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Analysis of the effects of a gerP mutation on the germination of spores of Bacillus subtilis

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Bacillus subtilis spores with a gerP mutation triggered spore germination via nutrient germinant receptors (GRs) slowly, although this defect was eliminated by spore coat removal. The gerP spores had longer lag times between nutrient germinant addition and initiation of rapid dipicolinic acid release than wild-type spores, but times for release of ~90% of spores' dipicolinic acid were identical. gerP spores were also defective in germination with the non-GR-dependent germinant DPA, but germinated faster than wild-type spores with dodecylamine. GR overexpression increased gerP spores' germination, but this was still slower than comparable wild-type spores' germination. Concentrations of L-alanine and L-valine giving maximal germination were ? 500 μM and ? 10 mM, while with gerP spores these values were 200-1000 mM and ? 100 mM. A high pressure of 150 megaPascals that triggers spore germination by activating GRs triggered wild-type and gerP spore germination identically. These data all suggest that GerP proteins facilitate access of nutrient germinants to their cognate GRs in spores.

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Analysis of the Effects of a *gerP* Mutation on the Germination of Spores of *Bacillus subtilis*

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As previously reported, *gerP Bacillus subtilis* spores were defective in nutrient germination triggered via various germinant receptors (GRs), and the defect was eliminated by severe spore coat defects. The *gerP* spores' GR-dependent germination had a longer lag time between addition of germinants and initiation of rapid release of spores' dipicolinic acid (DPA), but times for release of >90% of DPA from individual spores were identical for wild-type and *gerP* spores. The *gerP* spores were also defective in GR-independent germination by DPA with its associated Ca^{2+} divalent cation (CaDPA) but germinated better than wild-type spores with the GR-independent germinant dodecylamine. The *gerP* spores exhibited no increased sensitivity to hypochlorite, suggesting that these spores have no significant coat defect. Overexpression of GRs in *gerP* spores did lead to faster germination via the overexpressed GR, but this was still slower than germination of comparable *gerP*$^+$ spores. Unlike wild-type spores, for which maximal nutrient germinant concentrations were between 500 μM and 2 mM for L-alanine and ≤10 mM for L-valine, rates of *gerP* spore germination increased up to between 200 mM and 1 M L-alanine and 100 mM L-valine, and at 1 M L-alanine, the rates of germination of wild-type and *gerP* spores with or without all alanine racemases were almost identical. A high pressure of 150 MPa that triggers spore germination by activating GRs also triggered germination of wild-type and *gerP* spores identically. All these results support the suggestion that GerP proteins facilitate access of nutrient germinants to their cognate GRs in spores' inner membrane.

Spores of *Bacillus* species are metabolically dormant and extremely resistant to a wide variety of agents (38). As a consequence, these spores can remain viable for long periods. However, if specific nutrients termed germinants are present in the environment, spores can sense these compounds and rapidly return to active growth in the process of germination followed by outgrowth (37). Spores sense nutrient germinants when they bind to germinant receptors (GRs) located in the spore's inner membrane, with three important GRs existing in *Bacillus subtilis* spores (32, 37). The GerA GR triggers spore germination in response to either L-alanine or L-valine, while the GerB and GerK GRs together are required for germination with a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (AGFK). There is also a GerB GR variant termed GerB* that responds to L-asparagine alone (26). Binding of the various nutrient germinants to their respective GRs triggers the release of the spore core's large depot (~25% of dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) and its associated divalent cations, predominantly Ca^{2+} (CaDPA), and replacement of CaDPA by water. Release of the spore's CaDPA in turn triggers the hydrolysis of the spore's peptidoglycan cortex by cortex-lytic enzymes (CLEs) (9, 17, 37), and this allows core expansion and further water uptake that raises the spore core water content to that of a growing cell. This elevated core water content then allows progression into outgrowth with initiation of enzyme action in the spore core and, ultimately, the conversion of the germinated spore into a growing cell.

As noted above, the GRs are located in the spore's inner membrane. In *B. subtilis* spores, exogenous germinants must traverse the thick spore coat, the outer membrane, and the peptidoglycan cortex and germ cell wall to access the GRs. While the outer membrane may or may not be a significant permeability barrier in dormant spores, the coat is a permeability barrier to large molecules (18, 20). Thus, it is possible that there are special mechanisms to ensure that germinants can readily gain access to the GRs. Indeed, proteins encoded by the *gerP* locus may function to do this in spores of *Bacillus anthracis*, *Bacillus cereus*, and *B. subtilis* (3, 7). The *gerP* locus was originally identified by a mutation that decreased *B. cereus* spore germination with several nutrient germinants and also decreased apparent spore viability ~5-fold, presumably because germination was so defective in these spores. The original *gerP* mutation was in the *gerPC* gene, the third gene in the likely hexacistronic *gerP* operon; a similar operon is present in other *Bacillales* species. Mutations in individual genes of the *B. subtilis gerP* operon also reduce spore germination with nutrient germinants but do not reduce spore viability, and deletion of the whole operon gives the same general phenotype as do mutations in individual *gerP* genes. The *B. subtilis gerP* operon appears to be expressed only late in sporulation, most likely under the control of the RNA polymerase sigma factor σ^K, and this operon's expression is also negatively regulated by the GerE transcription factor. These findings are consistent with the GerP proteins being components of the spore coat. However, no gross coat defect has been observed in *gerP* spores, and the sequences of the various GerP proteins give no insight into their function. That the GerP proteins might be important in facilitating germinant access to GRs is further suggested by the suppression of the *gerP* mutations' effects

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TABLE 1 *B. subtilis* strains used

Strain	Genotype and phenotype[a]	Reference, source, or construction[b]
FB10	*gerB**	26
KGB73	Δ*cotE* Δ*gerE gerD-gfp* Δ*gerD* Km^r Sp^r Tc^r	16
PS533	Wild type Km^r	36
PS832	Wild type	Laboratory strain
PS3328	*cotE* Tc^r	48
PS3415	P*sspB*::*gerB** Sp^r	6
PS3476	P*sspD*::*gerA* MLS^r	6
PS3502	P*sspD*::*gerB** Sp^r	6
PS3521	Δ*gerA gerB** Sp^r	6
PS3665	Δ*gerA gerB** Δ*gerK* MLS^r Sp^r	1
PS4194	Δ*gerP* MLS^r	This work
PS4197	*gerB** Δ*gerP* MLS^r	PS4194 → FB10
PS4198	Δ*cotE* Δ*gerP* MLS^r Tc^r	PS4194 → PS3328
PS4202	Δ*gerP* P*sspB*::*gerB** MLS^r Sp^r	PS4194 → PS3415
PS4228	Δ*gerP* Tc^r	This work
PS4231	Δ*gerP* P*sspD*::*gerB** MLS^r Sp^r	PS4194 → PS3502
PS4232	Δ*alr* Tc^r	This work
PS4233	Δ*gerP* Δ*alr* MLS^r Tc^r	PS4232 → PS4194
PS4234	Δ*gerA gerB** Δ*gerP* MLS^r Sp^r	PS4294 → PS3521
PS4235	Δ*gerP* P*sspD*::*gerA* MLS^r Tc^r	PS4228 → PS3476
PS4236	Δ*gerA gerB** Δ*gerK* Δ*gerP* MLS^r Sp^r Tc^r	PS4228 → PS3665
PS4237	Δ*cotE* Δ*gerE gerD-gfp* Δ*gerD* Δ*gerP* Km^r MLS^r Sp^r Tc^r	PS4194 → KGB73
PS4247	Δ*dal* Tc^r	This work
PS4262	Δ*alr* Δ*dal* Cm^r Tc^r	PS4247 → PS4274
PS4263	Δ*alr* Δ*dal* Δ*gerP* Cm^r MLS^r Tc^r	PS4194 → PS4262
PS4274	Δ*alr* Cm^r	This work

[a] Abbreviations: Cm^r, resistance to chloramphenicol (5 μg/ml); Km^r, resistance to kanamycin (10 μg/ml); MLS^r, resistance to erythromycin (1 μg/ml) and lincomycin (25 μg/ml); Sp^r, resistance to spectinomycin (100 μg/ml); and Tc^r, resistance to tetracycline (10 μg/ml).

[b] Strains were constructed by transformation of strains to the right of the arrows with chromosomal DNA from the strains to the left of the arrows.

on spore germination either by a *cotE* mutation that eliminates assembly of much of the spore coat or by chemical removal of the spore coat, although both a *cotE* mutation and chemical decoating also eliminate the spores' outer membrane as a potential permeability barrier (3, 7). In the current work, we have further examined the properties of *gerP B. subtilis* spores, with the goal of providing additional evidence for or against a role for GerP proteins in allowing germinant access to the spore's GRs.

MATERIALS AND METHODS

***B. subtilis* strains used and spore preparation.** The wild-type *B. subtilis* strains used were PS832, a laboratory 168 strain, or PS533 (36), strain PS832 with plasmid pUB110 encoding resistance to kanamycin (10 μg/ml). All other *B. subtilis* strains are isogenic with strain PS832 (Table 1). All plasmid constructs were isolated in *Escherichia coli* strain DH5α. The *gerP* strain PS4194 has a deletion of the coding region of the *gerP* operon and its replacement with an MLS^r cassette. This strain was constructed as follows. The DNA region from +64 to +594 bp relative to the translation start site of *gerPF* was PCR amplified from chromosomal DNA of strain PS832 with primers containing 5′ XbaI and 3′ SacI sites (all primer sequences are available upon request). The PCR product was purified, digested with XbaI and SacI, and ligated to similarly cut plasmid pFE140 (27) to make plasmid pFE140*gerPF*. Another DNA region from −487 to +181 bp relative to the translation start site of *gerPA* was PCR amplified from chromosomal DNA of strain PS832 with primers containing 5′ XhoI and 3′ NsiI sites. The PCR product was purified, digested with XhoI and NsiI, cloned into similarly cut plasmid pFE140*gerPF*, and then sequenced, resulting in plasmid pPS4195. Strain PS832 was transformed to MLS^r with plasmid pPS4195, and the double-crossover event leading to the replacement of much of the *gerP* operon with the *ermC* cassette to make strain PS4194 was confirmed by PCR analysis.

Four additional deletion mutants, the *gerP*::*tet*, *alr*::*cam*, *alr*::*tet*, and *dal*::*tet* mutants, were generated for this work. In the majority of the mutants, the *alr* or *dal* gene or the *gerP* operon was replaced by Tc^r or Cm^r cassettes. The *alr* gene encodes what is predicted to be the *B. subtilis* spore-associated alanine racemase, while the *dal* gene likely encodes the alanine racemase essential for D-alanine synthesis in growing cells (30). Plasmid pXY1206 was used to generate the *gerP*::*tet* strain. The Tc^r cassette was PCR amplified from plasmid pFE149 (27). The upstream region of the *gerP* operon (−518 to +75 bp relative to the *gerPA* translation start site [+1 bp]) and the downstream region of the *gerP* operon (+1645 to +2270 bp) were PCR amplified from PS832 chromosomal DNA. A three-way overlap PCR was performed using the flanking regions and Tc^r cassette as the template, and the PCR product was purified and ligated to the pGEM-T Easy vector (Promega Corporation, Madison, WI) to generate plasmid pXY1206. The *alr*::*tet* strain was generated using plasmid pXY1202, which was constructed similarly to pXY1206. The upstream region of the *alr* gene (−566 to +49 bp relative to the translation start site [+1 bp]), the downstream region of the *alr* gene (+1035 to +1665 bp), and the Tc^r cassette were used as the template for a three-way overlap PCR. The PCR product was then ligated into the pGEM-T Easy vector to create pXY1202. The *alr*::*cam* strain was generated using plasmid pXY1248. This plasmid was constructed similarly to pXY1202, except that a Cm^r cassette from plasmid pDG268 (39) was used for the three-way overlap PCR. The *dal*::*tet* strain was generated using plasmid pXY1105. The upstream region of the *dal* gene (−440 to +63 bp relative to the *dal* translation start site [+1 bp]), the downstream region of the *dal* gene (+1081 to +1591 bp), and the Tc^r cassette were used as the template for the three-way overlap PCR. The PCR product was then ligated into the pGEM-T Easy vector to create pXY1105. Plasmids pXY1105, pXY1202, pXY1206, and pXY1248 were used to transform *B. subtilis* strains to Cm^r or Tc^r on LB medium plates by double-crossover events (1, 6, 27), and proper strain construction was verified by PCR. Transformants in which the *dal* gene was inactivated were selected on plates supplemented with 1 mM D-alanine, since *dal* mutants did not grow without D-alanine, as expected (30).

Spores of *B. subtilis* strains were prepared by sporulation at 37°C on 2× Schaeffer's glucose (SG) agar plates without antibiotics as described previously, except that D-alanine was present at 1 mM in plates for sporulation of *dal* strains (22, 25, 30). The presence of the D-alanine did not alter the sporulation of strains with an intact *dal* gene (data not shown). After 2 to 3 days at 37°C, plates were shifted to 23°C for 2 to 4 days, and then spores were harvested and purified, also as described previously (22, 25). All spores used in this work were free (>98%) from sporulating or growing cells, germinated spores, and cell debris, as determined by phase-contrast microscopy. Spores were chemically decoated as described previously (46).

Spore germination. Germination of spores with nutrient germinants was routinely preceded by a heat shock treatment of spores at an optical density at 600 nm (OD_{600}) of 10 to 20 in water at 75°C for 30 min, followed by cooling on ice. Spores were germinated at 37°C in 25 mM K-HEPES buffer (pH 7.4) with L-valine via the GerA GR, with AGFK via the GerB and GerK GRs, or with L-asparagine via the GerB* GR. Germination was assessed either by adding 50 μM $TbCl_3$ to the germination mix and measuring DPA release fluorometrically in a multiwell-plate reader with spores at an OD_{600} of 0.5 as described previously (47) or by examining ~100 spores by phase-contrast microscopy for those that had become phase dark and thus had germinated completely. Germination of spores with coat defects due to chemical decoating or a *cotE* mutation (18) is very strongly inhibited by $TbCl_3$ (46). Accordingly, with these spores there was no $TbCl_3$ in the initial germination mix, but $TbCl_3$ was added to 50 μM at

various times after the mixing of spores with germinants, and the Tb-DPA fluorescence was then measured immediately as described previously (47). Spores were also germinated without prior heat activation with 60 mM CaDPA, a GR-independent germinant (23), in 25 mM Tris-HCl (pH 7.5) at 30°C, and spore germination was measured microscopically as described above. Spores were germinated with dodecylamine, another GR-independent germinant (33), without prior heat activation in 25 mM K-HEPES buffer (pH 7.4) at 45°C with 0.4 mM dodecylamine, and germination was assessed by DPA fluorescence and confirmed by phase-contrast microscopy. All germination experiments with spore populations were carried out at least twice with essentially identical results.

The germination of multiple individual spores with 10 mM L-valine in 25 mM K-HEPES buffer (pH 7.4) was followed using differential interference contrast (DIC) microscopy as described previously (21, 49, 50). Briefly, heat-activated spores (1 μl; ~10^8 spores/ml in water) were spread on the surface of a microscope coverslip that was dried in a vacuum desiccator for ~10 min, and the coverslip was mounted on and sealed to a microscope sample holder kept at a constant temperature. The DIC images of hundreds of individual spores were recorded at a rate of 1 frame per 15 s for up to 150 min, and the image intensities of each individual spore were extracted (49, 50). In these analyses, a spore's DIC image intensity remains relatively constant after mixing with a germinant until the time, T_{lag}, when rapid CaDPA release begins. The spore's DIC image intensity then falls rapidly in parallel with CaDPA release that ends at time $T_{release}$. The parameter $\Delta T_{release}$ ($T_{release} - T_{lag}$) defines the time for release of ≥90% of a spore's CaDPA pool. The average values for these kinetic parameters of the germination of individual spores were determined from values for 58 individual spores that germinated.

For analysis of spore germination with high pressure (HP), frozen spore stocks (~10^9 spores/ml) were thawed and resuspended at 10^8 spores/ml in 40 ml of 50 mM K-HEPES buffer (pH 7.4). The spores were heat activated for 30 min at 70°C and cooled, and 1.5-ml aliquots were added to sterile plastic pouches (Labplas, Sainte-Julie, Quebec, Canada). Individual sample pouches were vacuum sealed using a Röschermatic vacuum packaging machine (Reiser, Canton, MA) and then double bagged in a second pouch and vacuum sealed to prevent the possibility of leaks that could contaminate the HP medium. Samples were stored in wet ice for ~24 h prior to HP treatments.

HP treatments were carried out using a PT-1 HP unit (Avure Technologies, Kent, WA) and water as the hydrostatic medium and to regulate the temperature of the thermal bath surrounding the pressure vessel. Sealed spore samples were placed inside the stainless steel HP vessel and rapidly reached thermal equilibrium with the surrounding water bath. Spores were treated under two sets of HP and temperature conditions: (i) HP of 150 MPa (22 kpsi) at 37°C and (ii) HP of 550 MPa (80 kpsi) at 50°C. The equipment come-up times for the increase from atmospheric pressure to the designated HP were 7 s for 150 MPa and 25 s for 550 MPa. The equipment come-up time was designated the new time zero for each experiment, and data were collected at various durations of hold times for up to 14 min after the come-up time. During the hold time, the pressure was essentially constant. After pressurization, the HP release was almost instantaneous, and the treated samples were stored at 4°C prior to analysis of spore germination by phase-contrast microscopy as described above.

Analytical procedures. Measurement of spore resistance to sodium hypochlorite was as described using spores at 23°C and an OD_{600} of 1 in 2.5 g/liter sodium hypochlorite at pH 11 (48). Fluorescence microscopy of spores of strains expressing functional GerD-green fluorescent protein (GFP) fusion proteins was as described previously (16).

RESULTS

Germination of wild-type and *gerP* spore populations or individual spores with various germinants. As reported previously (3, 7), *gerP B. subtilis* spores germinated more slowly and less completely than wild-type spores with nutrient germinants that targeted the GerA GR (L-valine) or the GerB plus GerK GRs (AGFK) (Fig. 1A). This was also seen with L-asparagine germination via the GerB* GR (see below). Also as reported previously (3, 7), the nutrient germination defect in *gerP* spores was suppressed by either a *cotE* mutation or chemical decoating (Fig. 1B and data not shown; see below), which eliminates much of the spore coat as well as spores' outer membrane. Germination of *gerP* spores was also significantly less efficient with the non-GR-dependent germinant CaDPA (Fig. 2A). However, germination of *gerP* spores was actually faster than wild-type spore germination with the non-GR-dependent germinant dodecylamine (Fig. 2B).

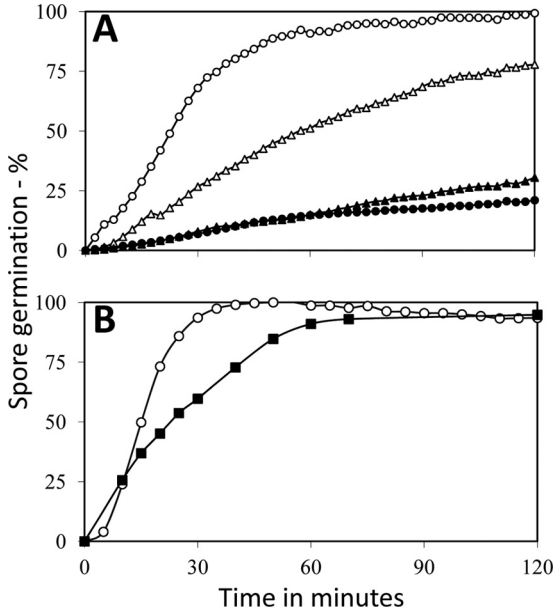

FIG 1 Germination of wild-type, *gerP*, and *cotE gerP* spores. (A) Spores of strains PS533 (wild type) and PS4194 (*gerP*) were germinated with either 10 mM L-valine or 10 mM in all AGFK components, and spore germination was followed by measuring Tb-DPA fluorescence as described in Materials and Methods. (B) Spores of strains PS533 (wild type) and PS4198 (*cotE gerP*) were germinated with 10 mM L-valine, and spore germination was monitored by measuring DPA release by Tb-DPA florescence with Tb added either from the beginning of the germination (wild-type spores) or adding Tb at various times to germination incubations (*cotE gerP* spores) and then measuring Tb-DPA fluorescence as described in Materials and Methods. Symbols: ○, wild-type spores germinating with L-valine; △, wild-type spores germinating with AGFK; ●, *gerP* spores germinating with L-valine; and ▲, *gerP* spores germinating with AGFK (A) and ○, wild-type spores, and ■, *cotE gerP* spores (B).

The GerP proteins are most likely spore coat proteins (3), and the effects of a *gerP* mutation on spore germination with CaDPA or dodecylamine were similar to those caused by a spore coat defect (23, 33). This suggests the possibility that *gerP* spores have a significant coat defect. However, *gerP* spores are reported to be resistant to lysozyme (3), a standard measure of the presence of a relatively intact spore coat (18). Another measure of the state of the spore coat is spore resistance to sodium hypochlorite, as spores with mutations in coat protein genes that remain lysozyme resistant can be somewhat sensitive to hypochlorite (20; R. Tennen, K. Ragkousi, and P. Setlow, unpublished results). However, while decoated wild-type spores were killed by ≥4 log units in 1 min with hypochlorite, intact wild-type and *gerP* spores exhibited quite similar resistance to this chemical (Fig. 3), and the difference between the wild-type and *gerP* spores' killing with hypochlorite was not statistically significant.

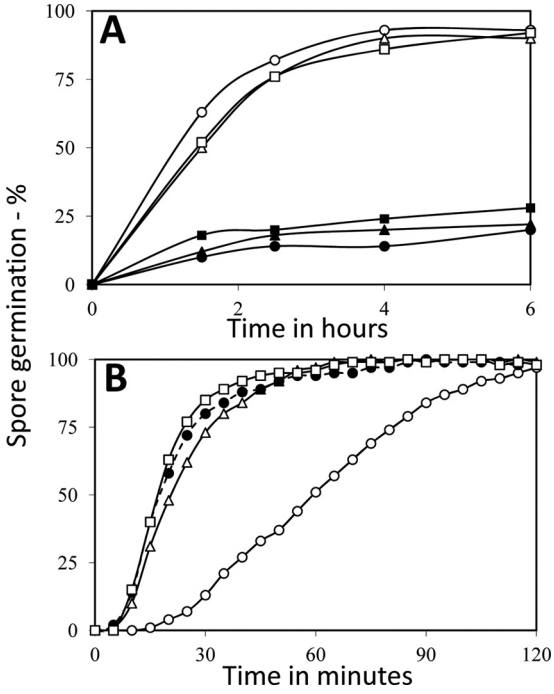

FIG 2 Germination of spores of wild-type and *gerP* strains with CaDPA and dodecylamine. Spores of various strains were germinated with either CaDPA (A) or dodecylamine (B), and spore germination with these agents was measured as described in Materials and Methods. Symbols: ○, PS533 (wild type); ●, PS4194 (*gerP*); △, FB10 (*gerB**); ▲, PS4197 (*gerB* gerP*); □, PS3502 (P*sspD*::*gerB**); and ■, PS4231 (*gerP* P*sspD*::*gerB**) (A) and ○, PS533 (wild type); ●, PS4194 (*gerP*); △, PS4228 (*gerP*); and □, PS4231 (P*sspD*::*gerB** *gerP*) (B).

The results noted above were all obtained with large spore populations. Recent work has indicated that additional information about spore germination can be obtained by examining the germination of large numbers of individual spores (21, 29, 49, 50), especially since the nutrient germination of spores in populations is extremely heterogeneous. Most of this heterogeneity is due to a highly variable lag period, termed T_{lag}, between mixing spores with germinants and the initiation of the rapid release of ≥90% of spore DPA (14, 19, 34, 49, 50). Accordingly, we compared the GR-dependent germination of large numbers of individual wild-type and *gerP* spores. While most individual *gerP* spores did not germinate during the observation period, germination of those *gerP* spores that did germinate proceeded according to the same general kinetic pattern exhibited by individual germinating wild-type spores (Fig. 4A and B; Table 2). Specifically, there was an initial T_{lag} period during which the spores' DIC image intensity remained relatively constant, followed by a rapid fall of 70 to 80% in the spores' normalized DIC image intensity in ~3 min ($\Delta T_{release}$), which is due to the release of >90% of spore DPA (21, 49). The T_{lag}s of individual spores were highly variable, ranging from a few minutes to >120 min. DPA release was complete at the time termed $T_{release}$, which was followed by a further slower decrease in spore DIC image intensity due to cortex hydrolysis and core water uptake, and then the DIC image intensity remained constant (21, 29, 49, 50). Analysis of the kinetics of multiple individual wild-type and *gerP* spores indicated that, as expected, the average T_{lag} for those spores that germinated was longer for *gerP* spores, and the median T_{lag} for the entire *gerP* spore population was even longer, since >70% of the *gerP* spores did not release

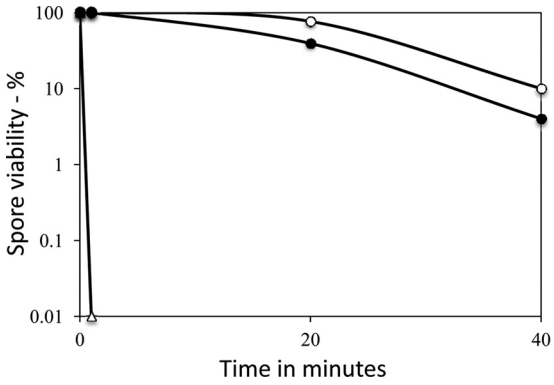

FIG 3 Inactivation of wild-type and *gerP* spores by sodium hypochlorite. Intact spores of strains PS4194 (*gerP*) (●) and PS533 (wild type) (○) and chemically decoated wild-type spores (△) were treated with sodium hypochlorite, and spore viability was measured as described in Materials and Methods.

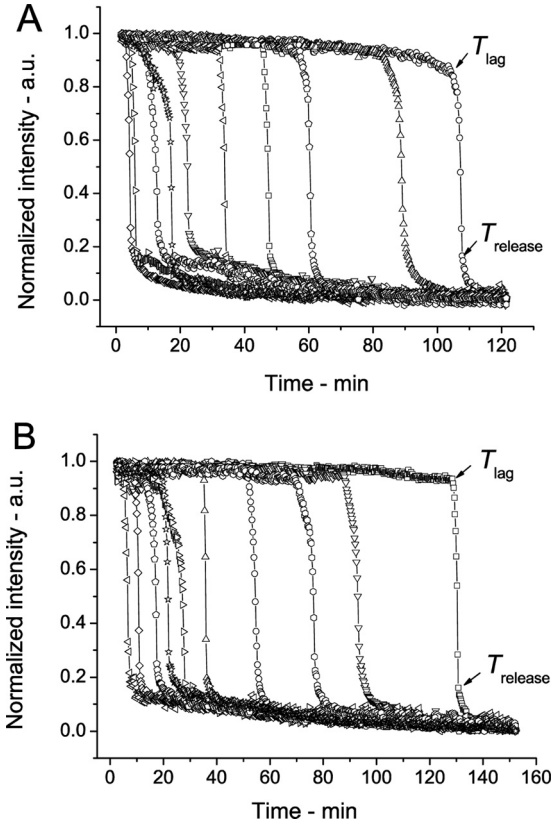

FIG 4 Analysis of the nutrient germination of individual wild-type and *gerP* spores. The intensities of the DIC images of 10 randomly chosen PS832 (wild type) (A) or PS4194 (*gerP*) (B) spores incubated in 10 mM L-valine that germinated in the 150-min observation period were measured as a function of germination time. Note that the sharp fall in DIC image intensity corresponds to the release of the great majority of spore DPA. The labeled arrows adjacent to the curves for the last spore to germinate denote the T_{lag}s and $T_{release}$s for these spores. An individual spore's DIC image intensities were normalized to 1.0 at 0 min and to 0 after the decrease in DIC image intensity was complete. The absolute values of wild-type and *gerP* spores' DIC image intensities at 0 min and after the decreases in DIC image intensities were complete were essentially identical. a.u., absorbance units.

TABLE 2 Kinetic parameters of the valine germination of individual wild-type and *gerP* spores[a]

Strain	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)
PS832 (wild type)	26 ± 32 (33)[b]	29 ± 32	3 ± 1.3
PS4194 (*gerP*)	44 ± 39 (>120)	48 ± 40	3.4 ± 1.6

[a] Average values of T_{lag}, $T_{release}$, and $\Delta T_{release}$ and their standard deviations were determined from measurements on 58 individual spores of strains PS832 (wild type) and PS4194 (*gerP*) that germinated with 10 mM L-valine in a 150-min observation period, as described in Materials and Methods. Ninety-three percent of the wild-type spores germinated during the observation period, but only 29% of the *gerP* spores germinated.

[b] Values in parentheses are the median T_{lag} values for the entire spore population, including the spores that did not germinate during the observation period in the experiment.

DPA in the 150-min observation period, while only 7% of the wild-type spores did not release DPA (Table 2). However, despite the notable difference in the T_{lag}s of wild-type and *gerP* spores, the average $T_{release}$ was essentially identical for both wild-type and *gerP* spores (Table 2).

Effects of increased GR levels on wild-type and *gerP* spore germination. The data given above indicated that *gerP* spores have a significant defect in GR-dependent spore germination. An obvious question is whether this defect can be suppressed by expression of higher levels of GRs, since GR overexpression results in faster germination via the overexpressed GR (1, 6, 42). As expected, GerA overexpression from the moderately strong forespore-specific *sspD* promoter (P*sspD*) that increases GerA levels in spores ~8-fold (42) increased the rate of otherwise wild-type spore germination at saturating L-valine concentrations and even more at subsaturating L-valine concentrations (Fig. 5A and data not shown). While the rate of L-valine germination of *gerP* spores was lower than that of wild-type spores, the rate of L-valine germination of *gerP* spores was increased significantly by GR overexpression, although this was still lower than that of wild-type spores with normal GerA levels (Fig. 5A).

Analysis of the effect of GerB* overexpression on L-asparagine germination of *gerP* or otherwise wild-type spores gave results generally similar to those seen with GerA overexpression, as GerB* overexpression in *gerP* spores increased the L-asparagine germination to a rate almost that observed in *gerB** spores (Fig. 5B). However, the *gerP* mutation alone had less of an effect on L-asparagine germination via the GerB* GR than on L-valine germination via the GerA GR (compare Fig. 5A and B).

Association of germination proteins in wild-type and *gerP* spores. Previous work has shown that GRs and GerD are colocalized in a small focus termed the germinosome in spores' inner membrane, and disruption of this cluster is associated with decreased rates of spore germination (16). Fluorescence microscopy revealed no differences in the fluorescent foci of wild-type and *gerP* spores carrying a functional GerD-GFP fusion (data not shown), suggesting that there is no defect in germinosome assembly in *gerP* spores. However, fluorescence microscopy to detect germination protein-GFP fusions succeeds only using spores with an extremely defective spore coat due to both *cotE* and *gerE* mutations, in order to reduce the extremely high autofluorescence due to the spore coat (16). Consequently, spore germination should be normal in *cotE gerE gerP* spores, and it was (data not shown).

Effects of nutrient germinant concentrations on germination of wild-type and *gerP* spores. The results given above are consistent with the suggestion that the effect of a *gerP* mutation is to slow the access of nutrient germinants to GRs in the spores' inner membrane. If this is the case, then a simple prediction is that increasing nutrient germinant concentrations might suppress the effects of a *gerP* mutation. Consequently, the germination of wild-type and *gerP* spores was followed with L-alanine concentrations ranging from 500 μM to 1 M (Fig. 6A and B). As expected (1, 6, 42), an L-alanine concentration between 500 μM and 2 mM was saturating for the germination of wild-type spores, while at >50 mM L-alanine, the germination of wild-type spores actually decreased. However, the results with the *gerP* spores were extremely different, as the germination of the *gerP* spores increased up to 1 M L-alanine, although increases were minimal after 50 mM. It was also notable that the germination of *gerP* spores at 1 M L-alanine was very similar to that of wild-type spores at this L-alanine concentration (compare Fig. 6A and B).

As noted above, the germination of wild-type spores decreased significantly at L-alanine concentrations of ≥50 mM. This decrease could be due to elevated levels of D-alanine when levels of L-alanine are high, since D-alanine is an extremely strong inhibitor of GerA GR function (1, 45). The D-alanine could be generated by a spore-associated alanine racemase (8, 31, 45) or be an impurity in the L-alanine itself. To attempt to decide between these possibilities and perhaps to eliminate the decrease in spore germination at elevated L-alanine concentrations, we examined the effect of loss of alanine racemases on wild-type and *gerP* spore germination. Almost all bacteria have an alanine racemase activity essential for the generation of the D-alanine needed for peptidoglycan

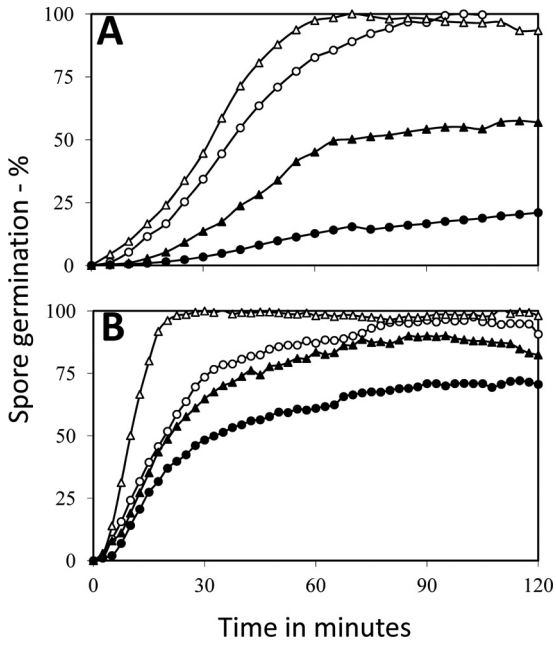

FIG 5 Effects of overexpression of the GerA or GerB* GRs on the germination of wild-type and *gerP* spores. Spores of strains PS533 (wild type) (○), PS3476 (P*sspD*::*gerA*) (△), PS4228 (*gerP*) (●), and PS4235 (*gerP* P*sspD*::*gerA*) (▲) (A) and FB10 (*gerB**) (○), PS4197 (*gerB** *gerP*) (●), PS3502 (*PsspD*::*gerB**) (△), and PS4231 (*gerP PsspD*::*gerB**) (▲) (B) were germinated with 10 mM L-valine (A) or 2 mM L-asparagine (B), and spore germination was monitored by Tb-DPA fluorescence as described in Materials and Methods.

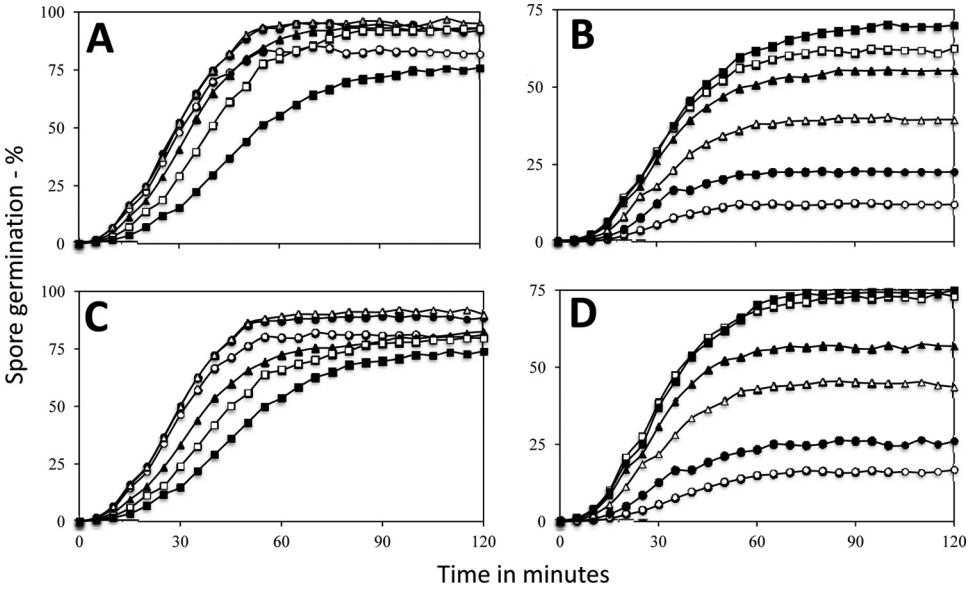

FIG 6 Germination of wild-type, *gerP*, *alr dal*, and *alr dal gerP* spores with various concentrations of L-alanine. Spores of strains PS533 (wild type) (A), PS4194 (*gerP*) (B), PS4262 (*alr dal*) (C), and PS4263 (*alr dal gerP*) (D) were germinated with various concentrations of L-alanine, and spore germination was measured by Tb-DPA fluorescence as described in Materials and Methods. The pH values of the germination incubations with 200 mM and 1 M L-alanine were checked to be sure that they were 7.4. The symbols for the various L-alanine concentrations are as follows: ○, 500 μM; ●, 2 mM; △, 10 mM; ▲, 50 mM, □, 200 mM; and ■, 1 M.

synthesis (30). However, *B. subtilis* has two alanine racemases, Alr and Dal. The *B. subtilis dal* gene is essential, while *alr* is not, although both genes are transcribed during sporulation under σ^E control, and there is evidence that *B. subtilis* spores have two associated alanine racemases (10, 11, 30, 41, 45). *B. cereus* and *B. anthracis* spores also have associated alanine racemase activity, although this appears to be due only to Alr, and Alr has been suggested to modulate spore germination by generating D-alanine (8, 10, 11, 30, 31, 37, 40, 41, 43, 45). Consequently, we generated strains carrying *alr* and *dal* mutations. These strains, including *alr dal* strains, all appeared to sporulate normally, with no evidence of premature germination of spores in the sporangia (data not shown), as is seen during sporulation of *B. anthracis* lacking Alr (8). Analysis of the germination of spores of *B. subtilis alr dal* strains with increasing L-alanine concentrations further showed that loss of either the *alr* gene alone or both the *alr* and *dal* genes did not significantly alter the germination of wild-type or *gerP* spores with high L-alanine concentrations (Fig. 6C and D and data not shown).

Since the possible presence of D-alanine might have complicated the assessment of the effects of high L-alanine concentrations on the germination of wild-type and *gerP* spores, as noted above, we also examined the effects of L-valine concentration acting via the GerA GR on these spores' germination (Fig. 7A and B). As expected, the maximal rate of wild-type spore germination required a higher concentration of L-valine than L-alanine (1, 42) (compare Fig. 6A and 7A). However, while wild-type spores' germination was almost maximal at 10 mM L-valine, the *gerP* spores' germination increased ~3-fold with between 10 and 100 mM L-valine (Fig. 7B). Unfortunately, L-valine solubility was not high enough to allow use of even higher concentrations of this germinant. It was also notable that a *cotE* mutation not only increased the germination of *gerP* spores but also resulted in a dependence on L-valine concentration for germination of *cotE gerP* spores that was very similar to that of *cotE* spores, becoming almost maximal at 10 mM L-valine (Fig. 8A and B). Note also that the *cotE* mutation had minimal effects, if any, on the response of the GerA GR in wild-type spores to L-valine concentrations of >10 mM (compare Fig. 7A and 8A).

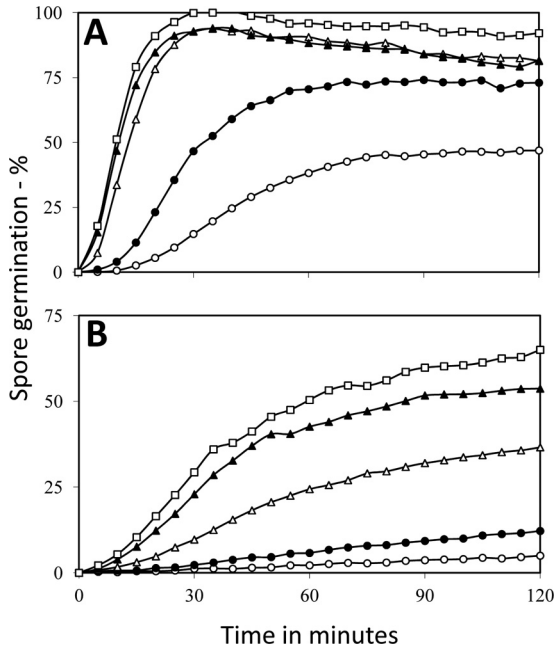

FIG 7 Germination of wild-type and *gerP* spores with various concentrations of L-valine. Spores of strains PS533 (wild type) (A) and PS4194 (*gerP*) (B) were germinated with various concentrations of L-valine, and spore germination was monitored by Tb-DPA fluorescence as described in Materials and Methods. The symbols for the various L-valine concentrations are as follows: ○, 1 mM; ●, 2 mM; △, 10 mM; ▲, 50 mM; and □, 100 mM.

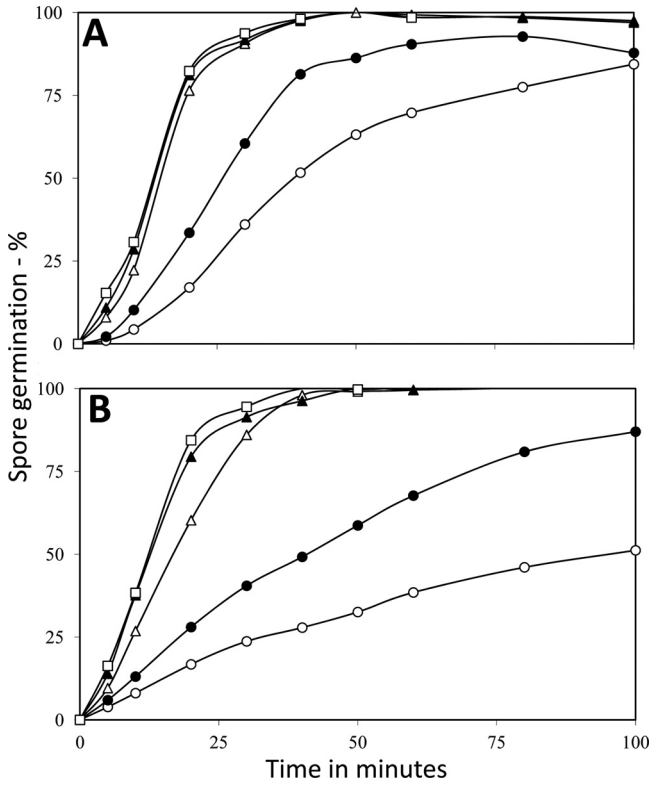

FIG 8 Germination of *cotE* and *cotE gerP* spores with various concentrations of L-valine. Spores of strains PS3328 (*cotE*) (A) and PS4198 (*cotE gerP*) (B) were germinated with various concentrations of L-valine, and germination of these coat-defective spores was monitored by measurements of fluorescence immediately after $TbCl_3$ was added at various times during germination as described in Materials and Methods. The symbols for the various L-valine concentrations are as follows: ○, 1 mM; ●, 2 mM; △, 10 mM; ▲, 50 mM; and □, 100 mM.

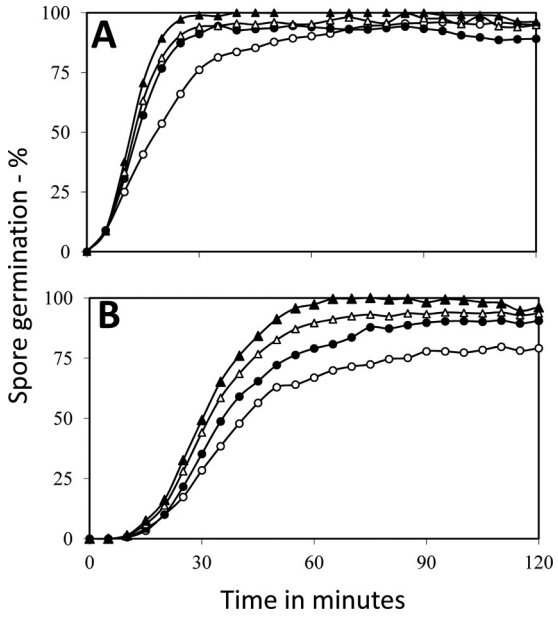

FIG 9 Effect of L-asparagine concentration on the germination of spores carrying the GerB* GR and with or without a *gerP* mutation. Strains FB10 (*gerB**) and PS4197 (*gerB* gerP*) were germinated with various L-asparagine concentrations, and spore germination was measured as described in Materials and Methods. The symbols for the various L-asparagine concentrations used are as follows: ○, 2 mM; ●, 10 mM; △, 50 mM; and ▲, 100 mM.

The effects of increasing L-asparagine concentrations on the germination of spores carrying the GerB* GR with or without the *gerP* mutation were also examined (Fig. 9A and B). In this case, the effects of increasing L-asparagine concentrations were not as dramatic as those with germination via the GerA GR. However, this may be because the *gerP* mutation had less of an effect on L-asparagine germination via the GerB* GR than on L-alanine or L-valine germination via the GerA GR. Even so, increasing L-asparagine concentrations from 10 to 100 mM gave more of an increase in *gerP* spore germination than on the germination of spores with a wild-type *gerP* operon.

HP germination of wild-type and *gerP* spores. The fact that the germination of *gerP* spores was increased significantly at nutrient germinant concentrations well above what is normally saturating for otherwise wild-type spores was consistent with the suggestion that the *gerP* mutation greatly slows access of nutrient germinants to their cognate GRs. If this is indeed the effect of a *gerP* mutation, then spore germination at an HP of ~150 MPa that triggers spore germination via activation of GRs (4, 24, 44) would be expected to be normal in *gerP* spores, since HP germination does not require that small-molecule germinants gain access to GRs. Indeed, this suggestion proved to be correct, as wild-type and *gerP* spores' germination with a pressure of 150 MPa was identical (Fig. 10A). The germination of wild-type and *gerP* spores at 550 MPa was also identical (Fig. 10B), as was expected, since germination at this pressure level does not require GR action (5, 24, 44).

DISCUSSION

The results in this communication are consistent with previous reports (3, 7) that *gerP* spores of *B. subtilis*, *B. cereus*, and *B. anthracis* are defective in nutrient-dependent germination and that this defect is eliminated by loss of much of the spore coat. Since nutrient germinants must pass through the spore's outer layers, including the coat, outer membrane, cortex, and germ cell wall, to access the GRs essential for nutrient germination, the defective nutrient germination of *gerP* spores has been suggested to be due to a role for GerP proteins in facilitating permeation of nutrient germinants through outer spore layers. *B. subtilis gerP* spores were also defective in CaDPA germination, as has been found with *B. anthracis gerP* spores (7). CaDPA triggers spore germination by activating the CLE CwlJ located in spores' outer layers, most likely at the outer edge of the spore cortex (2, 9, 15, 17, 23). Thus, the poor CaDPA germination of *gerP* spores is consistent with poor permeation of CaDPA through the spore coat or outer membrane, or both. In contrast to results with nutrients and CaDPA as germinants, the cationic surfactant dodecylamine was a more effective germinant for *gerP* spores. The target at which dodecylamine acts to trigger spore germination is likely the inner membrane, and decoating of wild-type spores increases their germination with dodecylamine (33). However, it is clear from their notable lack of sensitivity to lysozyme or hypochlorite that *gerP* spores have no major coat defect. Consequently, the reason for the more rapid dodecylamine germination of *gerP* spores is not clear.

Other new results in this work were also consistent with a role for GerP proteins in facilitating nutrient germinant access

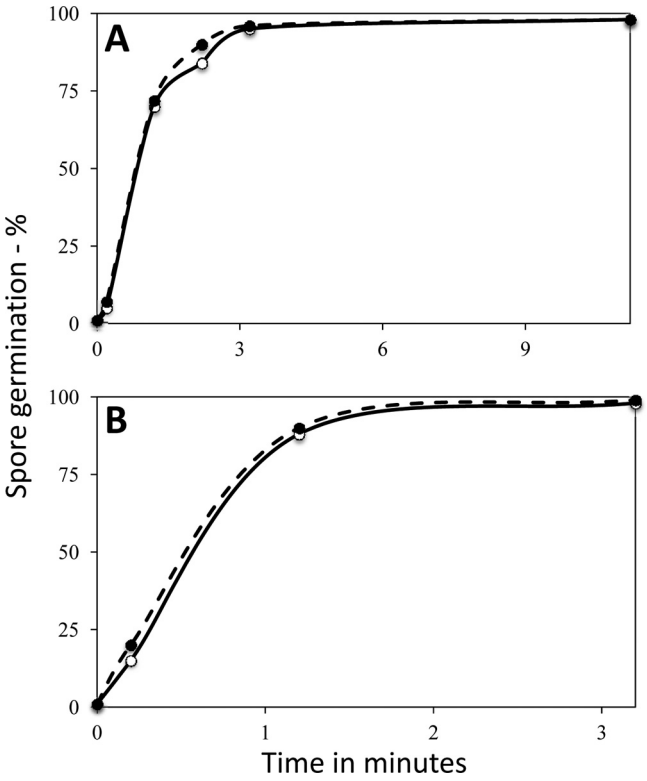

FIG 10 Germination of wild-type and *gerP* spores with HP. Spores of strains PS533 (wild type) (○) and PS4194 (*gerP*) (●) were germinated with either 150 MPa of pressure at 37°C (A) or 550 MPa of pressure at 50°C (B), and the extent of spore germination was determined by phase-contrast microscopy as described in Materials and Methods.

to their cognate GRs, as follows. (i) While overexpression of GRs increased germination by the relevant GR, a *gerP* mutation still reduced the rate of germination even by an overexpressed GR. (ii) Analysis of the nutrient germination of multiple individual *gerP* spores showed that of the spores that germinated, the pattern of their germination kinetics was essentially identical to that of wild-type spores, although *gerP* spores had longer average T_{lag} values than wild-type spores. Most importantly, the average $\Delta T_{release}$s for *gerP* spores' L-valine germination were essentially identical to those of wild-type spores, indicating that there was no defect in CaDPA release from the spore core in *gerP* spores, nor was there any defect in CwlJ action, as defective action of this specific CLE during germination increases $\Delta T_{release}$ values 5- to 15-fold (29, 35). (iii) Like wild-type spore germination (21, 29, 34, 49, 50), the nutrient germination of individual *gerP* spores was also extremely heterogeneous. The heterogeneity in *gerP* spore germination appeared to be almost completely in T_{lag}s prior to DPA release, as $\Delta T_{release}$s for wild-type and *gerP* spores were essentially identical, as noted above. Indeed, the heterogeneity in T_{lag}s of *gerP* spores appeared to be much greater than that of wild-type spores, as the majority of *gerP* spores did not germinate with 10 mM L-valine in 150 min. The reason for the extremely long T_{lag}s of the majority of *gerP* spores incubated with L-valine is not clear. However, the factors that increase average T_{lag}s in nutrient germination of wild-type *B. subtilis* spores include (34, 49) (*a*) low germinant concentrations, (*b*) low levels of relevant GRs, (*c*) the absence of GerD, and (*d*) a lack of heat activation prior to initiation of germination. Since *gerP* spores germinated normally after coat removal, the slow germination of intact spores cannot be due to the absence of GerD or low GR levels, and the heat activation temperature used in this work was a bit higher than that needed for rapid spore germination. The only known variable that is left is low germinant concentrations. Since *gerP* spores germinated much slower than wild-type spores at the same nutrient germinant concentration, *gerP* spores presumably require higher exogenous nutrient germinant concentrations to achieve the same level of GR activation as wild-type spores, and this is discussed further below. (iv) The foci of germination proteins seen by fluorescence microscopy of coatless spores carrying a GerD-GFP fusion appeared to be identical in both wild-type and *gerP* spores. This suggests that the decreased nutrient germination of *gerP* spores is not due to misassembly of the germinosome that is known to decrease GR-dependent germination markedly (16), although this conclusion was weakened by the necessity for examination of GerD-GFP fluorescence in severely coat-defective spores.

While the results summarized above were certainly consistent with a role for GerP in germinant permeation into spores, they did not prove this. However, other new results in this communication provide compelling evidence that GerP proteins are essential for rapid nutrient germinant access to GRs in the spore's inner membrane, including the findings that (i) germination at an HP of 150 MPa was identical for wild-type and *gerP* spores and (ii) the dependence of spore germination on nutrient germinant concentration was very different for wild-type and *gerP* spores. (i) An HP of 150 MPa triggers spore germination by activating GRs in some fashion and without the need for any small-molecule germinants, and germination at this HP is directly related to levels of both GRs and GerD (4, 24, 28). Therefore, spore germination at this pressure should be independent of rates of nutrient germinant access to GRs. Consequently, the essentially identical germination of wild-type and *gerP* spores at 150 MPa is further evidence that (*a*) GR and GerD levels are identical in wild-type and *gerP* spores and (*b*) germinant access to GRs is decreased in *gerP* spores. (ii) In nutrient germination of wild-type spores, germination was maximal at relatively low germinant concentrations, ≤10 mM for L-valine and ≤2 mM for L-alanine. Indeed, the L-alanine concentration needed for half-maximal wild-type *B. subtilis* spore germination under these conditions is ≤150 μM (1). In contrast, with *gerP* spores, rates of spore germination increased up to 100 mM L-valine or 1 M L-alanine. This difference is consistent with rates of germinant access to GRs not being rate limiting for wild-type spore germination, while germinant permeation has become rate limiting for germination of *gerP* spores. That this may be due to differences in germinant permeation through spores' outer layers in wild-type and *gerP* spores was further suggested by the elimination of the *gerP* germination defect by removal of the spore coat and outer membrane and the wild-type dependence of *cotE gerP* spore germination on L-valine concentration.

One surprising result in this work was that alanine racemase did not appear to be important in the slower germination of wild-type *B. subtilis* spores at high L-alanine concentrations. In addition, the *alr dal B. subtilis* strain exhibited no spore germination in developing sporangia, in contrast to results with *alr B. anthracis* (8). The reason for the lack of effects of loss of alanine racemase activity in sporulating *B. subtilis* is not clear, but perhaps in sporulating *B. subtilis* cells there is not sufficient free L-alanine to

trigger spore germination, or perhaps GR function is somehow inhibited until late in sporulation. Two possible explanations for the inhibition of wild-type spore germination at high L-alanine concentrations are (i) the presence of D-alanine in the L-alanine and (ii) nonspecific inhibition by the extremely high ionic strength at high L-alanine concentrations. These same two effects would also be exerted on *gerP* spores at high L-alanine concentrations, but the effects of inhibitory D-alanine might be mitigated by slow permeation of the D-alanine to the GerA GR. Thus, there might be three competing forces at play with *gerP* spores at very high L-alanine concentrations: (i) increased germination due to the higher L-alanine concentrations, (ii) decreased germination due to D-alanine in the L-alanine, and (iii) decreased germination due to nonspecific effects.

If, as seems likely, GerP proteins facilitate nutrient germinant access to GRs in spores' inner membrane, how might these proteins do this? As noted above, if the GerP proteins are present in the dormant spore, they are most likely present in the spore coat (3), although they have not yet been identified in spores. The amino acid sequences of the various GerP proteins are also not helpful in suggesting a function for these proteins. However, it is certainly possible that there is a permeability barrier to small hydrophilic molecules in spores' outer layers, as was suggested many years ago (12, 13), although whether this permeability barrier is the spore's outer membrane or some other structural feature of spores and how GerP proteins might facilitate germinants' ability to pass through spores' outer layers are not known. Perhaps detailed analysis of the permeability properties of wild-type and *gerP* spores will help resolve this issue.

ACKNOWLEDGMENTS

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