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

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*B. 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 encode 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 *gerB* gene, whereas the third affected the *gerK* gene. These results suggest that *gerB* and *gerK* encode components of the germinant receptor. Furthermore, genetic interactions between the wild-type *gerB* and the mutant *gerB* and *gerK* 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.

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 *gerA* and one of which affected the *gerK* operon. 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 DH5α F′ 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...
vegetative growth of *B. subtilis* (23). 2×SG medium was used for *B. subtilis* sporation 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 ΔgerA::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 gerAA-5′ (5′-CACGGCCGCACGATAATTTAGCATTGG) and gerA -C5 (5′-GATAATGAATTCTGACC), which hybridize starting at (underlined position) and (uninked position) respectively and thus are not lost by recombination with the dIII region and thus are not lost by recombination with the dIII sites in the plasmid vector denoted by the thick solid line and the solid region within the bar; the solid line represents flanking genomic DNA. The gerAA-5′′ and gerA -C5′′ primers were then used to amplify a DNA fragment from plasmid pFE11. The 3′ region of the gerA operon was amplified from genomic DNA with primers gerAA-5′′ (5′-CCCTACATGATAGATGGCAAC) and gerA -C5′′ (5′-AACGATGGAGCCAG) and once within the amplified sequence) and inserted at the PstI (which cuts once within primer gerAA-5′′ and once within the amplified sequence) and inserted at the PstI site. The amplified DNA was cut with HinI (which cuts at addition of 20% metrizoic acid) was layered on top of the gradient, which was centrifuged at 30,000 rpm in a TLS-55 rotor (TI100 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-mil aliquots which were either frozen for storage, plated out for screening individual colonies, or subcultured into 200 ml of 2×SG medium for sporulation.

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 (OD600) of 40 to 80 were heat activated at 70°C for 15 min and diluted to an OD600 of 0.5 to 0.7 in a plastic cuvette containing 1 ml of the germination mix (10 mM Tris-HCl [pH 8.4], 1 mg of 2,3,5-triphenyltetrazolium chloride per ml, 2.5 mM glucose, 10 mM potassium bicarbonate) 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.

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**Assays of sporulation.** A modification of a previously described filter assay (12, 15) was used to identify *B. subtilis* colonies whose spores germinated in 2×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 5MM 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 potassium bicarbonate) 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.

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

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

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<tr>
<th>Strain</th>
<th>Genotype</th>
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<tr>
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<td>FB57</td>
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<td>FB48→PS832</td>
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</table>

**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, BamHI; C, ClaI; H, HindIII; R, EcoRV; Ss, SstI; Stn, ApSP denotes the ampicillin resistance marker carried on the plasmid.
phage PSBl stock was obtained from Wayne Nicholson, University of Arizona. Standard procedures were used for phage PSBl 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′-GGGATCCATATGCGGTTGAGCTCCGATGACAACGCCGCG-3′) and gerBpET3 (5′-AATCCTAGTGATGAATCAG; the underlines indicate restriction sites) which hybridizes starting at (underlined) positions nt -279 and +950 relative to the gerB4 translation start site (+1). A 643-bp HindIII-EcoRV fragment containing the PCR fragment was subcloned between the same sites in plasmid pLT74 (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 their Southern blot analysis and are designated ΔgerB::pgerB because they contain a ΔgerB operon, which is truncated at the first EcoRI site in plasmid pFE16, followed by the sac cassette and then a full-length gerB operon with an intact promoter (2) (Fig. 1). Chromosomal DNA from those transformants was linearized with SrfI and ligated, and the ligation mix was used to transform E. coli TGI and Ap (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 FB9; pFE25, mut5 strain FB9; pFE26, stra1 strain FB10; pFE28, mut2 strain FB11; and pFE29, muta2 strain FB12.

**Site-directed mutagenesis.** The 1.5-kb BamHI fragment from the wild-type gerB operon was cloned at the BamHI site on pUC9 to generate plasmid pFE45, which was used as a template by using a Transfect-A-Clone site-directed mutagenesis kit (Clontech, Palo Alto, Calif.). The selection primer pUC19-RI/RV (5′-CGGCGATGGATATCATTCTCCAT-3′) or gerBMutb1 (5′-TCATTGAACGAATTGATTTGTTCTTACAG-3′), to introduce the gerBA1 mutation. The gerBA1* marker was then used to replace the gerB1 operon in plasmid pFE45 to construct the single-mutant gerB operon plasmids pH70 (GerBA1*), pH86 (GerBA2*), and pH89 (GerBB1*), respectively. The gerBA1* marker was introduced by replacing a 1.6-kb ClaI-BamHI fragment (Fig. 1) in pFE72 with the same fragment from pFE70. The gerBA2* and 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 BamHI fragment. In the second step, the 1.5-kb BamHI fragments from the wild-type (pFE24) and mutant (pFE70, pFE71, pFE72, pFE76, and pFE77) gerB plasmids were cloned in the correct orientation into pFE96 to generate plasmids pFE97 through pFE102, respectively. Each plasmid was linearized with BglII and used as a template by using a Transfect-A-Clone site-directed mutagenesis kit (Clontech, Palo Alto, Calif.), sequenced, recovered as an EcoRI fragment (EcoRI sites are present in vector pCR2.1), and inserted into the EcoRI site of plasmid pFE91 (a derivative of plasmid pUC18 lacking the Ecl136I-HincII region) to generate plasmid pFE92. The 4.1-kb Stul-SstI 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 BglII site at its 5′ end. The 1.6-kb HindIII-BglII fragment from pFE93 was inserted between the HindIII-BamHI sites in pDG364 to generate plasmid pFE95. A 2.1-kb HindIII-HindIII fragment (from pFE94 pFE24 lacking the 1.6-kb BamHI fragment) was cloned into the HindIII site of plasmid pFE95 to generate plasmid pFE96. The HindIII fragment in plasmid pFE96 was oriented to generate a gerB operon that lacked the 1.5-kb BamHI fragment.

**RESULTS**

**Isolation of d-alanine responsive mutants.** To identify spore germiant receptor(s), we decided to isolate B. subtilis mutants which 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 germiant 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 pool of mutants for those 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 (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 the red 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, mut1, mut2, 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 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
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 co-transduced 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 co-transduced with the MLS resistance markers from strains 1A644 and 1A645. Again, all four mutations co-transduced 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 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 co-transduced 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 co-transduced with the MLS resistance markers from strains 1A644 and 1A645. Again, all four mutations co-transduced 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.

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 ΔgerB::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.

**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

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* 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 congerion with the MLS resistance marker of strain 1A640 (10 ng of chromosomal DNA).

a By plate assay for d-alanine-induced spore germination.

b 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, mut1b, mut2b, and mut2a 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 congerion (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 BamHI fragment (Fig. 1), which spans part of the gerBA and gerBB cistrons. Chimeric plasmids were constructed by removing this 1.5-kb BamHI 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 congerion 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 BamHI fragment.

Sequences of the gerB operons from mutant and wild-type strains. Because the mutations mapped within the 1.5-kb BamHI 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 Gly347 (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 Pro326 (CCA)→Ser (UCA) alteration in the predicted GerBA protein (Fig. 5). The gerB operon from the mut1b mutant showed no alteration in the gerBA cistron but contained a single T→A transversion which produced a Phc269 (TTT)→Ile (ATT) substitution in the gerBB open reading frame (Fig. 5). The gerB operons from the mut2b and mut2a 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 mut1b 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 BamHI 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 BamHI fragment. The entire mutagenized DNA fragment was sequenced to ensure that it contained only the appropriate mutation and then used to replace the BamHI fragment in the wild-type gerB plasmid, pFE24. The resulting plasmid was introduced into the wild-type strain PS832 by congerion 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 of the three mutagenized BamHI 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-
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 produce 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 D gerB::spc and a wild-type strain, respectively. The strains were sporulated by resuspension, and the spores were assayed for germination.

FIG. 5. Locations of the mutations within the gerB operon. The DNA sequence of the 1.5-kb BamHI 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* (Gly297 [GGU] → Ser [AGU]), gerBA2* (Pro326 [CCA] → Ser [UCA]), and gerBB1* (Phe269 [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.
TABLE 3. Effect of wild-type and mutagenized \( \text{gerB} \) operons on germination of wild-type spores

<table>
<thead>
<tr>
<th>Mutation(^a)</th>
<th>MLS(^b) transforms</th>
<th>No. tested(^c)</th>
<th>% Germinated(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (no plasmid)</td>
<td>174</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>None (wild type)</td>
<td>123</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gly(_{297}) (GGU)→Ser (AGU)</td>
<td>68</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Pro(_{332}) (CCA)→Ser (UCA)</td>
<td>68</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Phe(_{209}) (UUA)→Ile (AUU)</td>
<td>40</td>
<td>52</td>
<td>0</td>
</tr>
</tbody>
</table>

\( ^a \) The mutations were introduced by site-directed mutagenesis into a plasmidborne, otherwise wild-type \( \text{gerB} \) operon. Plasmid DNA (300 to 500 ng) was introduced into the wild-type strain (PS832) by conjugation with the MLS resistance marker of strain 1A640 (10 ng of chromosomal DNA).

\( ^b \) By plate assay for \( \alpha \)-alanine-induced germination.

\( ^c \) Percentage that developed a red color in the plate assay for \( \alpha \)-alanine-induced germination.

\( ^d \) The mutations were introduced by site-directed mutagenesis into a plasmidborne, otherwise wild-type \( \text{gerB} \) operon. Plasmid DNA (300 to 500 ng) was introduced into the wild-type strain (PS832) by conjugation with the MLS resistance marker of strain 1A640 (10 ng of chromosomal DNA).

\( ^c \) By plate assay for \( \alpha \)-alanine-induced germination.

\( ^d \) Percentage that developed a red color in the plate assay for \( \alpha \)-alanine-induced germination.

Combination of mutations in \( \text{gerBA} \) and \( \text{gerBB} \). To determine the interaction between the \( \text{gerBA}^* \) and \( \text{gerBB}^* \) mutations, we examined the germination characteristics of spores containing mutations in both genes. Double-mutant \( \text{gerBA1}^* \text{gerBB1}^* \) or \( \text{gerBA2}^* \text{gerBB1}^* \) operons were derived from the single-mutant \( \text{gerB}^* \) plasmids and inserted at the \( \text{amyE} \) locus in the \( \text{gerB}_{:spc} \) 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 \( \text{gerBA} \) and \( \text{gerBB} \) 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 \( \alpha \)-alanine-induced germination.

To examine the effect of a wild-type \( \text{gerB} \) allele on the anomalous germination of double-mutant spores, the double-mutant \( \text{gerBA1}^* \text{gerBB1}^* \) and \( \text{gerBA2}^* \text{gerBB1}^* \) alleles were inserted at the \( \text{amyE} \) 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 \( \text{gerB} \) allele ameliorated the double-mutant phenotype. Because this effect permitted isolation of clean dormant double-mutant spores, we examined the interaction between the \( \text{gerBA}^* \) and \( \text{gerBB}^* \) mutations by comparing \( \alpha \)-alanine-induced germination of merodiploid double-mutant and single-mutant spores. In the presence of \( \alpha \)-alanine, the merodiploid double-mutant spores germinated faster than spores of either merodiploid single-mutant strains (Fig. 7), consistent with the idea that the \( \text{gerBA}^* \) and \( \text{gerBB}^* \) 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 \( \text{gerB} \) allele did not completely mask the anomalous germination phenotype of the double-mutant spores. Together, these studies showed that the \( \text{gerBA}^* \) and \( \text{gerBB}^* \) mutations enhance one another and that the wild-type \( \text{gerB} \) allele partially masks this interaction.

FIG. 6. Dominant/recessive nature of the \( \text{gerB} \) mutations. (A) Germination of the \( \Delta \text{gerB} \text{ amyE}_{:}\text{gerBA1}^* \) haploid spores (FB44) (○), \( \text{gerB} \text{ amyE}_{:}\text{gerBA1}^* \) merodiploid spores (FB50) (●), or \( \Delta \text{gerB} \text{ amyE}_{:}\text{gerB} \text{ operon} \) haploid spores (FB43) (□) in 10 mM \( \alpha \)-alanine–1 mM \( \alpha \)-glucose was assayed as described in the legend to Fig. 2. (B) Germination of \( \Delta \text{gerB} \text{ amyE}_{:}\text{gerBA2}^* \) haploid spores (FB45) (○) and \( \text{gerB} \text{ amyE}_{:}\text{gerBA2}^* \) merodiploid spores (FB51) (●) in 10 mM \( \alpha \)-alanine–1 mM \( \alpha \)-glucose. (C) Germination of \( \Delta \text{gerB} \text{ amyE}_{:}\text{gerBB1}^* \) haploid spores (FB46) (○) and \( \text{gerB} \text{ amyE}_{:}\text{gerBB1}^* \) merodiploid spores (FB52) (●) in 10 mM \( \alpha \)-alanine–1 mM \( \alpha \)-glucose. Germination curves of \( \text{gerB} \text{ amyE}_{:}\text{gerB} \text{ merodiploid spores} \) (FB49) in 10 mM \( \alpha \)-alanine–1 mM \( \alpha \)-glucose and of all spores in buffer alone were identical to that of the \( \Delta \text{gerB} \text{ amyE}_{:}\text{gerB} \) haploid (○ in panel A) and are not shown.
germinant receptor, and thus our genetically identify putative germinant receptor(s) in B. subtilis (14). In this report we have described a new strategy to perform aD-alanine-responsive receptor.

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 d-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. 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 merodiploids but would produce aD-alanine-responsive receptor.

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.

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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 germination 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 α-glucose in AFGK- and AlaFGK-induced germination (8). In addition, the Bacillus genome sequence (11, 17) has revealed two more operons, yndDEF and ykJQRT, 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 germination 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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