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Identification and characterization of the *Bacillus subtilis* spore germination protein GerY

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ABSTRACT In response to starvation, endospore-forming bacteria differentiate into stress-resistant spores that can remain dormant for years yet rapidly germinate and resume growth when nutrients become available. To identify uncharacterized factors involved in the exit from dormancy, we performed a transposon-sequencing screen taking advantage of the loss of spore heat resistance that accompanies germination. We reasoned that transposon insertions that impair but do not block germination will lose resistance more slowly than wild type after exposure to nutrients and will therefore survive heat treatment. Using this approach, we identified most of the known germination genes and several new ones. We report an initial characterization of 15 of these genes and a more detailed analysis of one (*ymaF*). Spores lacking *ymaF* (renamed *gerY*) are impaired in germination in response to both L-alanine and L-asparagine, D-glucose, D-fructose, and K^+. GerY is a soluble protein synthesized under σ^E control in the mother cell. A YFP-GerY fusion localizes around the developing and mature spore in a manner that depends on CotE and SafA, indicating that it is a component of the spore coat. Coat proteins encoded by the *gerP* operon and *gerT* are also required for efficient germination, and we show that spores lacking two or all three of these loci have more severe defects in the exit from dormancy. Our data are consistent with a model in which GerY, GerT, and the GerP proteins are required for efficient transit of nutrients through the coat to access the germination receptors, but each acts independently in this process.

IMPORTANCE Pathogens in the orders Bacillales and Clostridiales resist sterilization by differentiating into stress-resistant spores. Spores are metabolically inactive and can remain dormant for decades, yet upon exposure to nutrients, they rapidly resume growth, causing food spoilage, food-borne illness, or life-threatening disease. The exit from dormancy, called germination, is a key target in combating these important pathogens. Here, we report a high-throughput genetic screen using transposon sequencing to identify novel germination factors that ensure the efficient exit from dormancy. We identify several new factors and characterize one in greater detail. This factor, renamed GerY, is part of the proteinaceous coat that encapsulates the dormant spore. Our data suggest that GerY enables efficient transit of nutrients through the coat to trigger germination.

KEYWORDS sporulation, dormancy, germination, coat

Bacteria in the Bacillales and Clostridiales orders cause over a million infections annually and lead to significant financial losses in the food industry (1, 2). These pathogens can withstand diverse sterilization regimes by forming stress-resistant spores. While spores are metabolically inactive and can remain dormant for decades, they quickly resume growth upon exposure to nutrients, leading to food spoilage, food-borne illness, or severe diseases. Germination, the process by which spores exit dormancy, is a crucial target for combating these pathogens. Most spore-forming bacteria follow

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a common germination program involving a series of chemical steps catalyzed by a small set of conserved factors (3, 4). GerA family receptors in the spore membrane detect germinants, usually amino acids, sugars, or nucleosides. These receptors are ligand-gated ion channels and trigger ion release from the spore core upon detection of specific germinants (5). Ion release activates the SpoVA (Spo5A) transport complex, which releases dipicolinic acid (DPA) as a Ca^{2+} chelate from the spore (6, 7). Cell wall hydrolases, one of which is activated by DPA, then degrade the specialized peptidoglycan that encases the spore, enabling core rehydration, macromolecular synthesis, and resumption of growth (3, 4).

In addition to these broadly conserved factors that play central roles in spore germination, there is a set of genes that have been implicated in this process based on their requirement for efficient exit from dormancy. Only a subset of these factors has been characterized in detail. GerD is a lipoprotein that is required to cluster the germination receptors in the spore membrane (8). In its absence, germination occurs more slowly (9). SpoVAF and FigP form a membrane complex that releases ions in response to ion release by the germinant receptors (10, 11). Spores lacking this complex release ions more slowly and are delayed in the exit from dormancy. Finally, the *gerT* gene and the *gerP* locus that encode GerT and six GerP proteins (GerPA, PB, PC, PD, PE, and PF) are also required for efficient germination (12, 13). These proteins are produced in the mother cell and are part of the proteinaceous coat that surrounds the dormant spore (14). The current thinking is that these proteins enable efficient transit of nutrients across the coat, and in their absence, germination initiates more slowly. Recently, it was found that DUF421-containing proteins YetF and YdfS, which are produced in the developing spore, are required for normal germination kinetics (15). The molecular basis for the delay in germination in their absence is currently unknown. Similarly, studies focused on the YhcN family of spore lipoproteins that includes YhcN, YlaJ, YutC, and YrbB found these proteins to be required for timely germination (16, 17). The mechanism by which they promote efficient germination remains to be elucidated.

Finally, a recent high-throughput screen, in which spores from a transposon library were incubated with L-valine for 60 min and the spores that had not germinated were enriched by density gradient centrifugation, identified a collection of mutants that were delayed in germination (18). A subset of the factors identified in this screen was characterized further. Mutant spores lacking many of these genes appeared to reduce the levels of the germinant receptor GerA, which responds to L-valine. Thus, most of these factors were proposed to indirectly affect germination. Interestingly, although *gerD*, *spoVAF*, *figP* (*yqhR*), and the *gerP* locus were identified in this screen, *gerT* was not, suggesting the screen was not saturating.

Here, we performed a complementary transposon-insertion sequencing (Tn-seq) screen, in which we exposed spores from a transposon library to germinants and heat killed the germinated spores at various time points afterward. We then germinated the resistant spores on LB agar plates and collected the resulting colonies. Using this method, we identified a set of mutants with impaired germination that partially overlapped with the set reported in the original Tn-seq screen (18). We performed an initial characterization of 15 of these genes and a more detailed analysis of one (*ymaF*). We report that YmaF (renamed GerY) is a broadly conserved coat protein produced in the mother cell under SigE control. Spores lacking this coat protein are impaired in germination. We show that spores lacking several other inner and outer coat proteins have little impact on germination, arguing for more specialized roles for GerP, GerT, and GerY. Importantly, the removal of the spore coat largely suppressed the germination defects of Δ*gerP*, Δ*gerT*, and Δ*gerY* mutants. Our data are consistent with the model that GerY, like GerT and GerP, promotes efficient transit of germinants across the spore coat but that these factors act independently in this process.

MATERIALS AND METHODS

General methods

Bacillus subtilis strains were derived from the auxotrophic *trpC2* 168 strain (19). Sporulation was induced at 37°C by nutrient exhaustion in supplemented DS medium (DSM) (20) or by resuspension according to the method of Sterlini and Mandelstam (21). 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* knockout (BKE) collection (22) or were generated by isothermal assembly (23) and direct transformation into *B. subtilis*. All BKE mutants were backcrossed 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 (24). Tables of genes enriched for transposon insertions in the germination screens (Table S1), strains (Table S2), plasmids (Table S3), and oligonucleotide primers (Table S4), and a description of strains and plasmids construction can be found in the supplemental material.

Transposon-insertion sequencing

Tn-seq was performed as described previously (25). Briefly, a diverse transposon library was washed in DSM medium and diluted in 50 mL DSM to an OD_{600} of 0.03 and grown at 37°C. A sample was harvested at the onset of starvation (input), and the culture was allowed to sporulate for 36 h. The spore-containing culture was washed six times with 1× phosphate-buffered saline (PBS), resuspended in ddH_2O, heat activated at 70°C for 30 min, and then placed on ice for 20 min. The spore suspension was divided into three pools, each at OD_{600} of 2. Pool 1 was mixed with an equal volume of L-alanine (100 µM, final concentration). Pool 2 was mixed with an equal volume of AGFK (10 mM, final concentration of each component). Pool 3 was mixed with an equal volume of water. The mixtures with L-alanine and AGFK were divided into two glass test tubes and agitated at 37°C for 20 and 60 min (L-alanine) or 40 and 80 min (AGFK). The mixture lacking germinant was agitated at 37°C for 60 min. At the indicated times, each germination reaction was incubated at 80°C for 20 min to kill vegetative cells and germinated spores. The mixtures were then spread on LB agar plates. Furthermore, ~1,000,000 colonies from each condition were separately pooled. A fraction of the L-alanine (60 min) and AGFK (80 min) pools was used to inoculate 50 mL DSM. The resulting spores were subjected to a second round of 60-min (L-alanine) and 80-min (AGFK) germination, heat-kill, and plating. Genomic DNA (gDNA) was isolated from the pooled colonies from each condition. The gDNA was treated with the endonuclease MmeI, followed by adapter ligation. Transposon-chromosome junctions were PCR amplified in 18 cycles, and the 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 overrepresented were identified using the Mann-Whitney *U* test. Visual inspection of transposon insertion profiles was performed with the Sanger Artemis Genome Browser and Annotation tool (26). Some of the genes listed in Table S1 that were chosen for further characterization were based on visual inspection of the insertion profiles despite having a *P*-value >0.05 and/or a fold-enrichment of less than 3.

Spore preparation and purification

Spores generated by nutrient exhaustion on DSM agar plates were harvested after 96 h of incubation at 37°C. Spores were scraped off the plate, washed four times with ddH_2O, and resuspended in 350 µL of 20% Histodenz. The spore suspension was then loaded on top of 1 mL of 50% Histodenz in a microfuge tube and centrifuged at 16,000 × *g* for 35 min to separate dormant phase-bright spores from vegetative cells, germinated phase-dark spores, and cell debris. The pellet containing the denser dormant spores was

washed five times with ddH_2O and resuspended in 1 mL of ddH_2O. Spores were stored at 4°C and assayed for germination within 4 days.

Germination assays

Purified spores were diluted in ddH_2O to an OD_{600} of 1, heat activated at 70°C for 30 min followed by incubation on ice for 20 min. Furthermore, 100 µL of the spore suspension was transferred to a clear, flat-bottom 96-well plate, and an equal volume of L-alanine (100 µM, 1 mM, or 10 mM, final concentrations), AGFK (10 mM, final concentration of each component), or ddH_2O was added. The OD_{600} was recorded every 2 min for 3 h using an Infinite M Plex plate reader (Tecan). The 96-well plate was maintained at 37°C with constant agitation between measurements. Germination kinetics were analyzed in technical triplicate, and the values were averaged. All germination assays reported are representative of experiments that were performed on at least two biological replicates.

Chemical decoating of spores

Spores at an OD_{600} of 25 were resuspended in 1 mL decoating solution (0.1 M DTT, 10 mM Tris-HCl pH 8, 0.1 M NaCl, and 0.5% SDS) and incubated at 37°C for 90 min with vigorous vortexing at 15-min intervals (12). The spores were then pelleted and washed 10 times with ice-cold ddH_2O.

Microscopy

Sporulating cells induced by the resuspension method were collected by centrifugation at 8,500 × *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 and a monochrome CoolSnapHQ digital camera (Photometrics). Membranes were stained with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH; Molecular Probes) at a final concentration of 50 µM. Exposure times were 200, 400, and 1,000 ms for TMA-DPH, GFP, and YFP, respectively. Image analysis and processing were performed using MetaMorph software.

Immunoblot analysis

Purified spores were resuspended in 500 µL 1× PBS containing phenylmethylsulfonyl fluoride (1 mM final concentration). The spore suspension was transferred to 2 mL tubes containing Lysing Matrix B (MP Biomedicals) and chilled on ice. The spores were then ruptured mechanically using FastPrep (MP Biomedicals) at 6.5 m/s for 1 min. Immediately after lysis, 500 µL of 2× Laemmli sample buffer containing 10% β-mercaptoethanol was added to the tube. After centrifugation at 20,000 × *g* for 20 min, the supernatant was transferred to a fresh tube, and total protein was determined using a non-interfering protein assay (G-Biosciences). Protein concentrations were normalized, and ~10 µg of protein was separated by SDS-PAGE on 17.5% resolving gels, electroblotted onto Immobilon-P-membranes (Millipore), and blocked in 5% non-fat milk in 1× PBS-0.5% Tween 20. The blocked membranes were probed with anti-GerAA (1:5,000) (27), anti-GerBC (1:5,000) (28), and anti-GerKA (1:10,000) (28) diluted in 3% BSA in PBS with 0.5% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit antibodies (1:20,000) (Bio-Rad) and the Western Lightning ECL Reagents Kit (PerkinElmer). The figure showing the levels of the germinant receptors is representative of experiments that were performed on two biological replicates.

RESULTS

A screen for mutants that are delayed in spore germination

To investigate whether there were additional uncharacterized genes required for spore germination, we performed a transposon-sequencing (Tn-seq) screen taking advantage of the loss of heat resistance that accompanies the exit from dormancy. We reasoned

that after exposure to nutrients, spores with transposon insertions in genes that are required for efficient germination would remain heat resistant for a longer period of time compared to spores with insertions in unrelated genes. Accordingly, if a Tn mutant library of dormant spores is briefly exposed to nutrients and then immediately incubated at 80°C for 20 min, only the spores that had not yet germinated will survive. The surviving spores can then be enriched by plating on rich medium to promote germination and outgrowth.

We generated a mariner transposon library containing ~130,000 unique insertions (53% of all possible TA sites in the *B. subtilis* genome were hit ≥10 times). The library was sporulated by nutrient exhaustion in liquid sporulation medium. At the onset of starvation, a sample was removed, and the remaining culture was sporulated for 36 h. The resulting spores were washed and then incubated at 70°C for 30 min followed by 20 min on ice to promote synchronous spore germination. The culture was divided into three and separately mixed with ddH_2O, L-alanine (100 µM, final concentration), or a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (AGFK) at a final concentration of 10 mM for each component. The mixtures were then incubated at 37°C for 60 min (ddH_2O), 20 and 60 min (L-alanine), or 40 and 80 min (AGFK) to induce germination. At the indicated times, the mixtures were incubated at 80°C for 20 min to kill germinated spores and vegetative cells. The cultures were then plated on LB agar plates and incubated at 37°C overnight (Fig. 1A). Heat-resistant spores that were impaired but not blocked in germination formed colonies by the following morning. Furthermore, >1,000,000 colonies from each condition were separately pooled, and a fraction of this

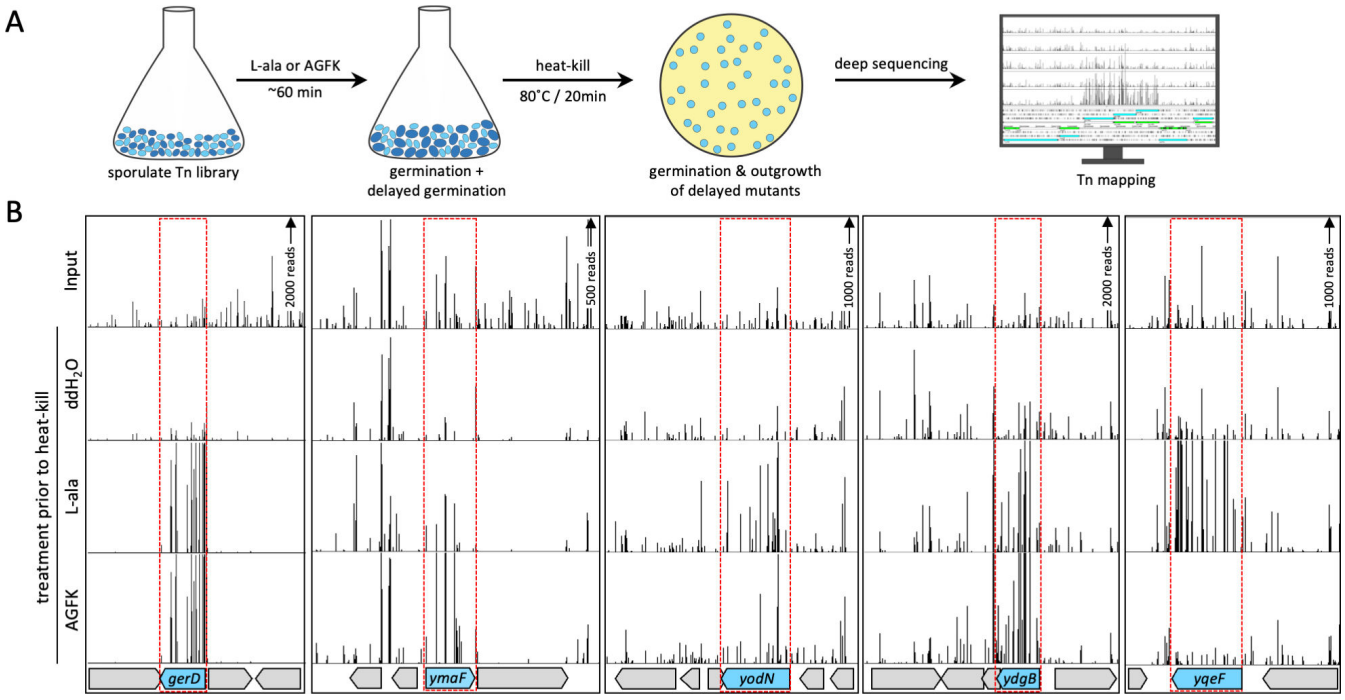

FIG 1 A genetic enrichment combined with transposon sequencing identifies new germination factors. (A) Schematic of the enrichment screen. A *B. subtilis* transposon library was sporulated in liquid medium. A sample was collected at the onset of starvation (not shown). The spore-containing culture was washed, divided into three, and incubated with ddH_2O, L-alanine, or AGFK to induce germination. After 60 min, the cultures were incubated at 80°C for 20 min to kill germinated spores and non-sporulated (vegetative) cells. The cultures were then plated on LB agar. Spores that did not germinate prior to heat-kill survived (light blue ovals) and formed colonies. Colonies from each condition were separately pooled. A fraction of the L-ala and AGFK mixtures was subjected to a second round of sporulation, germination, heat-kill, and plating (not shown). The transposon insertion sites were identified by deep sequencing and mapped to the *B. subtilis* 168 reference genome. (B) Transposon insertion profiles from five regions of the *B. subtilis* genome are depicted. Red boxes highlight *gerD, ymaF, yodN, ydgB,* and *yqeF* that were significantly enriched ($P < 0.05$) for transposon insertions after two rounds of sporulation and germination with L-ala and AGFK compared to water. Each vertical line indicates an insertion site, and the height represents the number of sequencing reads. The maximum number of reads shown is indicated in each panel. Additional insertion profiles can be found in Fig. S1.

pool was subject to a second round of enrichment. Genomic DNA from the pooled colonies from each condition and enrichment was isolated, and the transposon insertion sites and their abundance were determined by transposon sequencing. The Tn insertion profiles for each condition were compared to one another and to the profile from the library at the onset of starvation.

As anticipated, transposon insertions in the *gerA* operon required for germination in response to L-alanine (29) were highly enriched in the screen with L-alanine (Fig. S1A). As can be seen in Fig. S1A, transposons in this locus were enriched after 20-min incubation with L-alanine, and the enrichment increased further after 60 min and even more so after the second round of enrichment (>17-fold) (Table S1). By contrast, the transposon insertion profile across the *gerK* locus, which is required for germination in response to AGFK but not L-alanine (30), was unchanged after incubation with L-alanine under all conditions tested (Fig. S1A). Reciprocally, insertions in the *gerK* locus were enriched in the presence of AGFK (Fig. S1B). Unexpectedly, insertions in the *gerA* locus were also enriched in the AGFK condition, suggesting that the absence of the GerA receptor reduces or slows the response of spores to AGFK, at least under the conditions used for the screen. Finally, as can be seen in Fig. 1, insertions in the germination gene *gerD* that is required for efficient germination (9) were enriched for L-alanine- and AGFK-induced germination (Fig. 1B), further validating the approach. We identified 15 genes that were significantly (*P*-value ≤0.05) enriched in transposon insertions when the spore Tn library was induced to germinate with L-alanine compared to water. The fold-enrichment for these genes was ≥3.9 (Table S1). A total of 29 genes were significantly (*P*-value ≤0.05; fold-enrichment ≥3.9) overrepresented in transposon insertions when the spore library was induced to germinate with AGFK compared to water. In total, we identified 43 genes, of which 26 have been previously found to impair germination when mutated (Table S1). Figure 1B shows the transposon insertion profiles of four genes identified in our screen (*ymaF*, *yodN*, *ydgB, yqeF*). *ymaF* and *yqeF* were among the genes identified in the earlier Tn-seq screen (18). *ymaF* was not validated or characterized, but an initial characterization of *yqeF* was performed (18). *yodN* and *ydgB* were not previously implicated in germination.

Initial characterization of putative germination factors

We generated in-frame deletions in 26 of the hits in our screen, including all 14 new ones, and tested them for germination in response to L-alanine and AGFK. Of these mutants, 21 were impaired in germination, although most were modestly delayed (Table S1; Fig. S2). Four of the mutants from our screen (Δ*ymaF*, Δ*yodN*, Δ*ydgB*, and Δ*yqeF*) had more pronounced germination delays (Fig. 2). Importantly, all four mutants could be complemented in *trans*, indicating the absence of these genes was responsible for the defect rather than a polar effect on a neighboring gene. Interestingly, Δ*ydgB* spores were only delayed in germination in response to AGFK; and Δ*yqeF* spores were only delayed in response to L-alanine (Fig. 2; Fig. S3). The other two mutants (Δ*ymaF* and Δ*yodN*) were impaired in germination in response to both germinants (Fig. 2; Fig. S3).

The previous germination Tn-seq study that identified and characterized new germination factors found that, in most cases, the delay in germination could be explained by a reduction in the levels of the GerA receptor that responds to L-alanine and L-valine (18). Accordingly, we investigated the levels of the germinant receptors in spores from eight mutants from our screen, including Δ*ymaF*, Δ*yodN*, Δ*ydgB*, and Δ*yqeF*. Of these, only Δ*yqeF* was analyzed in the previous study. We purified spores from each mutant and generated lysates. Identical amounts of total protein were separated by SDS-PAGE and analyzed by immunoblot using anti-GerAA, -GerBC, and -GerKA antisera. In virtually all cases, the levels of the germinant receptor proteins were within 10% of wild type (Fig. S4). We tentatively conclude that the defects in spore germination are not due to a reduction in the levels of these receptors. Consistent with this idea, five of the eight genes tested are predicted to be expressed in the mother cell compartment and would

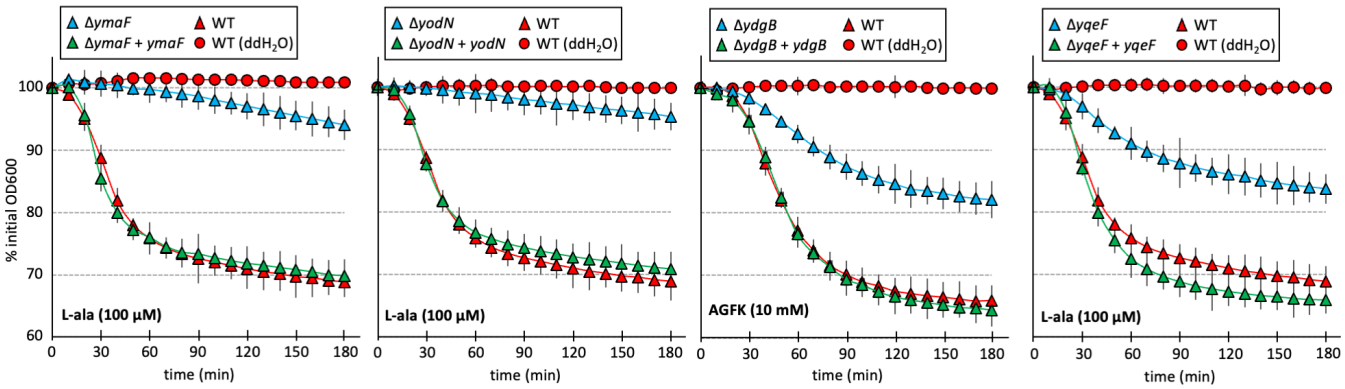

FIG 2 YmaF, YodN, YdgB, and YqeF are required for efficient spore germination. Germination assays of wild-type (WT), Δ*ymaF*, Δ*yodN*, Δ*ydgB,* and Δ*yqeF* spores in response to L-alanine or AGFK as assessed by the percent reduction in OD_{600} over time. Purified spores from the indicated strains were incubated with the germinant indicated at 37°C, and the drop in optical density was monitored over time. The germination defect of all four mutants could be complemented in *trans*. Representative data from one of three biological replicates are shown. Error bars indicate ±SD of three technical replicates.

therefore be unlikely to impact the levels of the germination receptors that are produced in the developing spore.

Characterization of YmaF, YodN, YdgB, and YqeF

Because spores lacking *ymaF*, *yodN*, *ydgB*, or *yqeF* had the strongest defects in spore germination among the hits from our screen, we characterized these factors further. Based on transcriptional profiling experiments, YmaF, YdgB, and YodN are predicted to be produced in the mother cell under the control of SigE, SigK, and GerE, respectively (31–33). The *yqeF* gene lacks a sporulation-specific promoter and is predicted to be transcribed by SigA (34). To experimentally validate these predictions, we generated promoter fusions to *yfp* and analyzed YFP fluorescence in a sporulation time course (Fig. S5). Sporulating cells harboring the P*ymaF-yfp* reporter exhibited fluorescence in the mother cell at an early stage of sporulation (Fig. 3A; Fig. S5). Cells lacking SigE failed to activate the reporter, while Δ*sigK*-sporulating cells produced YFP in the mother cell (Fig. S6), consistent with the transcriptional profiling studies (31–33). Sporulating cells that contained the P*ydgB-yfp* reporter had mother cell fluorescence at a late stage in sporulation that depended on SigK, while sporulating cells with P*yodN-yfp* produced YFP in the mother cell under the control of GerE at a very late stage in sporulation (Fig. 3A; Fig. S5 and S6). Thus, all three of these proteins are specifically produced in the mother cell compartment at different stages during spore maturation. By contrast, cells harboring the P*yqeF-yfp* reporter had weak YFP fluorescence at the onset of sporulation, and the signal remained low throughout sporulation (Fig. 3A; Fig. S5). These data support the idea that YqeF is produced during vegetative growth and is not under sporulation control. Based on our characterization of *ymaF* , we have renamed this gene *gerY*.

Next, we investigated where the proteins localize. We generated *yfp* fusions to each of the four genes and placed them under the control of their native promoters. However, we could only detect signal for two of the four proteins. YFP-GerY (YmaF) was enriched around the developing spore but was also present in the mother cell cytosol (Fig. 3B; Fig. S5). YdgB-YFP localized in puncta around the forespore (Fig. 3B; Fig. S5). We conclude that GerY, YdgB, and YodN are produced in the mother cell at different stages of spore development and that GerY and YdgB localize to the exterior of the spore, consistent with a potential role for these factors in spore germination.

GerY is a coat protein

To cast the widest net for mutants impaired in spore germination, we used a low concentration of L-alanine (100 µM) in our germination Tn-seq screen. To characterize

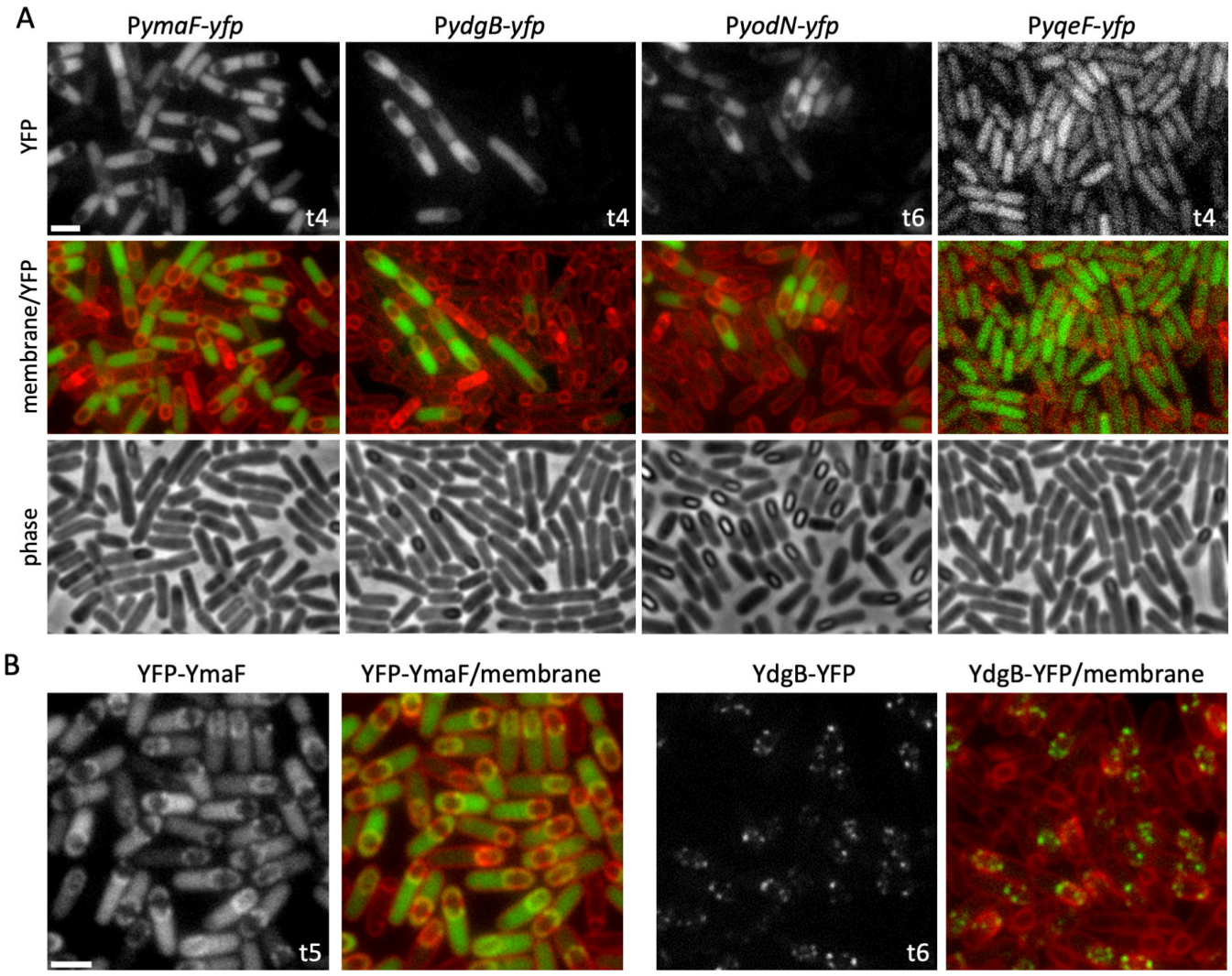

FIG 3 YmaF, YodN, and YdgB are synthesized in the mother cell during sporulation. (A) Representative images of sporulating strains harboring the indicated *yfp* fusion to the promoters of *ymaF*, yodN, *ydgB,* and *yqeF*. The sporulation time point (in hours) that was visualized for each strain is indicated in the low right corner of the YFP image. A sporulation time course for each strain can be found in Fig. S5. (B) Representative images of sporulating cells harboring YFP fusions to YmaF or YdgB. The sporulation time point (in hours) that was visualized for each strain is indicated in the low right corner of the YFP image. The membranes (false-colored red) were stained with the fluorescent dye TMA-DPH and merged with the YFP signal (false-colored green). Scale bars indicate 2 µm.

gerY more thoroughly, we performed germination assays on Δ*gerY* spores using 100 µM, 1 mM, and 10 mM L-alanine. For comparison, we selected 11 other mutants identified in our screen and 3 well-characterized germination mutants (Δ*gerD*, Δ*gerT,* and Δ*gerP*) (9, 12, 13) and performed germination assays under the same conditions. As anticipated, the germination defects for all 15 mutants were less pronounced with increasing concentration of L-alanine (Fig. 4A; Fig. S7). Nonetheless, several of the mutants, including Δ*gerY*, resembled the germination kinetics of the Δ*gerP* mutant (Fig. 4A; Fig. S7). Thus, several of these factors are likely to be *bona fide* germination factors.

Since Δ*gerY* was among the strongest hits in our screen and the YFP-GerY fusion was enriched around the developing spore, we investigated whether it was retained in mature spores. As controls, we analyzed spores harboring GerT-GFP (13) and GerPA-YFP fusions. As can be seen in Fig. 4B, all three factors were localized around the dormant spore. Furthermore, we found that YFP-GerY required both SafA and CotE for its localization in mature spores, similar to what was observed for GerT-GFP (13) (Fig. 4B). By contrast, the presence of GerPA-YFP was modestly disrupted in spores lacking SafA and

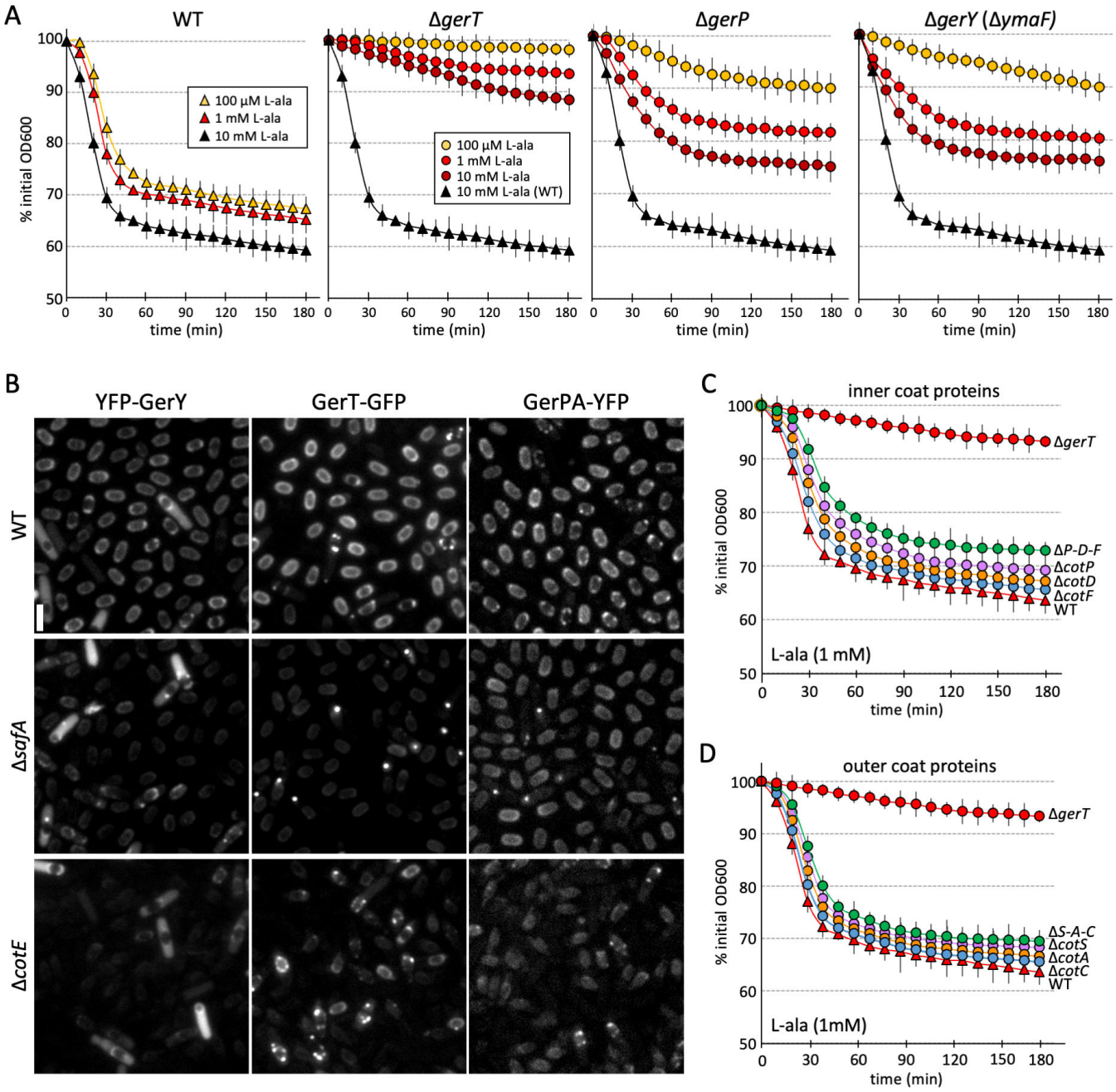

FIG 4 GerY is a coat protein. (A) Germination assays with a range of L-alanine concentrations. Purified spores from the indicated strains were incubated with L-alanine at 37°C, and the drop in optical density was monitored over time. (B) Representative images of purified spores harboring fluorescent fusions to GerT, GerPA, and GerY. In the absence of SafA or CotE, YFP-GerY and GerT-GFP are not retained in the spore coat. Scale bar indicates 2 µm. Spores lacking the inner coat proteins CotP, CotD, CotF (C) or the outer coat proteins CotS, CotA, CotC (D) have mild germination defects compared to wild type and Δ*gerT*. Purified spores from the indicated strains were incubated with 1 mM L-alanine at 37°C, and the drop in optical density was monitored over time. Representative data from one of three biological replicates are shown in panels A, C, and D. Error bars indicate ±SD of three technical replicates.

more substantially in the Δ*cotE* mutant. Altogether, these data argue that GerY, like GerP and GerT, is a coat protein.

The requirement for these coat proteins for efficient germination prompted us to investigate whether spores lacking other coat proteins might exhibit defective germination. Although our germination Tn-seq screen identified very few coat proteins, we tested three inner coat protein mutants (Δ*cotP*, Δ*cotD*, and Δ*cotF*) and three outer coat protein mutants (Δ*cotA*, Δ*cotC*, and Δ*cotS*) (14). All of the single mutants displayed minor

defects in germination compared to wild type and the Δ*gerT*-positive control (Fig. 4C and D; Fig. S8). Furthermore, triple mutants lacking all three inner coat proteins or all three outer coat proteins germinated nearly as well as the single mutants (Fig. 4C and D; Fig. S8). These experiments argue that the germination defects in the Δ*gerT*, Δ*gerP,* and *gerY* mutants are not due to a general disruption of the coat structure and suggest that these factors play more specific roles in the process.

GerT, GerY, and GerP have distinct roles in spore germination

Next, we investigated whether GerT, GerY, and GerP function together to mediate spore germination. We generated double and triple mutants and compared spore germination to the single mutants. As can be seen in Fig. 5A, spores lacking both *gerY* and *gerP* had a more severe germination defect than the individual mutants, and spores lacking *gerY* and *gerT* were modestly more impaired than the Δ*gerT* single mutant. Finally, the triple mutant was the most severely impaired (Fig. 5A). These data suggest that GerY, GerP, and GerT have independent roles in spore germination rather than functioning together in a single complex. Further support for this idea comes from double-mutant analysis with a mutation in *cotB*, encoding an outer coat protein (35). Spores lacking *cotB* have a modest germination delay (Fig. 5B). However, Δ*cotB* had distinct genetic interactions with Δ*gerT* compared to Δ*gerP* and Δ*gerY*. Δ*cotB* partially suppressed the germination defect of Δ*gerT*. By contrast, the germination defect of Δ*gerP* and Δ*gerY* was enhanced in the absence of *cotB* (Fig. 5B). Collectively, these data argue that GerT, GerP, and GerY function independently in promoting spore germination.

Finally, we investigated whether removing the spore coat can suppress the germination delay observed in the Δ*gerY* mutant, as was shown previously for Δ*gerP* spores (12, 36). Purified spores of all three single mutants and wild type were split into two. We chemically decoated half and left the coat on the half intact. As can be seen in Fig. 5C, decoating the spore largely suppressed the germination delays associated with spores lacking each of these factors. Collectively, these data are consistent with the model that all three proteins function to enable penetration of germinants through the coat but act independently in this process.

DISCUSSION

Here, we report the identification and characterization of a set of genes required for efficient spore germination in *B. subtilis*. Importantly, our Tn-seq screen and the one of Sayer and co-workers (18) identified an overlapping set of factors, suggesting the approach is robust. However, each screen also identified a subset of factors that were unique to each method. The screen of Sayer and co-workers used L-valine as the germinant (rather than L-alanine or AGFK) and separated spores that were delayed in germination from those that had germinated using density centrifugation. In addition, the screen did not involve a second round of enrichment. Consistent with the relatively minor experimental differences between the two screens, most of the mutants that were unique to each were the ones with relatively modest germination defects.

We characterized four of the genes identified in our screen in greater detail. Three of these factors (GerY, YdgB, and YodN) are produced in the mother cell at different stages of development, and one (YqeF) is expressed constitutively. While GerY and YodN are required for efficient spore germination in response to both L-alanine and AGFK, YqeF is only important in response to L-alanine and YdgB only in response to AGFK. GerY and YdgB specifically localize around the developing spore, and we show that GerY is associated with the mature spore in a manner that depends on CotE and SafA and is therefore likely to be a coat protein. Finally, our data suggest that GerY functions independently of two other coat-associated factors, GerT and GerP, that are also required for efficient germination.

The mechanisms by which GerY, GerP, and GerT promote germination are currently unknown. One hypothesis that has been suggested for the proteins encoded in the *gerP* operon is that they form a channel that spans the spore coat (12). Alphafold-Multimer

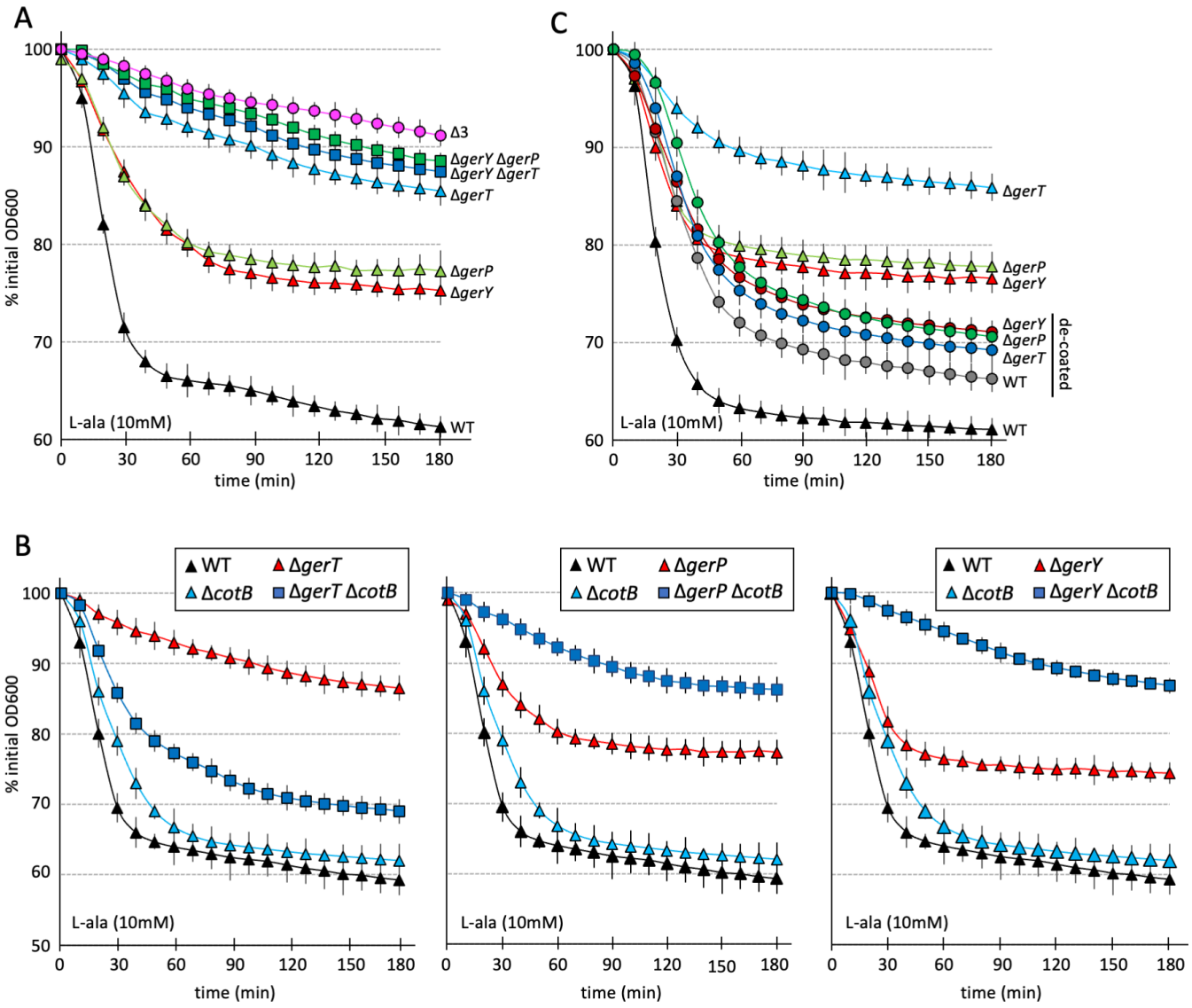

FIG 5 GerY, GerT, and GerP function in different genetic pathways. (A) Germination assays of single, double, and triple mutants of *gerY*, *gerT*, and *gerP* suggest each factor functions separately to promote efficient germination. Purified spores from the indicated strains were incubated with 10 mM L-alanine at 37°C, and the drop in optical density was monitored over time. (B) Germination assays of Δ*cotB* in the presence and absence of *gerT*, *gerP*, or *gerY* suggest each factor functions separately to promote efficient germination. (C) Germination assays with spores lacking their coat indicate that the reduced germination efficiency of Δ*gerT*, Δ*gerP*, and Δ*gerY* spores results from defects in the spore coat. Purified spores with or without their coats were incubated with 10 mM L-alanine at 37°C, and the drop in optical density was monitored over time. Representative data from one of three biological replicates are shown. Error bars indicate ±SD of three technical replicates.

(37) predicts that the GerP proteins assemble into a complex (Fig. S9); however, this complex does not contain a channel. Nonetheless, we envision that this protein complex promotes efficient transport across the coat by increasing the porosity of this thick proteinaceous layer (Fig. 6). Consistent with our genetic interaction data, neither GerY nor GerT is predicted to interact with the GerP complex nor with each other using AlphaFold3. Interestingly, GerY is conserved among *Bacilli* and *Clostridia*, while GerP is only present in *Bacilli* (Fig. S10), further supporting independent roles for these factors in spore germination. We hypothesize that GerY and GerT, like GerP, increase porosity of the coat to small molecules enabling efficient access to the germinant receptors. In the context of this model, we envision that each protein (or GerP complex) influences a distinct layer of the coat, contributing independently to the transit of germinants. The

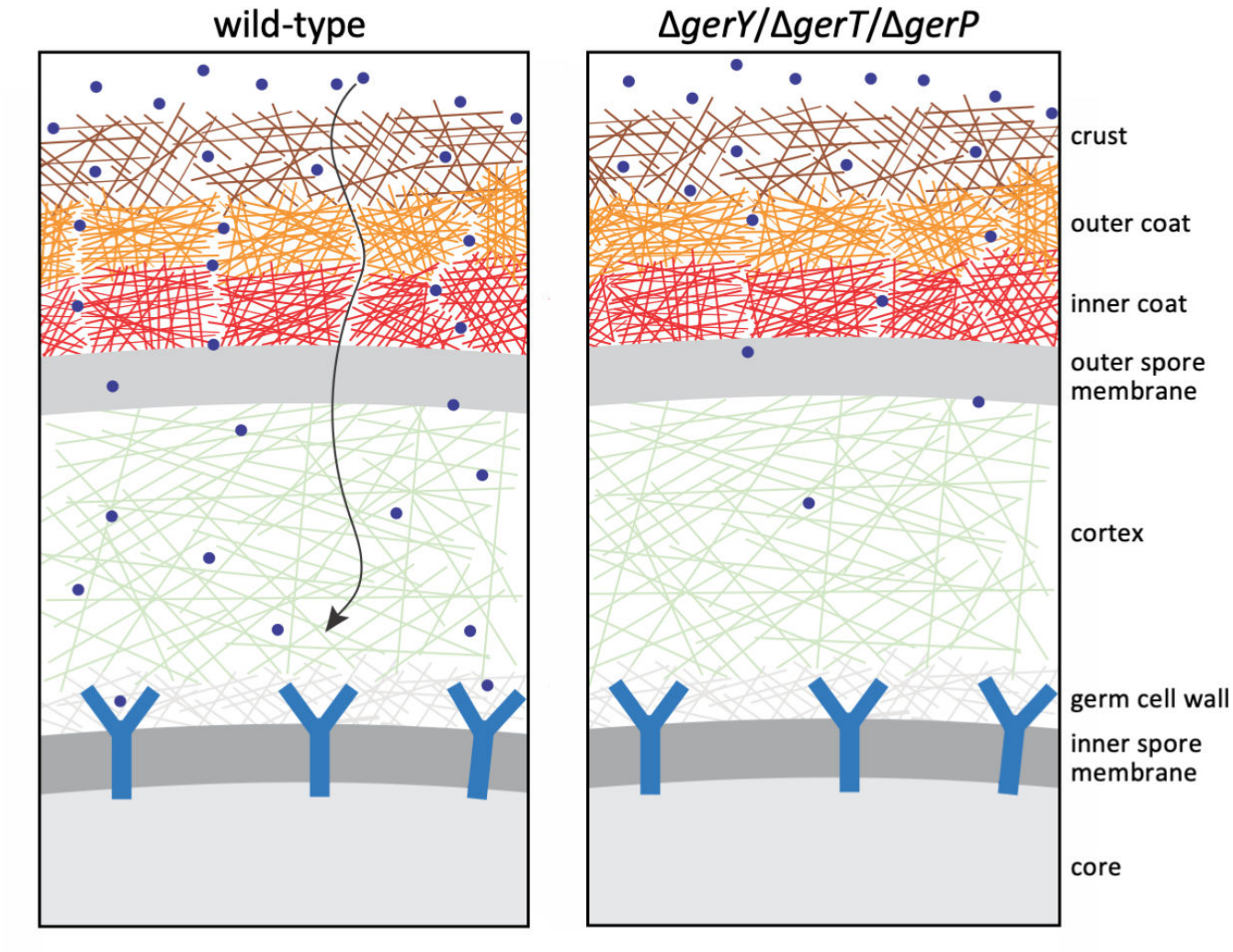

FIG 6 Schematic model for the role of GerY, GerT, and GerP proteins in the efficient transit of the germinants through the spore coat. Germinants (blue circles) must transit the crust, the outer and inner coat, the outer spore membrane, the cortex, and the germ cell wall to reach the germinant receptors in the inner spore membrane. GerY, GerT, and GerP are proposed to increase the porosity of the coat. In their absence, germinants are hindered in their access to the receptors.

structure of the coat and the contributions of these factors to the transit of germinants await future iterations of AlphaFold and improvements in cryo-electron microscopy methods.

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Fernando H. Ramírez-Guadiana, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft | Anna P. Brogan, Investigation, Software, Writing – review and editing | David Z. Rudner, Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (JB00399-24-s0001.pdf). Supplemental methods, Fig. S1 to S10, and Tables S2 to S4.
Table S1 (JB00399-24-s0002.xlsx). Genes enriched for transposon insertions in the germinations screens.

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