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Isolation and Characterization of Mutations in *Bacillus subtilis* That Allow Spore Germination in the Novel Germinant D-Alanine

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Bacillus subtilis **spores break their metabolic dormancy through a process called germination. Spore germination is triggered by specific molecules called germinants, which are thought to act by binding to and stimulating spore receptors. Three homologous operons, *gerA*, *gerB*, and *gerK*, were previously proposed to encode germinant receptors because inactivating mutations in those genes confer a germinant-specific defect in germination. To more definitely identify genes that encode germinant receptors, we isolated mutants whose spores germinated in the novel germinant D-alanine, because such mutants would likely contain gain-of-function mutations in genes that encoded preexisting germinant receptors. Three independent mutants were isolated, and in each case the mutant phenotype was shown to result from a single dominant mutation in the *gerB* operon. Two of the mutations altered the *gerBA* gene, whereas the third affected the *gerBB* gene. These results suggest that *gerBA* and *gerBB* encode components of the germinant receptor. Furthermore, genetic interactions between the wild-type *gerB* and the mutant *gerBA* and *gerBB* alleles suggested that the germinant receptor might be a complex containing GerBA, GerBB, and probably other proteins. Thus, we propose that the *gerB* operon encodes at least two components of a multicomponent germinant receptor.**

Upon starvation for one or more nutrients, cells of the gram-positive bacterium *Bacillus subtilis* differentiate into metabolically dormant spores which are adapted to resist environmental damage during dormancy (6, 27). The spore's dormancy and resistance properties ensure its survival through conditions that are not conducive to cell growth. When nutrient-rich conditions return, spore dormancy is broken and the spore is converted back to a vegetative cell through spore germination and outgrowth (9, 14). During that process, the spore loses its dormancy and resistance properties and consequently becomes vulnerable to its environment. Thus, before a spore initiates germination, it must ascertain that the environment is conducive to cell growth.

Many studies have shown that dormant spores use small molecules and ions as indicators of conditions that permit cell growth (35). These indicator molecules, called germinants, are by themselves sufficient to initiate spore germination, and their identity differs significantly between spores of different species. In *B. subtilis*, L-alanine or a combination of L-asparagine, D-fructose, D-glucose, and K^+ ions (AFGK) acts as a germinant to initiate spore germination (32–34). Because many germinants are metabolites, they were originally proposed to reactivate spore metabolism by supplying substrates for spore enzymes (7, 19). However, that hypothesis was challenged by subsequent work which showed that radiolabeled germinants are not significantly metabolized early in germination (25, 26) and that nonmetabolizable analogs of germinants also trigger germination (21, 28). Moreover, investigation of the germination-initiating properties of derivatives and isomers of the known germinants suggested that these molecules probably initiate germination by binding to and activating receptors that are present in the spore (35, 37).

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Candidates for the hypothesized spore germinant receptor(s) were identified in genetic screens for *ger* mutations that blocked spore germination (8, 15, 30). Of the *ger* mutations that were identified in those screens, mutations in *gerA*, *gerB*, and *gerK* conferred a germinant-specific defect in germination. For example, *gerA* mutants failed to germinate only in L-alanine, whereas *gerB* and *gerK* mutants exhibited a defect only in AFGK-induced germination (8, 15). These mutant phenotypes were best explained by a model in which the *gerA* product(s) were required for L-alanine recognition, while the *gerB* and *gerK* products were required for AFGK recognition (16). Subsequent work showed that *gerA*, *gerB*, and *gerK* are homologous tricistronic operons, indicating that these three loci might encode proteins with similar functions (3, 14, 38). In addition, the first two proteins in each operon are predicted to be integral membrane proteins (3, 38), which is consistent with them being receptors for environmental signals. Thus, it was proposed that the *gerA*, *gerB*, and *gerK* operons encode homologous components of distinct germinant receptors (16).

Although attractive, the idea that *gerA*, *gerB*, and *gerK* encode germinant receptors has not been substantiated, and it is not clear whether all three proteins encoded by each of these loci are required for recognition and binding of the germinant. In this work, we tried to address these issues by designing a genetic screen to specifically isolate mutations that affect the germinant receptor(s). We identified three mutations, two of which affected the GerBA protein and one of which affected the GerBB protein. Thus, our studies strongly support a model in which the *gerB* operon (and probably also the *gerA* and the *gerK* operons) encodes components of a spore germinant receptor.

MATERIALS AND METHODS

Strains, plasmids, and media used. *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* strains were constructed by transformation with either chromosomal DNA or plasmid DNA as previously described (1). *Escherichia coli* TG1 and DH5αF′ were used for production of plasmids as described elsewhere (23). The rich media LB and 2×YT were used for growth of *E. coli* and for

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Source or reference
PS832	Wild type	Laboratory stock
FB8	*mut4* isolate (PS832 *gerBA1**)	This study
FB9	*mut8* isolate (PS832 *gerBA2**)	This study
FB10	*mutb1* isolate (PS832 *gerBB1**)	This study
FB11	*mutb2* isolate (PS832 *gerBB1**)	This study
FB12	*muta2* isolate (PS832 *gerBB1**)	This study
FB22	FB8 Δ*gerA*::*spc*	FB8[pFE14]
FB25	FB8 Δ*gerB*:*spc*:*gerBA1**	FB8[pFE16]
FB34	FB8 *gerB*::*spc*	FB8[pFE19]
FB35	FB9 Δ*gerB*:*spc*:*gerBA2**	FB9[pFE16]
FB36	FB10 Δ*gerB*:*spc*:*gerBB1**	FB10[pFE16]
FB41	PS832 Δ*gerB*::*spc*	PS832[pFE106]
FB43	FB41 *amyE*::*gerB*	FB41[pFE97]
FB44	FB41 *amyE*::*gerBA1**	FB41[pFE98]
FB45	FB41 *amyE*::*gerBA2**	FB41[pFE99]
FB46	FB41 *amyE*::*gerBB1**	FB41[pFE100]
FB47	FB41 *amyE*::*gerBA1* gerBB1**	FB41[pFE101]
FB48	FB41 *amyE*::*gerBA2* gerBB1**	FB41[pFE102]
FB49	PS832 *amyE*::*gerB*	FB43→PS832
FB50	PS832 *amyE*::*gerBA1**	FB44→PS832
FB51	PS832 *amyE*::*gerBA2**	FB45→PS832
FB52	PS832 *amyE*::*gerBB1**	FB46→PS832
FB56	PS832 *amyE*::*gerBA1* gerBB1**	FB47→PS832
FB57	PS832 *amyE*::*gerBA2* gerBB1**	FB48→PS832

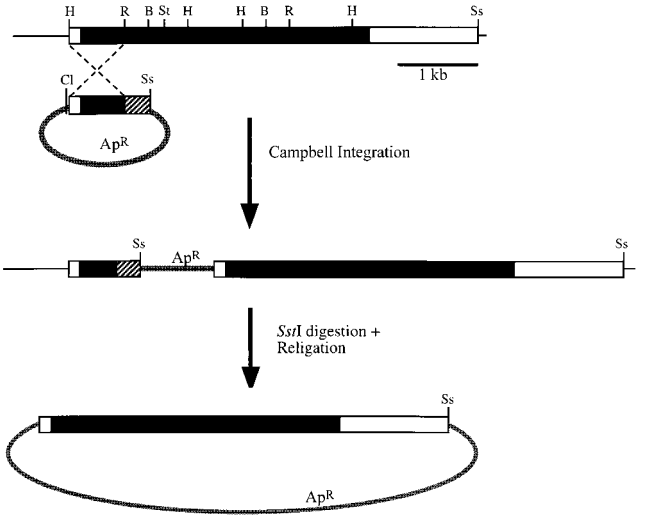

FIG. 1. Restriction map of the 5.3-kb genomic region which contains the *gerB* operon and the strategy used to clone that DNA fragment. The large bar denotes the 5.3-kb genomic region which includes the *gerB* operon demarcated by the solid region within the bar; the solid line represents flanking genomic DNA. The plasmid vector denoted by the thick solid line and the *spc* cassette represented by the hatched bar are not drawn to scale. Restriction enzyme sites: B, *Bam*HI; Cl, *Cla*I; H, *Hin*dIII; R, *Eco*RV; Ss, *Sst*I; St, *Stu*I. Ap^R denotes the ampicillin resistance marker carried on the plasmid.

vegetative growth of *B. subtilis* (23). 2×SG medium was used for *B. subtilis* sporulation at 37°C, and spores were harvested, cleaned, and stored as described elsewhere (18). *B. subtilis* spores that were used in the germination assays were prepared by the resuspension method at 30°C (29). When necessary, growth media were supplemented with (per liter) 50 or 100 mg of ampicillin; 100 mg of spectinomycin; 1 mg of erythromycin and 25 mg of lincomycin (MLS); or 5 mg of chloramphenicol.

The Δ*gerA*::*spc* plasmid was derived from plasmid pJL74 (13), which contains the spectinomycin resistance (*spc*) cassette. A DNA fragment containing the 5′ region of the *gerA* operon was PCR amplified from genomic DNA with primers gerAΔ-N5 (5′ CACGGCCGCACGATAATTTAGCATTGG) and gerAΔ-N3 (5′ CGGGATCCTCTACAAACGCTAC), which hybridize starting at (underlined position) nucleotides (nt) +31 and +422 relative to the translation start site (+1) of the *gerAA* gene. The PCR fragment was cut with *Eag*I and *Bam*HI (which cut within primers gerAΔ-N5 and gerAΔ-N3, respectively) and inserted between the *Eag*I-*Bam*HI sites of plasmid pJL74 (13) to create plasmid pFE11. The 3′ region of the *gerA* operon was PCR amplified from genomic DNA with primers gerAΔ-C5 (5′ AACTGCAGAACGATGGAGCCAG) and gerAΔ-C3 (5′ GAG GATAATGAATTCTGACC), which hybridize starting at (underlined position) nt +3347 and +3858 relative to the *gerAA* translation start site (+1). The resulting PCR fragment was cut with *Pst*I (which cuts once within primer gerAΔ-C5 and once within the amplified sequence) and inserted at the *Pst*I site in plasmid pFE11. The *Pst*I fragment in plasmid pFE14 was oriented such that it created Δ*gerA*::*spc*. Plasmid pFE14 was linearized with *Eco*RI prior to transformation into *B. subtilis*, and proper integration of the Δ*gerA*::*spc* fragment was confirmed by Southern blot analysis.

Plasmid pFE19 was used to introduce an insertional mutation in the *gerB* operon. A DNA fragment internal to the *gerB* operon was PCR amplified from genomic DNA with primers gerB15 (5′ GCTTGAACAGCTGATTGAAG) and gerB27 (5′ CCTACATGATAGATGGCAAC), which hybridize starting at (underlined position) nt +630 and +1861 relative to the *gerBA* translation start site (+1). The amplified DNA was cut with *Hin*dIII and *Stu*I (which cut within the amplified sequence [Fig. 1]) and inserted between the *Hin*dIII-*Eco*RV sites of plasmid pJL74 (13). The resulting plasmid pFE19 contained the region between the *Stu*I and *Hin*dIII sites in the *gerB* operon (solid bar in Fig. 1), and its insertion by Campbell integration (5) generated an insertional mutation in *gerB* designated *gerB*::*spc*. Note that the *gerBA1**, *gerBA2**, and *gerBB1** mutations (see below) lie outside the *Stu*I-*Hin*dIII region and thus are not lost by recombination with plasmid pFE19. Plasmid pFE106, which was used to introduce a Δ*gerB*::*spc* mutation, was constructed from plasmid pFE24 (see below) by removing the region between the *Bam*HI and *Pst*I sites in pFE24 and replacing it with a *Bam*HI-*Pst*I fragment containing the *spc* cassette from plasmid pJL74. The resulting plasmid pFE106 was linearized with *Sst*I prior to transformation. Correct insertion of pFE19 and pFE106 was confirmed by Southern blot analysis.

Mutagenesis. Mutagenized cultures of *B. subtilis* PS832 were generated by ethyl methanesulfonate mutagenesis of exponentially growing cells as described previously (5). The mutagenized cultures were sporulated by nutrient exhaustion, and the spores were harvested, cleaned, and stored as described elsewhere (18).

Separation of germinated and ungerminated spores. Germinated and ungerminated *B. subtilis* spores were separated on a metrizoic acid gradient on the basis of buoyant density (18). The gradient was prepared in a 2.5-ml ultraclear ultracentrifuge tube (Beckman, Fullerton, Calif.) by sequential layering of 0.1 ml of 70%, 0.5 ml of 60%, 0.2 ml of 50%, 0.2 ml of 40% and 0.2 ml of 30% metrizoic acid solutions. The spore suspension which was to be separated (in 0.2 ml of 20% metrizoic acid) was layered on top of the gradient, which was centrifuged at 13,000 rpm in a TLS-55 rotor (TL100 ultracentrifuge) for 45 min at 4°C. The deceleration was set at 8 to avoid disturbing the gradient at the end of the run. The dormant spores concentrated in the 70% layer at the bottom of the gradient, whereas germinated spores formed a band in the 50% layer. For purification of dormant spores, the 70% layer (0.1 to 0.2 ml) was recovered with a Pasteur pipette, diluted 10-fold in water, and centrifuged for 20 s to pellet the spores. The dormant spores were washed 10 times with 1 ml of water before use. For enrichment of germinated spores, the 50% layer (0.2 ml) was recovered with a Pasteur pipette and inoculated into 5 ml of 2×YT broth. After the culture had grown to saturation at 37°C, it was divided into 1-ml aliquots which were either frozen for storage, plated out for screening individual colonies, or subcultured into 200 ml of 2×SG medium for sporulation.

Assays of spore germination. A modification of a previously described filter assay (12, 15) was used to identify *B. subtilis* colonies whose spores germinated in D-alanine. Briefly, *B. subtilis* colonies were patched onto 2×SG agar plates (wrapped in a plastic bag to reduce drying) and sporulated by incubation at 37°C for 5 days. The sporulated colonies were lifted onto nitrocellulose filters, which were then baked at 65°C for 3 h to kill vegetative cells and heat activate dormant spores. After cooling to room temperature, the filters were placed on a Whatman 3MM paper disc soaked in germination solution (10 mM Tris-HCl [pH 8.4]), 1 mg of 2,3,5-triphenyltetrazolium chloride per ml, 2.5 mM glucose, 10 mM test germinant) and incubated at 37°C for 4 to 8 h. Colonies that contained germinating spores developed a red color because germinated but not dormant spores can reduce the tetrazolium dye (12, 15). Glucose was included in the germination solution because it enhanced red color development in the control studies which were used to standardize the protocol.

Liquid germination assays were used to more quantitatively compare the germination of spores from different strains (18). Spore suspensions at an optical density at 600 nm (OD_{600}) of 40 to 80 were heat activated at 70°C for 15 min and diluted to an OD_{600} of 0.5 to 0.7 in a plastic cuvette containing 1 ml of the germination mix (10 mM Tris-HCl [pH 8.4] plus 1 mM D-glucose, with or without 10 mM germinant) at room temperature. The cuvettes were covered with parafilm and mixed by inverting. The initial OD_{600} was recorded, the cuvettes were warmed to and maintained at 37°C, and the OD_{600} was read at 20- to 30-min intervals. The spores from different strains that were compared in these assays were prepared in parallel by the resuspension method using the same batch of medium.

Genetic mapping. The *B. subtilis* mapping strains, 1A627 to 1A645, were obtained from the *Bacillus* Genetic Stock Center, Ohio State University, and

phage PBS1 stock was obtained from Wayne Nicholson, University of Arizona. Standard procedures were used for phage PBS1 manipulation (4), except that 2× nutrient broth was used in place of brain heart infusion broth to culture *B. subtilis* cells for infection.

Recovery of the *gerB* operon from wild-type and mutant *B. subtilis* strains. To recover the *gerB* operon from *B. subtilis* strains, the 5′ region of the *gerB* locus was PCR amplified from strain PS832 chromosomal DNA with primers gerB06 (5′ GGTGATTGCGTCATGATCC) and gerB18 (5′ GAAATGGCCATTCTA GTCGG), which hybridize starting at (underlined positions) nt −279 and +950 relative to the *gerBA* translation start site (+1). A 643-bp *Hin*dIII-*Eco*RV fragment contained within the PCR fragment was subcloned between the same sites in plasmid pJL74 (13) to create plasmid pFE16. Plasmid pFE16 was used to transform the *B. subtilis* strain whose *gerB* operon was to be recovered to spectinomycin resistance. Transformants in which plasmid pFE16 had inserted at the *gerB* locus by Campbell integration (Fig. 1) were identified by Southern blot analysis and are designated Δ*gerB*::*spc*:*gerB* because they contain a Δ*gerB* operon, which is truncated at the first *Eco*RV site in Fig. 1, followed by the *spc* cassette and then a full-length *gerB* operon with an intact promoter (2) (Fig. 1). Chromosomal DNA from those transformants was linearized with *Sst*I and ligated, and the ligation mix was used to transform *E. coli* TG1 to Ap resistance (Fig. 1). Plasmids carrying the 5.3-kb *gerB* fragment from the different strains are designated as follows: pFE24, wild-type strain PS832; pFE23, *mut4* strain FB8; pFE25, *mut8* strain FB9; pFE26, *mutb1* strain FB10; pFE28, *mutb2* strain FB11; and pFE29, *muta2* strain FB12.

Site-directed mutagenesis. The 1.5-kb *Bam*HI fragment from the wild-type *gerB* operon was cloned at the *Bam*HI site in pUC19 to generate plasmid pFE45, which was mutagenized by using a Transformer site-directed mutagenesis kit (Clontech, Palo Alto, Calif.). The selection primer pUC19-RI/RV (5′ CGGCC AGTGATATCGAGCTCGG) was used in combination with one of three mutagenic primers, gerBMut4 (5′ CATTTATTTGCCCAGTCTGTATATTTCTC), gerBMut8 (5′ GCAGGCTTAACGTATCATTCCCGCC), or gerBMutb1 (5′ TATTGAACGAATTGATTTGTTCTTACAG), to introduce the *gerBA1**, *gerBA2**, or *gerBB1** mutation, respectively. Each mutagenized 1.5-kb *Bam*HI region was sequenced completely to ensure that it carried only the site-directed mutation. The *Bam*HI fragment from each mutant plasmid pFE67 (*gerBA1**), pFE68 (*gerBA2**), and pFE69 (*gerBB1**), was then used to replace the *Bam*HI fragment from the *gerB* operon in plasmid pFE24 to construct the single-mutant *gerB* operon plasmids pFE70 (*gerBA1**), pFE71 (*gerBA2**), and pFE72 (*gerBB1**). The *gerBA1* gerBB1** double-mutant plasmid (pFE76) was constructed by replacing a 1.6-kb *Cla*I-*Stu*I fragment (Fig. 1) in pFE72 with the same fragment from pFE70. The *gerBA2* gerBB1** double-mutant plasmid (pFE77) was similarly constructed from pFE72 and pFE71.

Integration of wild-type and mutant *gerB* operons at the *amyE* locus. The wild-type and mutant *gerB* operons were cloned into plasmid pDG364 (5) in two steps. Initially, we constructed pFE96, which is a pDG364 derivative containing a wild-type *gerB* operon (including its own promoter [2]) lacking the internal 1.5-kb *Bam*HI fragment. In the second step, the 1.5-kb *Bam*HI fragments from the wild-type (pFE24) and mutant (pFE70, pFE71, pFE72, pFE76, pFE77) *gerB* plasmids were cloned in the correct orientation into pFE96 to generate plasmids pFE97 through pFE102, respectively. Each plasmid was linearized with *Bgl*II and used to transform a Δ*gerB*::*spc* strain, FB41, to chloramphenicol resistance. Transformants in which the plasmid-borne *gerB* operon had integrated at the *amyE* locus were identified by their *amy* phenotype and Southern blot analysis.

Plasmid pFE96 was generated by a multistep process. Initially, a 1.3-kb fragment containing the 5′ end of the *gerB* operon was PCR amplified from wild-type genomic DNA with primers gerB06 (see above) and gerBpET3 (5′ GAAGATC TGAGCTCCGATGACAACGCCGCG), which hybridizes starting at (underlined position) nt +1099 relative to the *gerBA* translation start site (+1). This fragment was cloned into vector pCR2.1 (TA cloning kit; Invitrogen, San Diego, Calif.), sequenced, recovered as an *Eco*RI fragment (*Eco*RI sites are present in vector pCR2.1), and inserted into the *Eco*RI site of plasmid pFE91 (a derivative of plasmid pUC18 lacking the *Ecl*136II-*Hin*cII region) to generate plasmid pFE92. The 4.1-kb *Stu*I-*Sst*I fragment from plasmid pFE24 (Fig. 1) was inserted between the same sites in pFE92 to generate plasmid pFE93, which contains the wild-type *gerB* operon with a *Bgl*II site at its 3′ end. The 1.6-kb *Hin*dIII-*Bgl*II fragment from pFE93 was inserted between the *Hin*dIII-*Bam*HI sites in pDG364 to generate plasmid pFE95. A 2.1-kb *Hin*dIII-*Hin*dIII fragment from pFE94 (pFE24 lacking the 1.6-kb *Bam*HI fragment) was cloned into the *Hin*dIII site of plasmid pFE95 to generate plasmid pFE96. The *Hin*dIII fragment in plasmid pFE96 was oriented to generate a *gerB* operon that lacked the 1.5-kb *Bam*HI fragment.

RESULTS

Isolation of D-alanine responsive mutants. To identify spore germinant receptor(s), we decided to isolate *B. subtilis* mutants whose spores germinated in the novel germinant D-alanine because we expected such mutants to arise as the result of mutations in a gene encoding a preexisting germinant receptor. As it is difficult to identify rare mutant spores that germinate in D-alanine within a population of wild-type spores, we initially enriched a spore population for mutants that could germinate in D-alanine. The enrichment was achieved by separating germinated and dormant spores on the basis of their differential migration in a buoyant density gradient (18). The separation protocol was standardized for spores of our wild-type strain PS832 by centrifuging a mixture of germinated (in 10 mM L-alanine) and ungerminated spores in a 20 to 70% metrizoic acid gradient. After centrifugation, the spores were concentrated in two major bands (data not shown); the dormant spores migrated to the 70% metrizoic acid layer, while the germinated spores concentrated in the 50% metrizoic acid layer. The resolution of the two bands was further improved by increasing the height of the intervening 60% metrizoic acid layer (Materials and Methods).

To isolate mutant spores that germinated in D-alanine, we started with spores obtained from ethyl methanesulfonate-mutagenized cells. The spores were incubated in a germination mix containing 10 mM D-alanine as the sole germinant for 1 h at 37°C, concentrated in a microcentrifuge, and centrifuged in a metrizoic acid gradient (Materials and Methods). As expected, most of the spores did not germinate in D-alanine and formed a single band at the position of the dormant spores. Although we did not observe a band of germinated spores in the 50% metrizoic acid layer, we inoculated that fraction in 2×YT broth to recover any spores that might have germinated in D-alanine. The culture was then sporulated in 2×SG medium, and the spores were used for a subsequent round of enrichment. After the third round of enrichment, the enriched culture was plated on LB agar plates to recover individual colonies. One thousand of these colonies were then sporulated on 2×SG plates and individually tested for spore germination in D-alanine by the plate assay (Materials and Methods). Two colonies, called *mut4* and *mut8*, developed a red color indicative of spore germination in D-alanine. To confirm that color development was the result of spore germination, spores from both red colonies and colonies without red color were inspected by phase-contrast microscopy. Whereas spores from colonies without red color appeared bright under phase-contrast optics, spores from the red colonies were dark, suggesting that *mut4* and *mut8* spores had indeed germinated in D-alanine. Interestingly, the *mut8* spores took longer to develop the red color than the *mut4* spores, suggesting that the two mutants were not identical. Three additional mutants, *mutb1*, *mutb2*, and *muta2*, were recovered when the overall screen was repeated with a second batch of independently mutagenized cells.

Response of the mutants to different germinants. While we hoped that the mutant spores were germinating specifically in D-alanine, it was possible that they were simply unstable and had a tendency to germinate nonspecifically. To address this possibility, wild-type and mutant spores were purified, heat activated, and incubated at 37°C in a germination mix (10 mM Tris-HCl [pH 8.4], 1 mM D-glucose) with or without added D-alanine. Germination of the spore suspensions was followed by measurement of the OD_{600}, which decreases as the phase-bright dormant spores germinate and become phase dark. In the germination reaction lacking D-alanine, neither wild-type nor mutant spore suspensions showed a significant change in OD_{600} (<2%) (Fig. 2A and data not shown), indicating that none of those spores germinated in the absence of D-alanine. When 10 mM D-alanine was added to the germination reaction, spores from all five mutants but not wild-type spores germinated (Fig. 2A and data not shown). The requirement for D-alanine seemed to be saturable since germination of the mutant spores in 10 mM D-alanine was comparable to that in

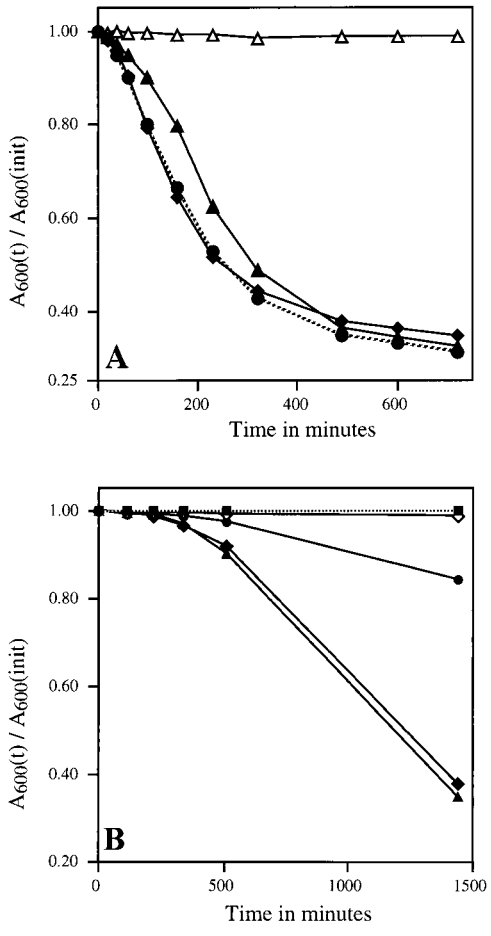

FIG. 2. Germination of mutant spores in the novel germinant, D-alanine, in the presence (A) or absence (B) of D-glucose. Spores from wild-type strain PS832 (■) or mutant strain FB8 (*mut4*) (◇, ◆), FB9 (*mut8*) (●), or FB10 (*mutb1*) (△, ▲) were heat activated and subsequently incubated in 10 mM Tris-HCl (pH 8.4) buffer (open symbols) or buffer supplemented with 10 mM D-alanine (closed symbols) with (A) or without (B) 1 mM D-glucose at 37°C. The OD_{600} (shown here as A_{600}) of each sample was measured periodically and plotted as a fraction of the initial OD_{600} [$A_{600}(t)/A_{600}(init)$] versus time. Spores from all strains produced overlapping, reasonably flat curves when incubated in buffer alone, and only one representative curve is shown (△ in panel A and ◇ in panel B). Wild-type PS832 spores produced identical curves in D-alanine in the presence or absence of D-glucose, and only one representative curve (■ in panel B) is shown.

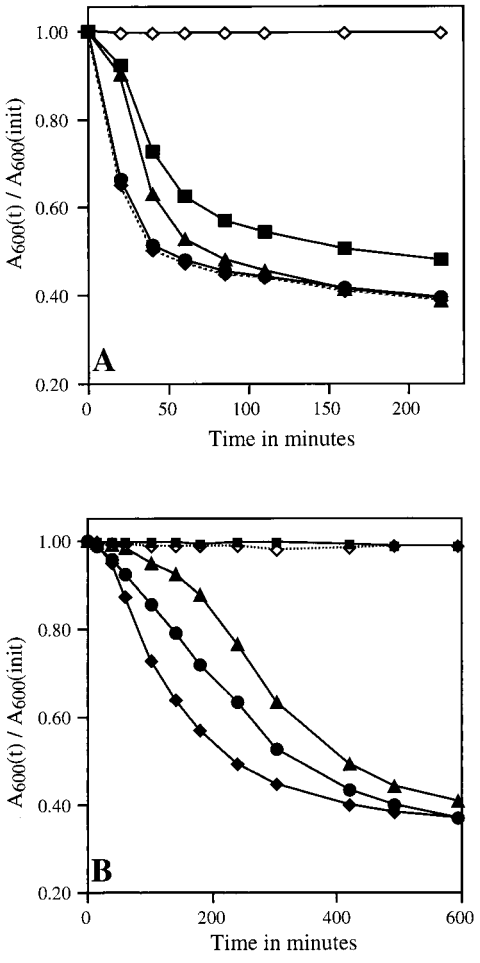

FIG. 3. Germination of mutant spores in the germinants L-alanine (A) and L-asparagine (B). Spores from wild-type strain PS832 (■) or mutant strain FB8 (*mut4*) (◇, ◆), FB9 (*mut8*) (●), or FB10 (*mutb1*) (▲) were assayed for germination in buffer alone (open symbols), 10 mM L-alanine (closed symbols in panel A), or 10 mM L-asparagine (closed symbols in panel B) as described in the legend to Fig. 2. Note that D-glucose was not present in these germination reactions. Spores from all strains produced overlapping, reasonably flat curves when incubated in buffer alone, and only one representative curve is shown (◇ in both panels).

20 mM D-alanine but faster than that in 1 mM D-alanine (data not shown). These observations suggested that germination of the mutant spores in D-alanine was not due to spore instability and was dependent on the presence of D-alanine in the germination reaction. Nevertheless, germination of the mutant spores in D-alanine was slower than in L-alanine (Fig. 3A; see below), suggesting that D-alanine was not an optimal germinant.

The germination mix used above contained D-glucose, which was included because it enhanced color development in the plate assays (Materials and Methods). As D-glucose is a known germinant in certain *Bacillus* spp. (20), we assessed its contribution to germination in D-alanine. When D-glucose was excluded from the germination mix, all mutant spores germinated in the presence of D-alanine, albeit at a considerably lower rate (Fig. 2B). Thus, D-glucose enhanced, but was not necessary for, germination of the mutant spores in D-alanine.

To determine if the mutant phenotype could be attributed to a change in a germinant receptor, we examined the response of the mutant spores to two known germinants, L-alanine and AFGK (32, 34). We reasoned that if the mutant spores possessed a mutant germinant receptor(s), then they might respond differently to these germinants. As shown in Fig. 3A, the patterns of L-alanine-induced germination of wild-type and mutant spores were comparable. However, spores from all mutants germinated much faster than wild-type spores in AFGK (data not shown). Moreover, mutant spores also germinated in L-asparagine (Fig. 3B), which does not normally induce germination of wild-type spores (Fig. 3B) (32). Thus, the mutant spores exhibited an altered response to AFGK and L-asparagine, suggesting that the mutations might have altered the germinant receptor(s) that normally sense AFGK.

The above observations also argued against the possibility that the mutant spores germinated in D-alanine by efficiently converting it to the germinant L-alanine. A D-alanine racemase activity, which interconverts D-alanine and L-alanine, is associated with spores (10), and its upregulation presents a simple explanation for the mutant phenotype. However, that explana-

tion could not easily account for the ability of the mutant spores to germinate in L-asparagine. Furthermore, genetic linkage studies (see below) showed that the mutations were not linked to the *dal* locus (~44.2 degrees on the chromosome), which encodes the D-alanine racemase, nor the *yncD* locus (~162 degrees), which encodes a hypothetical protein that is homologous to D-alanine racemase.

Genetic mapping of the mutant loci. Because preliminary characterization of the mutants suggested that they might contain mutations in a germinant receptor, we genetically mapped the mutations. Initially, we used PBS1-mediated generalized transduction to determine the linkage between the *mut4* mutation in strain FB8 and the MLS resistance marker in 19 *B. subtilis* mapping kit strains, each of which carries the MLS resistance marker at a unique chromosomal location (31). PBS1 transducing lysates made in each mapping strain (1A627 to 1A645) were used to transduce strain FB8 to MLS resistance, and spores from at least 50 MLS-resistant transductants were tested for germination in D-alanine by the plate assay. We found that 85% of the MLS-resistant transductants obtained from lysates made in strain 1A644 had lost the mutant phenotype. Thus, the MLS resistance marker and the wild-type allele of the *mut4* mutation from strain 1A644 cotransduced 85% of the time, suggesting that the two loci were linked. Consistent with this finding, none of the MLS resistance markers in the 18 other mapping strains showed any linkage to the *mut4* locus. To determine if the remaining four mutations (*mut8*, *mutb1*, *mutb2*, and *muta2*) mapped within the same region, we measured the frequency at which they cotransduced with the MLS resistance markers from strains 1A644 and 1A645. Again, all four mutations cotransduced 80 to 90% of the time with the MLS resistance marker in strain 1A644 but showed no significant cotransduction with the MLS resistance marker in strain 1A645. Thus, all five mutations were linked to the MLS resistance marker located at 316 degrees on the chromosome in strain 1A644.

To refine the genetic mapping, we examined the linkage of the mutations to the MLS resistance marker in strains 1A644 and 1A645 by cotransformation. Genomic DNA from strains 1A644 or 1A645 was used to transform each mutant to MLS resistance, and at least 50 of those transformants were sporulated and tested for spore germination in D-alanine. A very low DNA/cell ratio (<10 ng/transformation) was used for transformation to prevent congression which results when a single cell takes up two different pieces of DNA. When 1A644 chromosomal DNA was used to transform the mutants, 8 to 10% of the MLS-resistant transformants exhibited a wild-type germination phenotype. In contrast, no detectable cotransformation was observed between the mutant loci and the MLS resistance marker in strain 1A645. Thus, the mutant loci cotransformed with the MLS-resistance marker in strain 1A644, suggesting that the mutations were located very near 316 degrees on the chromosome.

Because the *gerB* operon, which is required for AFGK-induced germination, maps close to 315 degrees on the chromosome (3), we further examined the linkage of the *mut4* mutation to the *gerB* locus. The *gerB* locus in the *mut4* strain FB8 was marked with a spectinomycin resistance cassette as described in Materials and Methods to create a *mut4* Δ*gerB*::*spc*: *gerB* strain FB25. Chromosomal DNA from strain FB25 was then transformed into a wild-type strain, PS832, to determine cotransformation linkage between the *mut4* mutation and the *spc*-marked *gerB* locus. Out of 100 spectinomycin-resistant transformants tested, spores from 92 transformants germinated in D-alanine. Thus, the *mut4* mutation was very tightly linked to the *gerB* locus.

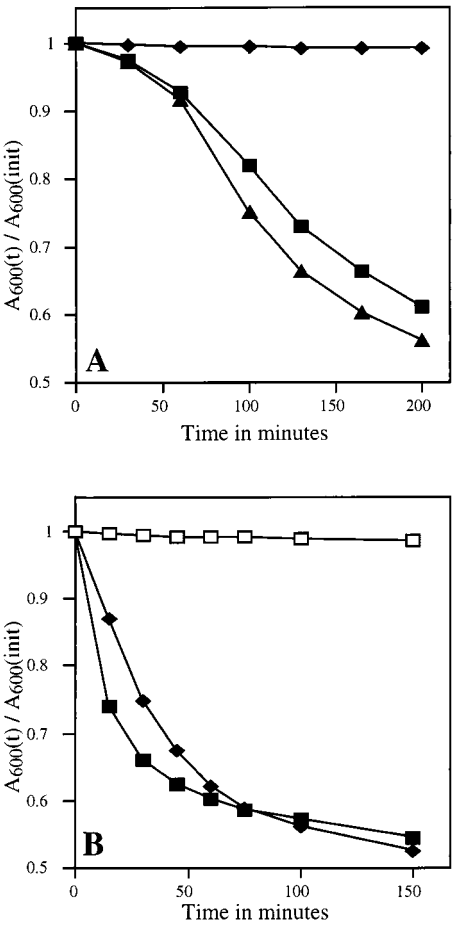

FIG. 4. Effect of *gerA* or *gerB* disruption on *mut4* spore germination in D-alanine (A) and L-alanine (B). Spores from the *mut4* strain FB8 (□, ■), *mut4* Δ*gerA*::*spc* strain FB22 (▲), or *mut4 gerB*::*spc* strain FB34 (◆) were assayed for germination in buffer (open symbols) with either 10 mM D-alanine and 1 mM D-glucose (closed symbols in panel A) or 10 mM L-alanine alone (closed symbols in panel B) as described in the legend to Fig. 2. Spores from all strains produced overlapping, flat curves when incubated in buffer alone, and only one representative curve is shown (□ in panel B).

Effect of a *gerB* mutation on the mutant germination phenotype. The tight linkage of the *mut4* mutation to the *gerB* locus suggested that the mutation might affect a *gerB* cistron. This idea was also consistent with the response of the mutant spores to AFGK and L-asparagine. We reasoned that if a mutant GerB protein was indeed responsible for the *mut4* phenotype, then disruption of the *gerB* operon would eliminate the mutant phenotype. To test this prediction, the *gerB* operon was disrupted in the *mut4* mutant, and spores from the *mut4* strain FB8 and its *gerB* derivative strain FB34 were tested for germination in various germinants. Unlike the *mut4* spores, the *mut4 gerB* double-mutant spores failed to germinate in D-alanine (Fig. 4A). The germination defect of the double-mutant spores was specific to D-alanine and was not apparent in other germinants such as L-alanine (Fig. 4B) or a rich medium (data not shown). Thus, the *mut4* spores required an intact *gerB* operon for germination in D-alanine. Moreover, that requirement was specific to the *gerB* operon because disruption of *gerA*, which is highly homologous to *gerB* (3), did not affect germination of *mut4* spores in D-alanine (Fig. 4A).

Because the other four mutations mapped very close to the *mut4* mutation, we also examined their interaction with *gerA*

TABLE 2. Effect of *gerB*-containing 5.3-kb genomic fragment from wild-type or mutant donor strains on germination of wild-type spores

Donor strain[a]	MLS^r transformants	
	No. tested[b]	% Germinated[c]
PS832	245	0
FB8 (*mut4*)	384	21
FB9 (*mut8*)	49	33
FB10 (*mutb1*)	98	59
FB11 (*mutb2*)	49	67
FB12 (*muta2*)	98	48

[a] Donor strain from which the *gerB*-containing 5.3-kb genomic fragment was cloned. The cloned DNA (300 to 500 ng) was introduced into the wild-type strain (PS832) by congression with the MLS resistance marker of strain 1A640 (10 ng of chromosomal DNA).

[b] By plate assay for D-alanine-induced spore germination.

[c] Percentage that developed a red color in the plate assay for D-alanine-induced germination.

and *gerB* disruptions. Spores from *gerA* and *gerB* derivatives of the *mut8*, *mutb1*, *mutb2*, and *muta2* mutants were tested for germination in D-alanine by the plate assay. Whereas sporulated colonies of all single *mut* mutant and double *mut gerA* mutants developed a red color in the presence of D-alanine, those of the *mut gerB* double mutants failed to develop a red color (data not shown). Thus, the D-alanine-induced germination of spores from all five mutant strains was dependent on GerB but not GerA function, consistent with the idea that the mutations affected GerB function.

Recovery of the *gerB* operon from the mutant strains. Because a variety of criteria suggested that the mutations which allowed spore germination in D-alanine affected the *gerB* operon, we decided to localize the mutations within the *gerB* operon. For this purpose, a 5.3-kb genomic DNA fragment, which contained the *gerB* operon and 1.6 kb of downstream DNA, was recovered from wild-type and mutant strains by a two-step integration-recovery method (Fig. 1). The recovered DNA, which was not linked to a selectable marker, was then introduced into the wild-type strain PS832 by congression (Table 2) with the unlinked, chromosomal MLS resistance marker from strain 1A640. Spores from at least 49 MLS-resistant transformants were tested for germination in D-alanine by the plate assay. As expected, all transformants obtained by introduction of the wild-type 5.3-kb DNA fragment produced only wild-type spores (Table 2). However, when the genomic fragment derived from the *mut4* mutant was used, 21% of the MLS-resistant transformants produced spores that germinated in D-alanine (Table 2). Thus, the genomic fragment containing the *gerB* operon and some downstream DNA from the *mut4* strain conferred the mutant phenotype on an otherwise wild-type strain. Similar experiments showed that the same 5.3-kb genomic fragment from each mutant was sufficient to confer the mutant phenotype in a wild-type strain (Table 2), suggesting that all five mutations lay within the same 5.3-kb region of the chromosome.

To more precisely map the mutations within the 5.3-kb fragment, we generated wild-type–mutant chimeric fragments and tested their effect on spore germination. The 5.3-kb DNA fragment contains an internal 1.5-kb *Bam*HI fragment (Fig. 1), which spans part of the *gerBA* and *gerBB* cistrons. Chimeric plasmids were constructed by removing this 1.5-kb *Bam*HI fragment from the wild-type *gerB* operon in plasmid pFE24 and substituting the same fragment from each mutant *gerB* operon. The resulting five chimeric plasmids were transformed into a wild-type strain by congression as described above, and spores from the transformants were scored for germination in D-alanine by the plate assay. Each chimeric plasmid, but not the wild-type plasmid, conferred a mutant phenotype in at least 30% of the MLS-resistant transformants, indicating that all of the mutations were located within the 1.5-kb *Bam*HI fragment.

Sequences of the *gerB* operons from mutant and wild-type strains. Because the mutations mapped within the 1.5-kb *Bam*HI region in the *gerB* operon, we identified the mutations at the DNA level by sequencing that region of the *gerB* operon from wild-type and mutant strains. Compared to the wild-type sequence, the *gerB* operon from the *mut4* mutant showed a single G→A transition which resulted in a Gly_{297} (GGU)→Ser (AGU) substitution in the *gerBA* open reading frame (Fig. 5). The sequence of the *gerB* operon from the *mut8* mutant differed from the wild-type sequence by a single C→T transition in the *gerBA* open reading frame (Fig. 5). This transition produces a Pro_{326} (CCA)→Ser (UCA) alteration in the predicted GerBA protein (Fig. 5). The *gerB* operon from the *mutb1* mutant showed no alteration in the *gerBA* cistron but contained a single T→A transversion which produced a Phe_{269} (TTT)→Ile (ATT) substitution in the *gerBB* open reading frame (Fig. 5). The *gerB* operons from the *mutb2* and *muta2* mutants contained the same T→A transversion, indicating that these three mutants probably arose as the result of a single mutagenic event. Thus, the screen yielded three independent mutations in the *gerB* operon, two in the *gerBA* cistron and one in the *gerBB* cistron, that allowed spores to germinate in D-alanine. The mutant alleles from the *mut4*, *mut8*, and *mutb1* strains will be referred to as *gerBA1**, *gerBA2**, and *gerBB1**, respectively, in the remainder of the text.

While comparing the sequence of the 1.5-kb *Bam*HI fragment obtained from the wild-type PS832 strain with the sequence in the *Bacillus* genome database, we observed several differences (Fig. 5). Each of these changes was present in six independently isolated genomic clones and thus probably reflects a polymorphism between strain PS832 and the *B. subtilis* strain from which the *gerB* was previously sequenced.

Introduction of each *gerB mutation into a wild-type strain.** Although the data presented above strongly indicated that the mutations which we had identified by DNA sequence analysis were solely responsible for the mutant phenotype, we felt it important to prove this point conclusively. To this end, each mutation was first engineered by site-directed mutagenesis into a plasmid containing the wild-type 1.5-kb *Bam*HI fragment. The entire mutagenized DNA fragment was sequenced to ensure that it contained only the appropriate mutation and then used to replace the *Bam*HI fragment in the wild-type *gerB* plasmid, pFE24. The resulting plasmid was introduced into the wild-type strain PS832 by congression with the chromosomal MLS resistance marker from strain 1A640, and spores from at least 50 MLS-resistant transformants were scored for germination in D-alanine. When a plasmid carrying any one of the three mutagenized *Bam*HI fragments was used, 15 to 50% of the colonies yielded spores that germinated in D-alanine (Table 3). By comparison, none of the MLS-resistant transformants obtained by introduction of the wild-type *gerB* plasmid, pFE24, exhibited the mutant phenotype (Table 3). Thus, each of the three mutations allowed otherwise wild-type spores to germinate in D-alanine.

To further demonstrate that each mutation was sufficient to confer the mutant phenotype, we constructed strains that contained a single, ectopic copy of either the wild-type or a mutant *gerB* operon at the *amyE* locus. The strains were sporulated by the resuspension method, and the spores were tested for germination in D-alanine. While spores from strain FB43, which contains the wild-type *gerB* operon, failed to germinate in D-

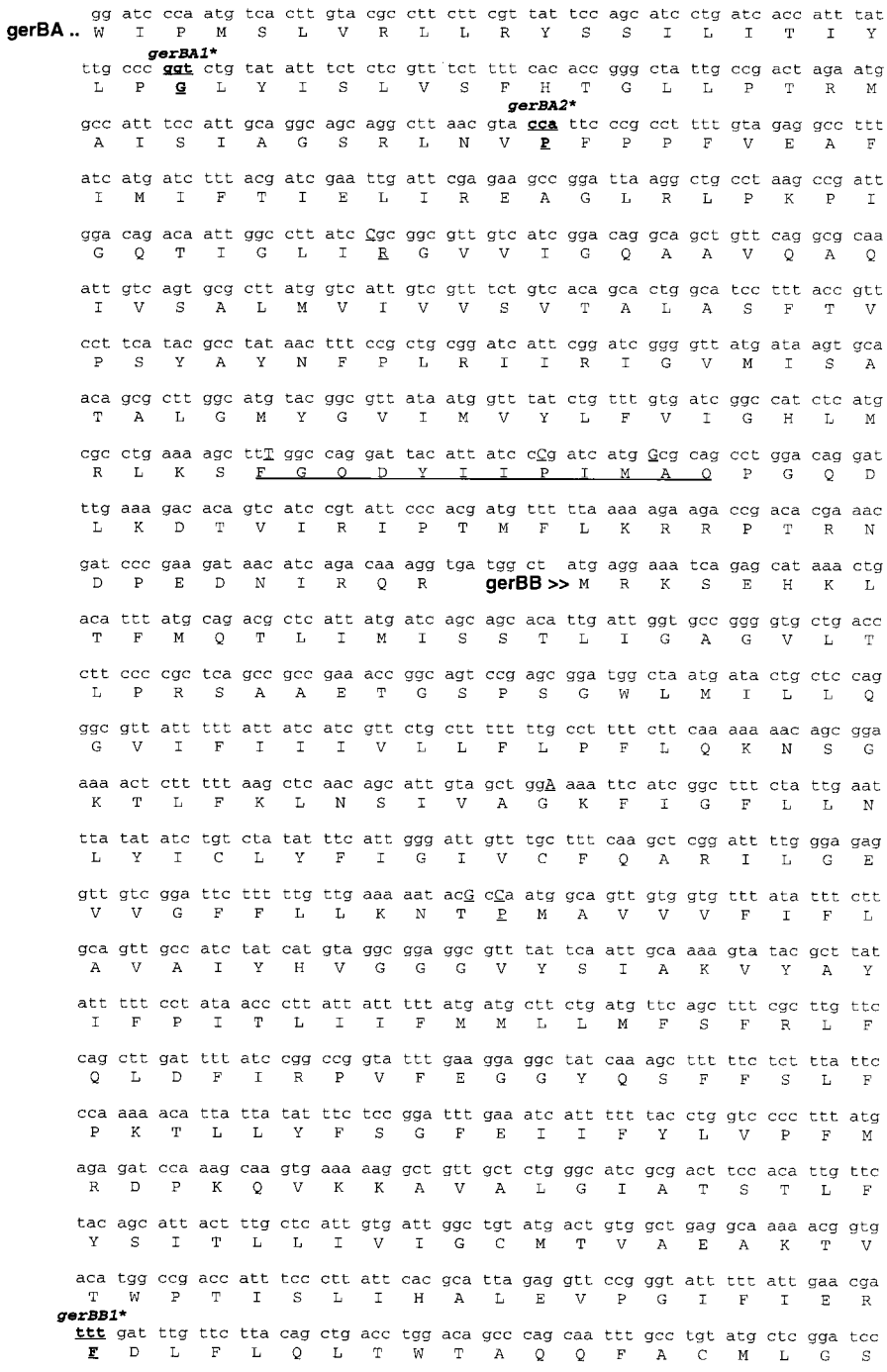

FIG. 5. Locations of the mutations within the *gerB* operon. The DNA sequence of the 1.5-kb *Bam*HI fragment from strain PS832 is shown together with the predicted protein sequences of the *gerBA* and *gerBB* open reading frames. The locations of the *gerBA1** (Gly$_{297}$ [GGU]→Ser [AGU]), *gerBA2** (Pro$_{326}$ [CCA]→Ser [UCA]), and *gerBB1** (Phe$_{269}$ [UUU]→Ile [AUU]) mutations are represented by boldface underlined letters. Deviations of the *gerB* sequence from that of our wild-type strain PS832 and the published sequence (11, 17) and the resulting amino acid changes (if any) are underlined.

alanine, spores from strains that contained either *gerBA1** (FB44), *gerBA2** (FB45), or *gerBB1** (FB46) mutant operons germinated in D-alanine (Fig. 6). Thus, the single-amino-acid changes were indeed sufficient to product the mutant phenotype.

Dominant/recessive nature of the *gerBA* and *gerBB* mutations. To determine if the phenotype conferred by the *gerB** mutations could be attributed to a new function gained by the mutant GerBA* and GerBB* proteins, we examined if the *gerB** mutations were dominant over the wild-type *gerB* allele. Haploid strains (which contained a single-mutant *gerB** operon) and merodiploid strains (which contained a wild-type *gerB* and a mutant *gerB** allele) were constructed by inserting mutant *gerB* operons at the *amyE* locus in either a Δ*gerB*::*spc* and a wild-type strain, respectively. The strains were sporulated by resuspension, and the spores were assayed for germi-

TABLE 3. Effect of wild-type and mutagenized *gerB* operons on germination of wild-type spores

Mutation[a]	MLS[r] transformants	
	No. tested[b]	% Germinated[c]
None (no plasmid)	174	0
None (wild type)	123	0
Gly_{297} (GGU)→Ser (AGU)	68	25
Pro_{326} (CCA)→Ser (UCA)	68	15
Phe_{269} (UUU)→Ile (AUU)	40	52

[a] The mutations were introduced by site-directed mutagenesis into a plasmid-borne, otherwise wild-type *gerB* operon. Plasmid DNA (300 to 500 ng) was introduced into the wild-type strain (PS832) by congression with the MLS resistance marker of strain 1A640 (10 ng of chromosomal DNA).

[b] By plate assay for D-alanine-induced germination.

[c] Percentage that developed a red color in the plate assay for D-alanine-induced germination.

nation in D-alanine. Spores from all three merodiploids germinated in D-alanine (Fig. 6), indicating that all three mutations were dominant over the wild-type *gerB* allele. Thus, the mutant phenotype probably results from a function gained by the mutant GerBA* and GerBB* products.

Although merodiploid spores containing a wild-type *gerB* allele and any one of the three mutant *gerB** alleles germinated in D-alanine, their germination was slower than that of the corresponding haploid mutant spores (Fig. 6). This effect was most striking in the *gerBA1** mutant and was less so in the *gerBA2** and *gerBB1** mutants. The effect of the wild-type *gerB* allele was also detected with the plate assays, in which the merodiploid spores turned red more slowly than the haploid spores. Further, the effect of the wild-type *gerB* allele on the phenotype of the *gerB** mutant spores was independent of the chromosomal location of the two alleles; merodiploid spores that contained the mutant allele at the *gerB* locus and the wild-type allele at the *amyE* locus also germinated more slowly in D-alanine than did haploid spores that contained the mutant allele at the *gerB* locus (data not shown). Together, these results suggest that the wild-type GerB proteins can dilute the effect of the mutant proteins on spore germination.

Combination of mutations in *gerBA* and *gerBB*. To determine the interaction between the *gerBA** and *gerBB** mutations, we examined the germination characteristics of spores containing mutations in both genes. Double-mutant *gerBA1* gerBB1** or *gerBA2* gerBB1** operons were derived from the single-mutant *gerB** plasmids and inserted at the *amyE* locus in the Δ*gerB*::*spc* strain FB41. While preparing spores from the double-mutant strains, we observed that 20 to 30% of the spores germinated in the distilled water used to wash the spores. This anomalous germination of the double-mutant spores was independent of the sporulation conditions and was not apparent in any of the single-mutant spores. Thus, the mutations in *gerBA* and *gerBB* seemed to enhance one another. Consistent with this idea, the double-mutant spores turned red much faster (in less than one-fifth the time) than the single mutants (data not shown) in the plate assay for D-alanine-induced germination.

To examine the effect of a wild-type *gerB* allele on the anomalous germination of double-mutant spores, the double-mutant *gerBA1* gerBB1** and *gerBA2* gerBB1** alleles were inserted at the *amyE* locus in strain PS832, and the resulting merodiploid strains were sporulated by resuspension. These merodiploid double-mutant spores showed very low anomalous germination during cleaning, suggesting that the wild-type *gerB* allele ameliorated the double-mutant phenotype. Because this effect permitted isolation of clean dormant double-mutant spores, we examined the interaction between the *gerBA** and *gerBB** mutations by comparing D-alanine-induced germination of merodiploid double-mutant and single-mutant spores. In the presence of D-alanine, the merodiploid double-mutant spores germinated faster than spores of either merodiploid single-mutant strains (Fig. 7), consistent with the idea that the *gerBA** and *gerBB** mutations enhanced one another. In addition, we observed that the merodiploid double-mutant spores showed significant germination in buffer alone (Fig. 7), suggesting that the wild-type *gerB* allele did not completely mask the anomalous germination phenotype of the double-mutant spores. Together, these studies showed that the *gerBA** and *gerBB** mutations enhance one another and that the wild-type *gerB* allele partially masks this interaction.

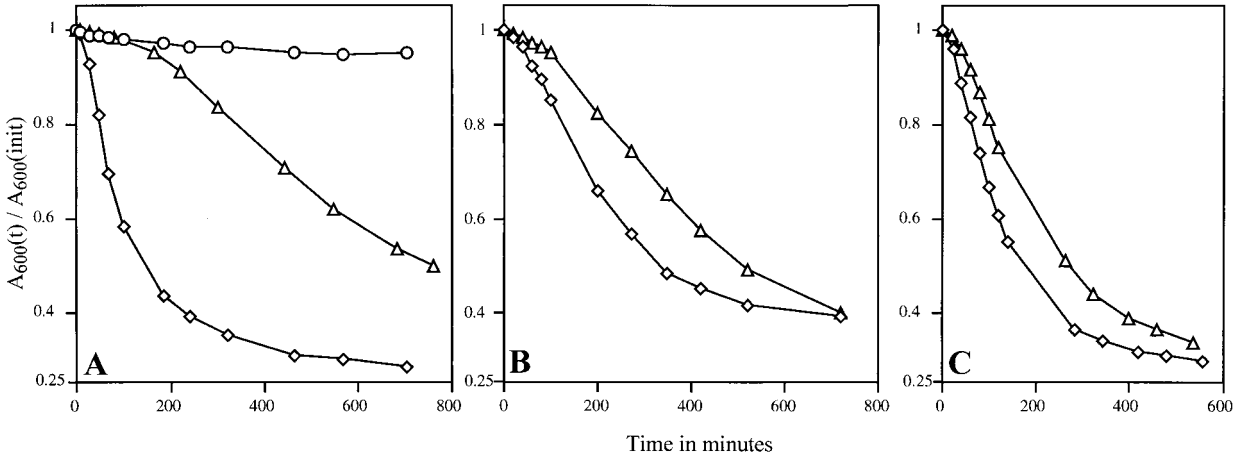

FIG. 6. Dominant/recessive nature of the *gerB* mutations. (A) Germination of the Δ*gerB amyE*::*gerBA1** haploid spores (FB44) (◇), *gerB amyE*::*gerBA1** merodiploid spores (FB50) (△), or Δ*gerB amyE*::*gerB* haploid spores (FB43) (○) in 10 mM D-alanine–1 mM D-glucose was assayed as described in the legend to Fig. 2. (B) Germination of Δ*gerB amyE*::*gerBA2** haploid spores (FB45) (◇) and *gerB amyE*::*gerBA2** merodiploid spores (FB51) (△) in 10 mM D-alanine–1 mM D-glucose. (C) Germination of Δ*gerB amyE*::*gerBB1** haploid spores (FB46) (◇) and *gerB amyE*::*gerBB1** merodiploid spores (FB52) (△) in 10 mM D-alanine–1 mM D-glucose. Germination curves of *gerB amyE*::*gerB* merodiploid spores (FB49) in 10 mM D-alanine–1 mM D-glucose and of all spores in buffer alone were identical to that of the Δ*gerB amyE*::*gerB* haploid (○ in panel A) and are not shown.

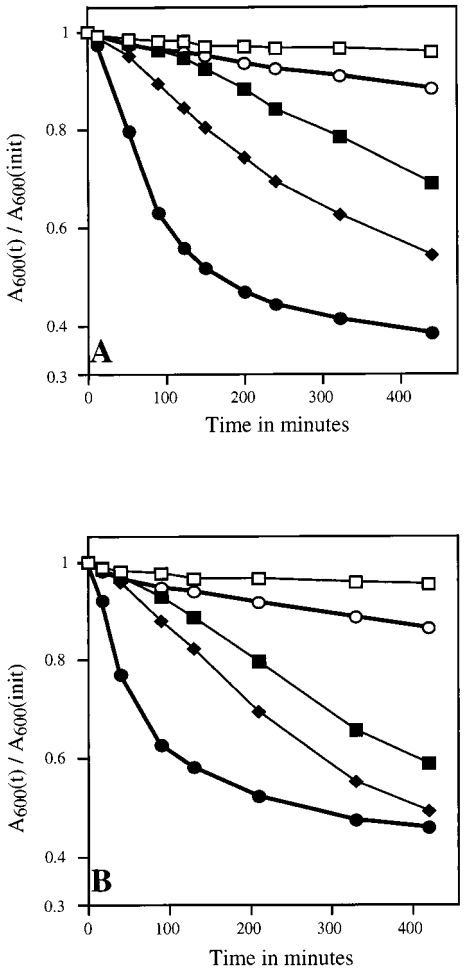

FIG. 7. Combinations of *gerBA** and *gerBB** mutations. (A) Germination of *gerB amyE*::*gerBA1** (FB50) (□, ■), *gerB amyE*::*gerBB1** (FB52) (◆), and *gerB amyE*::*gerBA1* gerBB1** (FB56) (○, ●) spores in 10 mM Tris-Cl (pH 8.4) in the absence (open symbols) or presence (solid symbols) of 10 mM D-alanine and 1 mM D-glucose was assayed as described in the legend to Fig. 2. (B) Germination of *gerB amyE*::*gerBA2** (FB51) (□, ■), *gerB amyE*::*gerBB1** (FB52) (◆), and *gerB amyE*::*gerBA2* gerBB1** (FB57) (○, ●) spores was assayed as described above.

DISCUSSION

Accurate recognition of germinants is critical to ensure that dormant spores germinate only under favorable environmental conditions. In *B. subtilis* spores, recognition of the germinant L-alanine or AFGK is thought to be mediated by specific receptors (14). In this report we have described a new strategy to genetically identify putative germinant receptor(s) in *B. subtilis*. Our findings suggest that two proteins encoded by the *gerB* operon are components of a germinant receptor, and thus our work supports previous studies (16, 24) which had proposed a role for *gerB* in germinant recognition. In addition, our studies suggest that the germinant receptor is a complex of at least two proteins, both of which are most likely integral membrane proteins.

The *gerB* locus was originally implicated in AFGK recognition because inactivating mutations at that locus specifically blocked AFGK-induced germination (24). In this study, we identified three dominant mutations in the *gerB* operon which allowed spores to germinate in the novel germinant D-alanine. Whereas loss of *gerB* function blocked germination in AFGK (3, 15, 16), gain-of-function *gerB* mutations allowed spores to germinate in D-alanine. These findings are best explained by a model in which *gerB* encodes one or more components of a receptor required for AFGK-induced germination. In this model, a dysfunctional AFGK receptor could account for the germination defect of *gerB* mutant spores, whereas a subtle structural alteration of the receptor could explain why our dominant *gerB** mutations allow spores to germinate in D-alanine (see below). But why would alterations in the AFGK receptor allow it to recognize D-alanine? In addition to AFGK, *gerB* was shown to mediate germination in a mixture of L-alanine, D-fructose, D-glucose, and K^+ ions (AlaFGK) (24). Moreover, in both mixtures, AFGK and AlaFGK, *gerB* was implicated in recognizing the amino acid (3). This ability of the *gerB* receptor to recognize a range of amino acids could account for its repeated isolation in our screen for mutations that produce a D-alanine-responsive receptor.

The *gerB* operon encodes three putative proteins, GerBA, GerBB, and GerBC, all of which are required for AFGK-induced germination (3). However, it is not clear which, if any, of these proteins are part of the germinant receptor. In this study, we identified mutations in *gerBA* and *gerBB* that allowed spores to germinate in D-alanine. All of these mutations were dominant, indicating that both mutant GerBA* and mutant GerBB* proteins could affect germinant recognition. Thus, both GerBA and GerBB seem to be components of the germinant receptor, suggesting that the receptor is actually a complex of several proteins. Such a model would account for the genetic interaction between the *gerB* and *gerB** alleles, as the ability of the wild-type *gerB* allele to partially mask the phenotype of *gerBA** and *gerBB** could result from competition between wild-type and mutant proteins for incorporation into the receptor complex. For example, if the receptor was a GerBA-GerBB dimer, then all of the GerBA* and GerBB* molecules would be incorporated into GerBA*-GerBB* double-mutant receptors in *gerBA* gerBB** haploid spores. However, only one-half of the mutant products would form double-mutant receptors in merodiploids because the remaining molecules would be incorporated into GerBA*-GerBB or GerBA-GerBB* receptors, and thus the merodiploids would have fewer double-mutant receptors. On this basis, we propose that the germinant receptor is a complex of GerBA and GerBB proteins, both of which play a role in recognition of the germinant. It is possible that the receptor complex also contains products of genes which were not identified in the screen because of a low frequency of gain-of-function mutations, and further studies will be needed to elaborate the constitution of the receptor complex.

The predicted GerBA and GerBB proteins contain 5 and 10 putative membrane-spanning domains, respectively (3), suggesting that they are probably integral membrane proteins. Thus, it is tempting to speculate that the germinant receptor complex is associated with and transduces a germinant signal across a spore membrane. The spore is surrounded by an inner membrane that is derived from the forespore and an outer membrane which originates from the mother cell (16). Because the integrity of the outer membrane is questionable (16), it is not clear which of the two membranes forms the outermost barrier across which the germinant signal must be transduced (16). Thus, it is not currently possible to predict the location of the germinant receptor. Moreover, recent studies attempting to localize the GerA proteins, which are also proposed to constitute a germinant receptor (38), gave contradictory results about the membrane in which those proteins are located (14, 22, 36). Thus, identification of the membrane that harbors the germinant receptor, and presumably marks the site where the

germination reaction is initiated, remains an important issue to be addressed.

In addition to *gerB*, previous genetic studies identified two other operons, *gerA* and *gerK*, that were implicated in germinant recognition (8, 24). Both of these operons encode proteins that are homologous to the *gerB* products and therefore could perform a similar function (14). The *gerA* operon is required for germination in L-alanine and might encode a germinant receptor that is dedicated to L-alanine recognition. Consistent with *gerA* and *gerB* encoding two distinct receptors, we found that a *gerA* disruption did not affect the *gerB** mutant phenotype. The *gerK* operon probably encodes a distinct glucose receptor, as *gerK* was proposed to mediate the effects of D-glucose in AFGK- and AlaFGK-induced germination (8). In addition, the *Bacillus* genome sequence (11, 17) has revealed two more operons, *yndDEF* and *yfkQRT*, that share sequence homology with the *gerB* operon. Thus, it is likely that *B. subtilis* spores contain a family of germinant receptors that mediate responses to diverse germinants.

In conclusion, we propose that the *gerB* operon and its homologues encode a family of multicomponent receptors that recognize environmental germinants and trigger germination. Further biochemical studies of the proteins encoded by *gerB* should allow us to test various predictions of the model presented here and refine our understanding of the germinant receptor. In addition, the dominant *gerB* mutations identified here can be used in genetic epistasis tests to define the *ger* loci that act downstream of the receptor. The identification of those loci should provide us with insights into how the receptor ultimately triggers germination.

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