature germination is shown. Scale bar indicates 2 μm.

We note that on this microscope the germinated phase-dark spores have a greyish appearance. By hour 1.5, spore outgrowth had begun and continued over the next 1.5 h (Fig. 6A). Consistent with the idea that the prematurely germinated spores are sensitive to heat, the dull phase-grey spores present at the start of the experiment failed to grow out over the 3-h experiment. To analyze a larger population of prematurely germinated spores, we used density gradient centrifugation to separate phase-bright and phase-dark/grey and hollow spores (Supporting Information Fig. S13). The prematurely germinated spores were $>95\%$ pure with a small percentage of vegetative cells and contaminating phase-bright spores (Fig. 6B and Supporting Information Fig. S13). We analyzed this purified fraction in an outgrowth time course. The purified phase-dark, dull phase-grey and hollow spores were incubated with LB medium and then monitored every 30 min by phase-contrast microscopy. In the absence of heat treatment, a subpopulation of phase-dark spores initiated outgrowth after $\sim$ 1 h (Fig. 6B and Supporting Information Fig. S14). This subpopulation appeared to continue growing over the next 2 h.

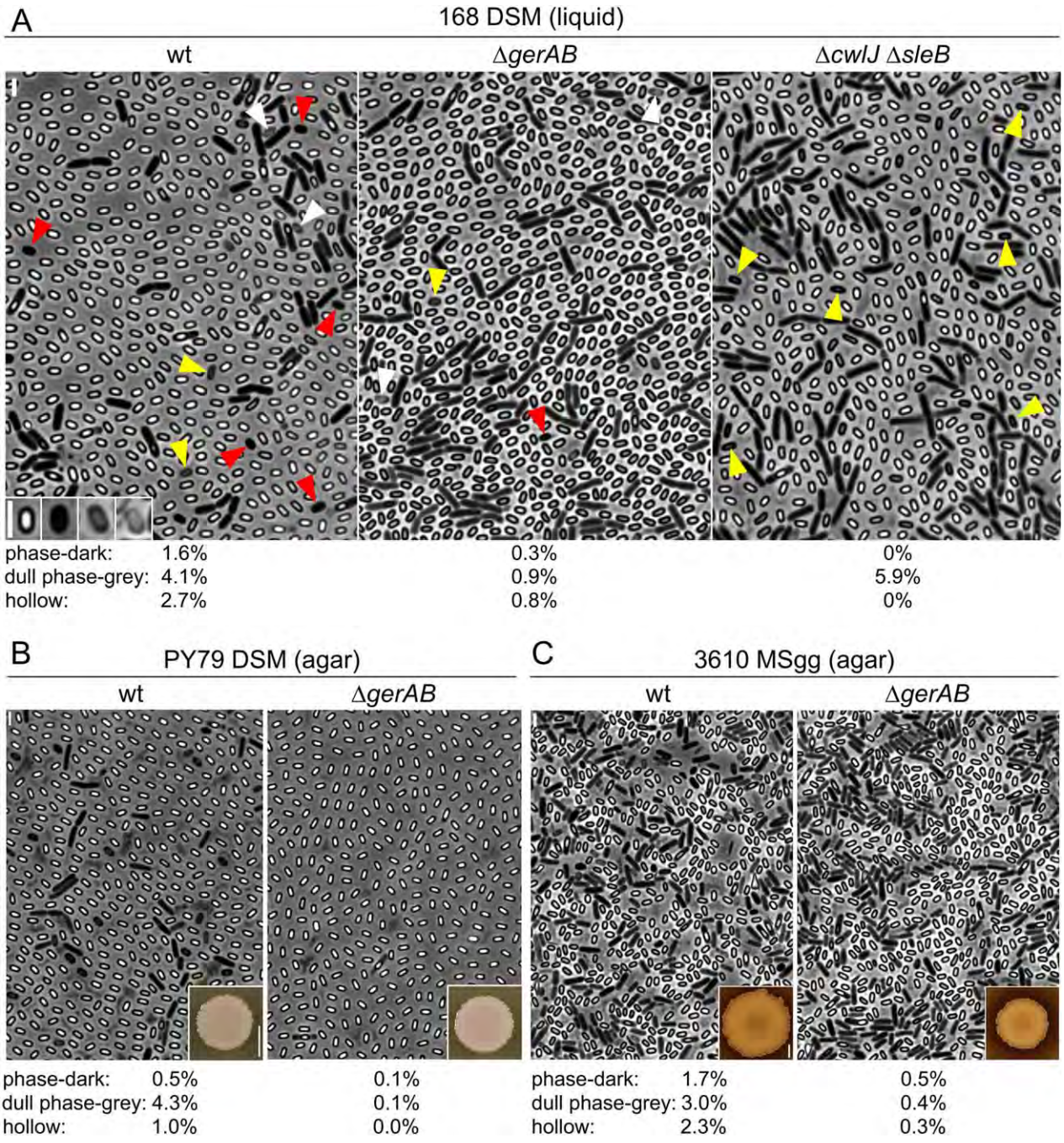

Fig. 5. GerA-dependent premature germination in a subset of wild-type sporulating cells.
A. Representative phase-contrast images of sporulated cells. Wild-type (wt), Δ*gerAB* and Δ*cwlJ* Δ*sleB* mutants of *B. subtilis* 168 were induced to sporulate by nutrient exhaustion at 37°C in liquid DSM. After 24 h, the cells were imaged by phase-contrast microscopy and spore phenotypes were quantified. > 3000 spores were scored for each strain. Examples of phase-dark (red carets), dull phase-gray (yellow carets) and hollow (white carets) spores are highlighted. A representative phase-bright, phase-dark, dull phase-grey and hollow spore is shown (insert). For the Δ*cwlJ* Δ*sleB* mutant, phase-grey spores with dark halos (yellow carets) are highlighted. Scale bars indicate 2 μm.
B. Wild-type (wt) and the Δ*gerAB* mutant from *B. subtilis* PY79 were sporulated on solid DSM agar. After 96 h at 37°C, one spot (insert) was scraped, washed in 1× PBS and then visualized by phase-contrast microscopy. The spore phenotypes were quantified as in (A). > 2000 spores were scored for each strain. Scale bars indicate 2 μm (spores) and 5 mm (spots of sporulating cells).
C. Wild-type (wt) and Δ*gerAB* cells of *B. subtilis* 3610 were grown sporulated on solid MSgg agar. After 96 h at 37°C, one spot (insert) was scraped, resuspended in 1× PBS, disrupted by sonication and washed three times before visualization by phase-contrast microscopy. The spore phenotypes were quantified as in (A). > 2000 spores were scored for each strain. Scale bars indicate 2 μm (spores) and 1 cm (spot of biofilm).

Importantly, a large percentage of the prematurely germinated spores remained dull phase-grey in appearance for the entire 3-h incubation. Thus, the vast majority of prematurely germinated spores were inviable. Furthermore, and as expected, virtually all of the prematurely germinated spores failed to grow out after incubation at 80°C for 20 min (Fig. 6B and Supporting Information Fig. S14). We suspect that the few spores that transitioned to vegetative growth after heat treatment were contaminating phase-bright spores in the purified fraction. Collectively, these results indicate that the majority of spores that trigger GerA-dependent premature germination lose viability or their resistance properties.

Discussion

Here, we have shown that a large set of mutants that are predicted to impact the cortex, the coat, or spore core dehydration trigger GerA-dependent germination during spore morphogenesis. Furthermore, we report that a surprisingly large population of wild-type cells activate GerA during sporulation triggering premature germination and loss of resistance properties or viability. Importantly, premature germination was observed in domesticated and undomesticated wild-type strains sporulated in liquid and on solid media. These data suggest that maintaining a germinant receptor that sensitively detects and responds to L-alanine has been positively selected despite the repeated loss of a subset of the sporulating population from premature germination. Based on the types of mutants that increased premature germination, we propose that errors or mistakes that are made during morphogenesis trigger premature germination in wild-type cells.

It remains an open question why the GerA receptor and not the other two principal receptors GerB and GerK is so prone to activation. Quantitative immunoblots indicate that the levels of the GerA receptor in the spore are at most twofold higher than that of GerB or GerK (Stewart and Setlow, 2013), arguing against the idea that GerA levels can account for this GerA-specific phenomenon. We envision three possible explanations for this specificity that are not mutually exclusive. In the first, GerA is intrinsically more sensitive than GerB and GerK (Venkatasubramanian and Johnstone, 1993). This sensitivity would ensure a rapid response by the dormant spore to low concentrations of L-alanine and would suggest that L-alanine serves as the principal signal for nutrient availability. In this scenario, defects to the envelope layers or a failure to dehydrate the core could inappropriately trigger the receptor in an L-alanine-dependent or -independent manner. Thus, inappropriate activation would be the cost of this heightened sensitivity. It is noteworthy that *B. subtilis* and other endospore formers encode a sporulation-specific alanine racemase (AlrB) that is thought to reduce the sensitivity of cells to L-alanine by converting it into D-alanine. Although a Δ*alrB* mutant causes premature germination in *Bacillus anthracis* (Chesnokova *et al.*, 2009), the analogous mutant in *B. subtilis* had no impact on the phenotypes reported here (Supporting Information Fig. S15) (Kanda-Nambu *et al.*, 2000). In the second model, defects due to mutations or errors in morphogenesis could result in metabolites (derived from mother cell or forespore) gaining access to the intermembrane space. The concentration of these metabolites is not known but based on analysis in *E. coli* (Yuan *et al.*, 2006) the concentration of L-alanine is likely to be higher than the germinants (asparagine, glucose, fructose, K^+) required to activate GerB and GerK (Venkatasubramanian and Johnstone, 1993). In the third model, the L-alanine in the stem peptides of the spore peptidoglycan might be the source of the inducing signal. Approximately 50% of these peptides are removed in the formation of muramic delta lactams in the spore cortex (Gilmore *et al.*, 2004). If endopeptidases act upon these peptides, L-alanine could be liberated in the intermembrane space. In the context of this model, mutations or errors in morphogenesis could lead to a rise in the L-alanine concentration to a level sufficient to trigger GerA. Unfortunately, it is currently not possible to test this model because removal of the stem peptides is required to generate the substrate for SleB and CwlJ (Popham *et al.*, 1996). Accordingly, mutations that block cortex maturation prevent its degradation during germination. Future experiments will be directed at distinguishing among these models.

Our findings that many sporulation mutants lose viability during differentiation by triggering the GerA-dependent germination pathway have interesting parallels with a newly discovered pathway that becomes activated when the spore coat is improperly assembled (Tan *et al.*, 2015). Defects in coat assembly were recently shown to trigger the degradation of the SpoIVA protein that forms the basement layer of this multiprotein structure. In situations where these defects are due to mutations and are therefore chronic, this degradation pathway causes loss of spore viability. Based on these findings, it was proposed that this pathway functions to eliminate mutants with mild sporulation defects that might accumulate in the population. Although the loss of spore viability in response to mutations in spore morphogenesis that we report here could similarly serve to cleanse the genome, we suspect this was not the evolutionary driving force for either pathway. In the case of coat assembly, we propose that SpoIVA degradation is a quality control mechanism that allows the cell to correct errors in the assembly of the SpoIVA layer. Since SpoIVA polymers, once assembled, appear to be inert

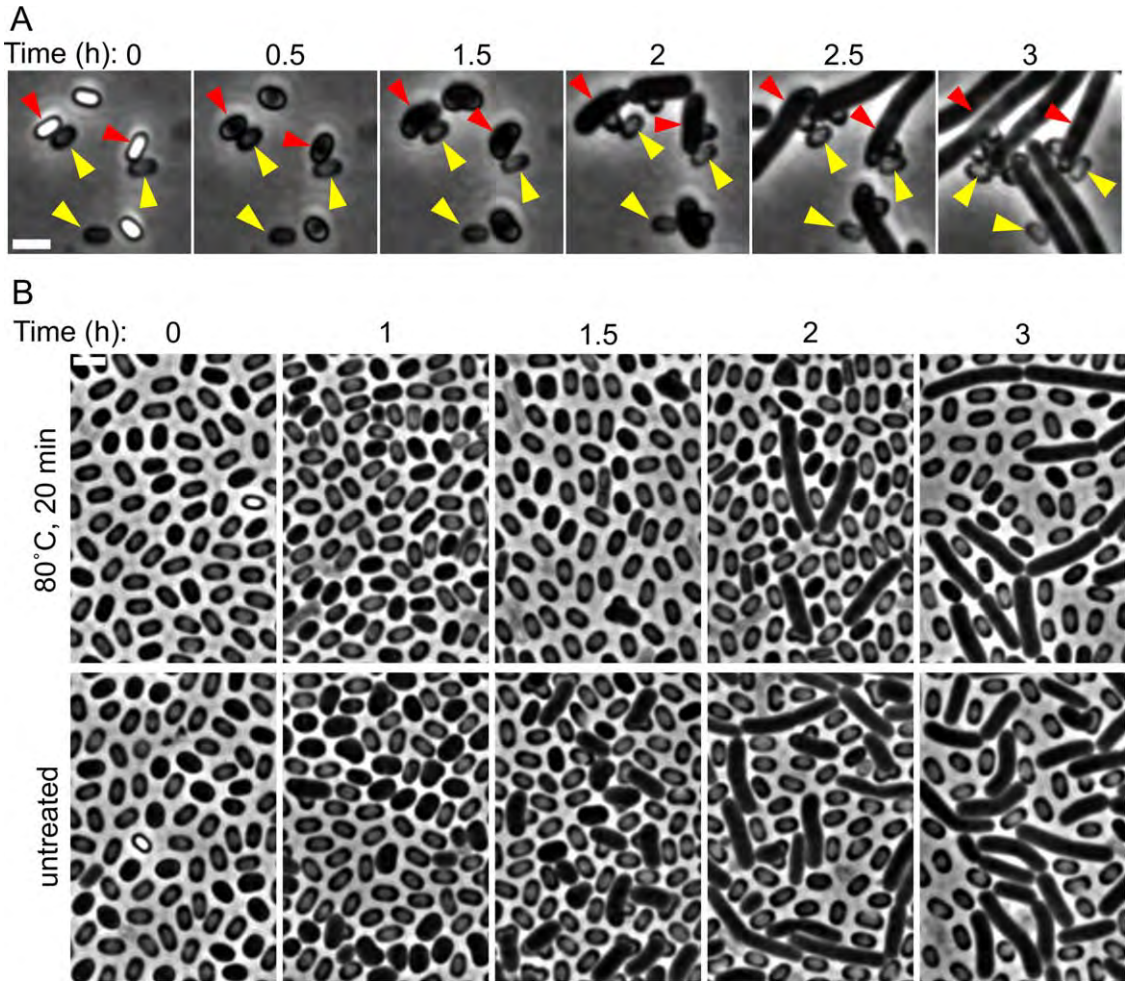

Fig. 6. Prematurely germinated spores are inviable or lack resistance properties.
A. Representative time-lapse microscopy of spores induced to germinate and outgrowth. Spore germination and outgrowth was monitored on a LB agarose pad at 37°C by phase-contrast microscopy. Phase-bright spores (red caret) germinate (T0.5) and initiate outgrowth (T1.5). Phase-grey spores (yellow carets) fail to grow during the 3-h experiment.
B. Purified prematurely germinated spores from wild-type were heat-treated (80°C for 20 min) or left untreated and then resuspended in 2 ml of LB. Spores were incubated at 37°C and then visualized by phase-contrast microscopy at the indicated times (Larger fields can be found in Supporting Information Fig. S14). Virtually, all prematurely germinated spores were sensitive to heat and most failed to grow during the 3-h experiment. Scale bar indicates 2 μm.

(Ramamurthi and Losick, 2008; Castaing *et al.*, 2013), a mechanism to degrade off-pathway assembly products might be necessary for these corrections. In the case of GerA, as we have argued above, we suspect that the evolutionary pressure to respond quickly to nutrients in the environment outweighed the loss of 2–8% of the spore population due to errors. Nonetheless, the ability to remove modestly deleterious mutations from the genome seems to be an added benefit of both pathways.

Another outcome of our study is a new appreciation that the primary defects of many sporulation mutants are more modest than previously reported. This is best exemplified by the *ylbJ* mutant, which sporulates at 0.6% rather than at 0.00002% when the GerA receptor is absent. Similarly, mutations in *pdaB*, *uppP*, *spoVT*, *spoVR* and others all have more modest phenotypes in a Δ*gerAB* background. A more detailed study of all of these mutants in a strain that cannot induce premature germination has the potential to shed new light on the specific roles of these factors in spore morphogenesis. Reciprocally, our discovery that >25 mutations inappropriately activate GerA suggests a functional link among their gene products. A deeper understanding of the mechanism that triggers GerA activation could reveal the common defect shared by these mutants.

Finally, we note that several of the genes we identified in our Tn-seq screen had previously been found to have unusual or unexplained phenotypes that can now be interpreted in the context of our data. For example, developing spores lacking *pdaB* (*spoVIE*) had been reported

to transition from phase-grey to phase-bright and then back to phase-grey or phase-dark (Fukushima *et al.*, 2004). Based on the data presented here, the transition from phase-bright back to grey/dark is likely due to the inappropriate activation of GerA and exit from dormancy. Similarly, mutations in the *spoVT* gene in *B. cereus* were recently shown to display phenotypes that suggested premature germination (Eijlander *et al.*, 2016). We suspect that, as in the case in *B. subtilis*, this mutant inappropriately triggers spore germination by one or several of the germinant receptors in *B. cereus*. It will be interesting to see if this response is also mediated by the L-alanine receptor (Barlass *et al.*, 2002). Lastly, Popham and coworkers have reported that *B. subtilis* spores lacking *spmA* or *spmB* appear to germinate faster than wild-type (Popham *et al.*, 1995) and in *Clostridium perfringens* these mutants are unstable (Orsburn *et al.*, 2008). We wonder whether the morphological defects caused by these mutants prime the GerA receptor to respond even more rapidly to nutrient exposure. We suspect that the ability to germinate faster can similarly explain why transposon insertions were over-represented in so many of the genes identified in our Tn-seq screen in the Δ4 *gerK*$^+$ library compared to the wild-type library.

In summary, we have discovered that the germinant receptor GerA is poised on a knife's edge and triggers premature germination in a significant portion of wild-type cells leading to spores lacking resistance properties or viability. We hypothesize that GerA is activated by errors in spore morphogenesis and the challenge for the future is to define what these errors are and how they activate GerA.

Experimental procedures

General methods

B. subtilis strains were derived from 168, PY79 or 3610. Sporulation in liquid medium was induced at 37°C by nutrient exhaustion in supplemented DS medium (DSM) (Schaeffer *et al.*, 1965) or by resuspension according to the method of Sterlini–Mandelstam (Sterlini and Mandelstam, 1969). For sporulation on solid media, strains were grown in DSM or MSgg to an OD_{600} of 0.5 and 5 μl were spotted on DSM or MSgg agar plates. Plates were incubated for 96 h at 37°C. Sporulation efficiency was determined in 24–30 h cultures as the total number of heat-resistant (80°C for 20 min) colony-forming units (CFUs) compared to wild-type heat-resistant CFUs. Insertion-deletion mutants were from the *Bacillus* knock-out (BKE) collection (Koo *et al.*, 2017) or were generated by isothermal assembly (Gibson, 2011) of PCR products followed by direct transformation into *B. subtilis*. All BKE mutants were back-crossed twice into *B. subtilis* 168 before assaying and prior to antibiotic cassette removal. Antibiotic cassette removal was performed using a temperature-sensitive plasmid that constitutively expresses Cre recombinase (Meeske *et al.*, 2015). Unless otherwise indicated, *B. subtilis* strains were constructed using genomic DNA and a 1-step competence method. Tables of strains (Supporting Information Table S5) and oligonucleotide primers (Supporting Information Table S6) and a description of strains constructions can be found in Supplemental Material.

Transposon insertion sequencing (Tn-seq)

Transposon insertion sequencing (Tn-seq) was performed on independently generated libraries as described previously (Meeske *et al.*, 2016). Briefly, transposon libraries were generated in wild-type, Δ4 *gerA*$^+$ and Δ4 *gerK*$^+$ strains in which GerA or GerK was the only functional germinant receptor. The libraries were washed in DSM and diluted into 50 ml DSM at an OD_{600} of 0.05. Samples were harvested at the onset of starvation (T0) and 24 h later (T24). The T24 samples were incubated at 80°C for 20 min and plated on LB agar. Approximately 500,000 colonies from the germinated spores were pooled. Genomic DNA (gDNA) was extracted from the samples and digested with MmeI, followed by adapter ligation. Transposon-chromosome junctions were amplified in 16–18 PCR cycles. PCR products were gel-purified and sequenced on the Illumina HiSeq platform using TruSeq reagents (Tufts University TUCF Genomics facility). Reads were mapped to the *B. subtilis* 168 genome (NCBI NC_000964.3), tallied at each TA site, and genes in which reads were statistically underrepresented were identified using the Mann–Whitney *U* test. Visual inspection of transposon insertion profiles was performed using Artemis software (version 16.0; Sanger Institute, Hinxton, Cambridge, CB10 1SA, UK). Some of the genes in Table 1 were chosen for analysis based on visual inspection of the insertions profiles despite having high *P*-values.

Microscopy

Sporulating cells and spores were collected by centrifugation at 6500 × *g* for 1 min and immobilized on 2% agarose pads. Phase-contrast microscopy was performed using an Olympus BX61 microscope equipped with an UplanF1 100× phase contrast objective. Time-lapse microscopy was performed using a Nikon TE2000 inverted microscope with a Nikon CFI Plan Apo VC 100× objective. LB agarose pads were placed on a H401 plate, where temperature was maintained at 37°C with an H401-T-Single temperature controller (Okolab, Burlingame, CA 94010, USA). Images were acquired every 15 min. Image analysis and processing were performed using MetaMorph software (version 7.7; Molecular Devices, Sunnyvale, CA 94089, USA).

Spore scoring

Four to six fields of sporulating cells (400–800 spores/field) were analyzed per strain. The number of phase-bright, phase-dark, dull phase-grey and hollow spores was determined using the Manual Count Objects command in MetaMorph software. For sporulation induced on DSM agar plates, the entire spot of sporulated cells was scraped, washed three times with 1× PBS and analyzed by phase-contrast microscopy. For sporulation induced on MSgg agar plates, the complete colony was scraped, washed three times with 1× PBS and the biofilm was disrupted by sonication (two rounds of 15 1-s pulses with an amplitude of 80% and 30 s of incubation on ice between the rounds). The cell suspension was washed three more times with 1× PBS before visualization by phase-contrast microscopy.

Spore preparation and purification

Spores produced by nutrient exhaustion on DSM agar plates were harvested after 96 h of incubation at 37°C and suspended in 5 ml of ddH_2O. Spores from two agar plates were pooled washed three times with ddH_2O and then resuspended in 200 μl of 20% Histodenz. The spore suspension was then loaded on top of 1 ml of 50% Histodenz and centrifuged at 16,000 × g for 30 min to separate phase-dark, dull phase-grey and hollow spores from dormant phase-bright spores. Both fractions were isolated, washed five times with 1× PBS and resuspended in 100 μl of 1× PBS.

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