

Effect of Amino Acid Substitutions in the GerAA Protein on the Function of the Alanine-Responsive Germinant Receptor of *Bacillus subtilis* Spores[∇]

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Spores of *Bacillus subtilis* require the GerAA, GerAB, and GerAC receptor proteins for L-alanine-induced germination. Mutations in *gerAA*, both random and site directed, result in phenotypes that identify amino acid residues important for receptor function in broad terms. They highlight the functional importance of two regions in the central, integral membrane domain of GerAA. A P324S substitution in the first residue of a conserved PFPP motif results in a 10-fold increase in a spore's sensitivity to alanine; a P326S change results in the release of phase-dark spores, in which the receptor may be in an "activated" or "quasigerminated" state. Substitutions in residues 398 to 400, in a short loop between the last two likely membrane-spanning helices of this central domain, all affect the germination response, with the G398S substitution causing a temperature-sensitive defect. In others, there are wider effects on the receptor: if alanine is substituted for conserved residue N146, H304, or E330, a severe defect in L-alanine germination results. This correlates with the absence of GerAC, suggesting that the assembly or stability of the entire receptor complex has been compromised by the defect in GerAA. In contrast, severely germination-defective mutants such as E129K, L373F, S400F, and M409N mutants retain GerAC at normal levels, suggesting more local and specific effects on the function of GerAA itself. Further interpretation will depend on progress in structural analysis of the receptor proteins.

In endospore-forming bacteria, the *gerA* family of operons generally encode three proteins that are likely to interact to form specific receptors for individual chemical germinants (15, 23). Most species encode a number of such operons, which have evolved to provide responses to different germinants by a process of gene duplication and divergence. The first example of this type of operon described was the *gerA* operon of *Bacillus subtilis*; the GerA proteins are required for germination in L-alanine as the sole germinant (18, 21, 28), whereas the GerB and GerK receptor proteins are required for an alternative germination in a mixture of L-asparagine, D-glucose, D-fructose, and K⁺ (AGFK). The *gerA* operon is expressed at a low level during sporulation, in the developing forespore, and is under the control of the forespore-specific sigma factor sigma G; the levels of expression are downregulated by AbrB-like

protein SpoVT (3) and dependent on activity of the PrpE phosphatase (10).

The proteins encoded (GerAA, GerAB, and GerAC) are all predicted to be targeted to the spore inner membrane, and direct evidence for this has been obtained for GerAA and GerAC (11). The GerAA protein is predicted to contain an integral membrane domain of five predicted membrane spans, following a large hydrophilic N-terminal domain, and followed in turn by a relatively short hydrophilic C-terminal domain. As *gerA* is expressed in the forespore, it is likely that the large N-terminal domain of GerAA would be located on the inside of the inner spore membrane. Germination-defective point mutants were isolated, following enrichment for spores that failed to lose heat or chemical resistance following exposure to alanine as the germinant, and were mapped to the *gerA* locus (18, 21, 25). Cloning of the adjacent *citG* gene (16) allowed classification of a selection of point mutations into three complementation groups (27), corresponding to the three operons *gerAA*, *gerAB*, and *gerAC* (28). In general, most of the point mutants examined had an extreme defect in germination in L-alanine without seriously interfering with germination in the AGFK combination of germinants. With the exception of the two *gerAB* mutations that reduced the sensitivity of germination to L-alanine (17, 21), the amino acid changes in the point mutants had not been examined.

In an attempt to explore functionally important residues, albeit in the absence of any direct structural information, point mutations in several *gerA* random mutants have been identified and additional site-directed mutants constructed. These studies have defined several regions of the GerAA protein impor-

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TABLE 1. Mutant strains used in this study^a

Strain	<i>gerAA</i> allele ^b	AA change ^b	Mutation type(s) ^c	Phenotypic group ^d	GerAC status ^e
BH4951	80	E129K	R	III	Present
AM1671	108	N146A	D	III	None
AM1666	103	V252A	D	I	
AM1667	104	S294P	D	II	
AM1672	109	H304A	D	III	None
AM1673	110	P324S	D	I*	
AM1674	111	F325A	D	I	
AM1681	113	P326S	D		
AM1683	114	E330A	D	III	None
AM1684	115	E335A	D	III	5%
BH4819	7	P349S	R	II	
AM1878	84	V369	R, FS	III	None
AM1668	105	L373F	D	III	Present
BH4818	16	G398S	R	III	Present
AM1675	112	L399N	D	II	
BH4810	56	S400F	R	III	Present
AM1669	106	M409N	D	III	Present
AM1670	107	H430A	D	II	

^a Strains from the mutant collection of D. A. Smith contained random mutations. Strain AM1681 had phase-dark spores, and strain BH4818 was temperature sensitive.

^b The *gerAA* allele and the consequent amino acid (AA) change are shown.

^c D, site-directed mutation; R, random mutation; FS, frameshift mutagenesis.

^d The phenotypic groups are based on L-alanine-dependent germination. Group I strains showed normal or near-normal germination. The group I* strain was more sensitive to germinant. Group II strains showed a slower decrease in the optical density, while group III strains showed little or no response to germinant.

^e The GerAC status is shown only for group III mutants and reports the presence or absence and the level of GerAC protein detected in Western blots of spore extracts.

tant in receptor function and distinguish mutations that disrupt receptor organization from those likely to have a more specific defect.

MATERIALS AND METHODS

Strains and culture conditions. *B. subtilis* mutant strains used in this study are listed in Table 1. The routine culture media were L broth for *Escherichia coli* and Oxoid nutrient broth for *B. subtilis*. Antibiotic resistance was selected by adding the following antibiotics to the nutrient agar: ampicillin (50 $\mu\text{g ml}^{-1}$) for *E. coli* and kanamycin (5 $\mu\text{g ml}^{-1}$) and chloramphenicol (3 $\mu\text{g ml}^{-1}$) for *B. subtilis*. Spores of *B. subtilis* were generally prepared in CCY medium (24) containing 20 $\mu\text{g ml}^{-1}$ tryptophan and washed as previously described (18). For two strains, those containing *gerAA56* and *gerAA107* alleles, sporulation in CCY medium was asynchronous, with some lysis; therefore, SG liquid medium (13) containing 20 $\mu\text{g ml}^{-1}$ tryptophan, in which they sporulated to completion, was used instead. Spores of the parental strain 1604 prepared in both media showed similar germination behavior, although the rate of germination in AGFK was a little slower for spores prepared in SG liquid medium.

Transformation of *E. coli* was accomplished by standard methods, and transformation of *B. subtilis* was brought about by the method of Kunst and Rapoport (12). Minimal medium (1) contained tryptophan (40 $\mu\text{g ml}^{-1}$) with sodium D,L-lactate (0.5% [wt/vol]; Fisher Scientific, United Kingdom) as the carbon source for selection of Cit⁺ transformants. Cells from the transformation mix were washed three times in Spizizen minimal salts before plating on minimal medium containing lactate.

Spore germination. Spores were heat activated at 70°C for 30 min and cooled in ice before the addition of germinants. Spores were diluted to an optical density at 600 nm (OD₆₀₀) of 0.6 in 1 mM L-alanine, 20 mM KCl, and 10 mM Tris-HCl (pH 7.6) or in 30 mM L-asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, 20 mM KCl, and 50 mM Tris-HCl (pH 8.4) for AGFK (L-asparagine, D-glucose, D-fructose, and K⁺) germination. Germination was carried out at 37°C, and the change in OD₄₉₀ of the suspension was measured every 2 min for 120 min using a VICTOR² 1420 multilabel counter (Wallac). Spores were germinated in 20-ml volumes, and aliquots were removed for measurement of dipicolinic acid (DPA)

release (22). The proportion of heat-resistant organisms during germination was measured after 10⁻⁴ dilution in H₂O by heating the organisms at 70°C for 60 min. L-Alanine, D-alanine, L-valine, cycloleucine, and γ -aminobutyric acid were from Sigma (>99% purity), and DL- β -aminobutyric acid and L- α -aminobutyric acid (>98%) were from MP Biomedicals Inc. The pH values of the germinants were adjusted before use.

Plasmid templates for mutagenesis. The 11-kb pAAM53 plasmid (27) contains most of *citG*, as well as *gerAA* and *gerAB*, in the shuttle vector pHV33, a hybrid of pBR322 and pC194. This plasmid was digested with HindIII in order to eliminate the pC194 vector. Digested DNA was diluted, religated, and used to transform *E. coli* DH5 α (*dam*⁺) to ampicillin resistance, yielding the 8-kb pAAM201 plasmid. Plasmid pAAM210 was constructed by cloning the 5-kb EcoRI fragment from pAAM3 (27), which contains most of *citG*, all of *gerAA* and *gerAB*, as well as approximately 900 bp of *gerAC*, into pBR322.

Recipient *B. subtilis* strains for introduction of site-directed mutations. The closely linked *citG* gene provided a selection for introduction of linked *gerA* alleles from mutagenized plasmids. Three different *citG::kan* mutants were made at different stages of the work; strain AM1651 carried an insertion in *citG*, requiring screening of Cit⁺ transformants for the linked *gerA* mutation, whereas in strains AM1679 and AM1720, sections of the *citG-gerA* region were replaced with a *kan* cassette, requiring simultaneous introduction of *citG* and the *gerA* allele to reconstruct the region.

Strain AM1651 (*citG::kan*). A kanamycin resistance cassette was amplified from pDG792 (9) by PCR with NcoI sites at both ends and cloned into the unique NcoI site within *citG* in pAAM201 to generate pAAM202. The pAAM202 (*citG::kan*) plasmid was linearized with EcoRI and introduced into competent cells of *B. subtilis* 1604, selecting kanamycin-resistant transformants. A typical transformant, AM1651, failed to grow on minimal medium with lactate as the sole carbon source and, as expected for *citG* mutants, was asporogenous.

Strain AM1697 (*citG-gerAB::kan*). The NcoI-StuI region of *citG-gerA*, including *gerAA* and half of *gerAB*, in pAAM201 was replaced by the *kan* cassette from pDG792, as a NcoI-StuI fragment to generate pAAM220. DNA was linearized at the ScaI site in the *bla* gene and used to transform strain 1604 to kanamycin resistance; transformants were checked for failure to grow on lactate.

Construction of site-directed mutations in the *gerAA* gene of *B. subtilis*. Thirteen *gerAA* mutations generated by site-directed mutagenesis of pAAM201 were introduced individually into the chromosome of *B. subtilis* (Table 1). Some changed conserved amino acid residues, often alanine substitutions, while others were changes that introduced an amino acid found at that position in a homolog. For example, the changes in *gerAA103(V252A)* and *gerAA104(S294P)* were made to introduce residues present at that position in GerBA, the changes in *gerAA110(P324S)* and *gerAA113(P326S)* were in conserved residues, and the changes in *gerAA105(L373F)* and *gerAA112(L399N)* introduced the residues at that position in GerXA of *Bacillus anthracis* and GerIA of *Bacillus cereus* ATCC 10876, respectively.

Mutations in *gerAA* were introduced into pAAM201 using a QuikChange II XL site-directed mutagenesis kit (Stratagene) and appropriate complementary oligonucleotide primers (primer details available on request). Mutagenized plasmids were isolated from *E. coli* transformants, the mutation was checked by sequencing, and the plasmid DNA, linearized at the unique ScaI site in the *bla* gene, was introduced into *B. subtilis* AM1651 (*citG::kan*) competent cells by transformation (12). CitG⁺ transformants were selected on minimal medium with 0.5% (wt/vol) D,L-lactate (Fisher) and then tested for kanamycin resistance. The CitG⁺ Kan^s transformants were then examined for their spore germination phenotype on rich medium by the tetrazolium (Tzm) overlay test (18), and PCR-amplified DNA was sequenced to check for the presence of the mutation; cotransformation of the *gerAA* mutation in most cases with *citG*⁺ was 20 to 40%, depending on the position of the mutation in *gerAA*. In the case of group I mutants, displaying near-wild-type germination and red in the tetrazolium test, direct sequencing of PCR products from random transformants was the only screening approach possible.

The revised more efficient strategy used pAAM210 as the template for mutagenesis and used linearized DNA from sequenced, mutagenized plasmids to transform *citG* mutant strains AM1679 and AM1720, selecting for growth on lactate. All Cit⁺ transformants in this case carried the *ger* mutation, which was also checked by sequencing. One checked colony of each mutant was retained, and spores were prepared and stored at 4°C.

Western blotting of proteins from crude spore extracts. Spores (10 mg [dry weight]) were suspended in 0.5 ml prechilled breakage buffer (50 mM Tris-HCl [pH 7.5] containing 0.5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Spore breakage, preparation of extracts, SDS-PAGE, and Western blotting were performed by the method of Cooper and Moir (6a).

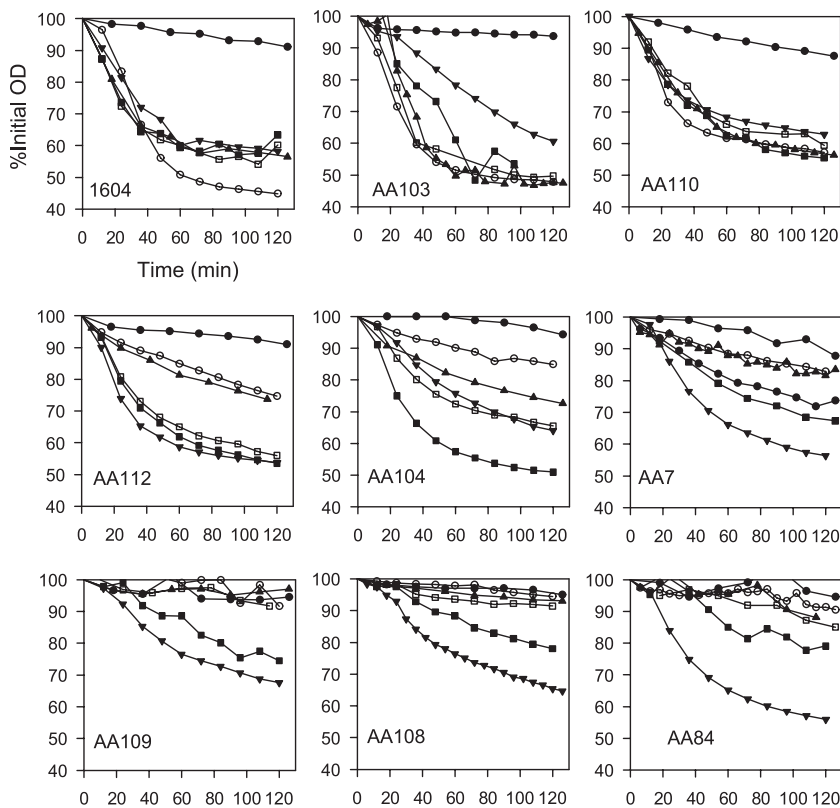


FIG. 2. Effects of mutations on spore germination. Group I mutants, represented by *gerAA103* (AA103) and AA110 mutants, showed an approximately wild-type phenotype. Group II mutants, represented by *gerAA112* (AA112), AA104, and AA7 mutants, had a partial defect in L-Ala germination. Group III mutants, represented by *gerAA109* (AA109), AA108, and AA84 mutants, had extreme defects in L-Ala germination. For each graph, time (in minutes) is shown on the x axis, and the percentage of the initial optical density is shown on the y axis. Symbols: ●, buffer; ○, L-alanine; □, L-alanine plus Glc; ▲, L-alanine plus Fru; ■, L-alanine plus Glc plus Fru; ▼, AGFK.

this group, spores germinate only slowly and fractionally in L-alanine with added glucose and fructose, presumably via the GerB/K receptor combination only; they do not attain the wild-type rates or extent seen for some of the point mutants in group II. This suggests that the response to L-alanine of the Ala-GFK receptor is not maximal, in the absence of some contribution from the GerA receptor. The absence of functional GerA receptor may slow asparagine-GFK germination rates a little, but as there was more variability between germination rates in wild-type spore preparations in this combination, this was difficult to define.

In two mutants with intermediate levels of germination of spore suspensions, measurements were made of other germination parameters—loss of heat resistance and release of dipicolinic acid (DPA) (Fig. 3). For both the *gerAA111*(F325A) mutant (group I but with a lower maximal rate; Fig. 4) and the *gerAA107*(H430A) mutant (group II), heat resistance loss was incomplete and significantly preceded release of DPA, within the time scale of the experiment. The data suggest that those spores that did respond by losing heat resistance took longer to release their DPA than was the case for wild-type spores. The significance of this is not clear and would require analysis of DPA release from single spores.

An amino acid change in a proline-rich loop (PFPP) of GerAA can increase the responsiveness to germinants. The *gerAA110* and *AA111* mutants (group I; P324S and F325A,

respectively), germinate well in L-alanine, but they do show some important differences from wild-type behavior. Spore germination of these *gerAA* mutants was tested in various concentrations of L-alanine and analogues, including L-valine, cy-

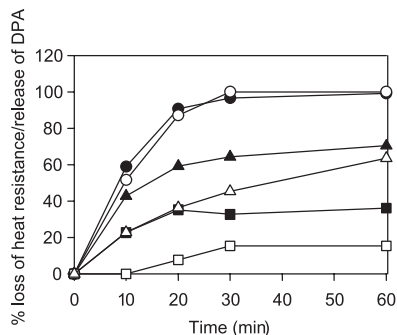


FIG. 3. Effects of the *gerAA107* and *gerAA111* mutant alleles on loss of heat resistance and DPA release during germination. Spores germinating at 37°C in 10 mM Tris-HCl (pH 7.5) containing 1 mM L-alanine and 20 mM KCl were sampled at intervals, and loss of heat resistance and release of DPA were measured. Values for the loss of heat resistance (black symbols) and release of DPA (white symbols) in strain 1604 (*ger*⁺) (circles), *gerAA111* mutant (triangles), and *gerAA107* mutant (squares) are shown. The percentages of OD loss at 60 min were 27 for the *gerAA111* mutant and 20 for the *gerAA107* mutant.

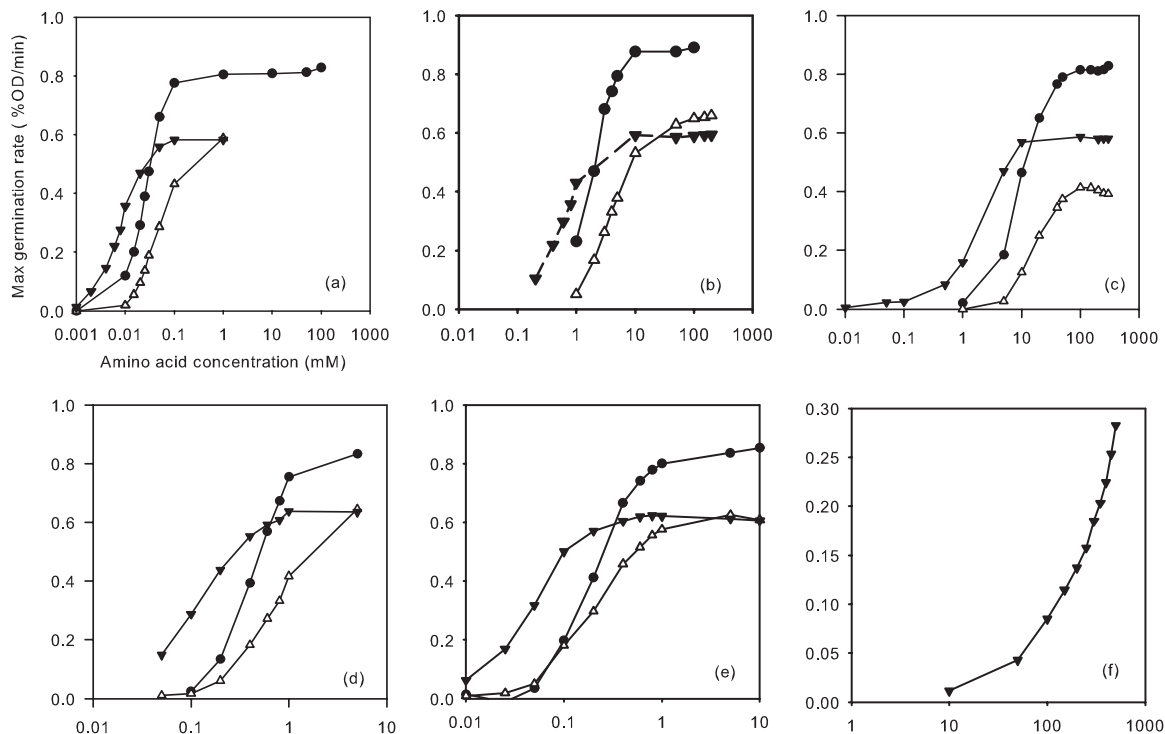


FIG. 4. Dependence of maximum germination rate on germinant concentration for the wild type and two mutants. L-alanine (a), L-valine (b), cycloleucine (c), β -aminobutyrate (d), α -aminobutyrate (e), and γ -aminobutyrate (f) were used as germinants for the wild type (●), *gerA110* mutant (▼), and *gerA111* mutant (△). In each graph, the amino acid concentration (in millimolar) is shown on the x axis, and the maximum germination rate (percent optical density per minute) is shown on the y axis. Only the *gerA110* mutant gave a significant rate in panel f.

cloleucine, β -aminobutyrate, α -aminobutyrate, and γ -aminobutyrate. As described previously (21), the maximum slope of each curve (germination rate) was determined. The germination rate was then plotted against the concentration of germinant on a logarithmic scale (Fig. 4), and C_{50} , the concentration for half-maximal germination rate, was calculated.

Spores of the *gerA110* mutant were more responsive than the wild type to low concentrations of alanine and its analogues. However, the maximum germination rate of the *gerA110* mutant was lower than the wild type, reflecting a less synchronous response. This suggests that the amino acid substitution in this mutant alters the receptor protein so that it will respond to a smaller germination stimulus, but paradoxically, the average spore takes longer to germinate, even in excess germinant (see Fig. 2 for an alanine germination curve). Spores of the *gerA110* mutant also germinated in very high concentrations of γ -aminobutyrate as the sole germinant, whereas the wild type will only do so in combination with sugars (26). In contrast, spores of the *gerA111* mutant germinated slightly slower than the wild type in L-alanine and its analogues; even at high concentrations of germinants, the maximum rate of germination was at least 25% lower than the wild type, and the concentration of germinant required for a half-maximal response was increased (Fig. 4). Like the wild type, *gerA111* spores did not germinate in γ -aminobutyrate up to 0.5 M. This amino acid substitution has only a minor effect on spore germination, but the spores are consistently less responsive to L-alanine or its analogues than those of the parent.

D-Alanine inhibition. D-Alanine is a competitive inhibitor of germination initiated by L-alanine and its analogues (26), though it does not inhibit germination in L-alanine plus sugar adjuncts. Spore germination of the wild type and *gerA110* and *gerA111* mutants was determined in the presence of L- and D-alanine. L-Alanine was used at 1 mM, as this would give the maximum germination rate for the wild type and mutants (Fig. 4a), and the ratio of L- to D-alanine was varied by reducing the D-alanine concentration (Table 2). At a 1:1 ratio, germination of wild-type and *gerA111* spores was inhibited by D-alanine by

TABLE 2. Inhibition of germination of wild-type, *gerA110*, and *gerA111* spores by D-alanine^a

Ratio of alanine isomers (L-alanine/D-alanine)	Alanine isomer concns (mM) (L-alanine/D-alanine)	Inhibition (%)		
		Wild type	<i>A110</i> mutant	<i>A111</i> mutant
1:1	1:1	>95	37	88
2:1	1:0.5	62	12	78
4:1	1:0.25	24	<5	53
8:1	1:0.125	8	<5	31
10:1	1:0.1	<5	<5	28
20:1	1:0.05	<5	<5	16

^a All conditions contained 20 mM KCl in 10 mM Tris-HCl (pH 7.5). Germination was measured by the loss of optical density (OD), and the percentage of inhibition was calculated as $100 \times [(\text{maximum rate of the decrease in OD in the presence of D-alanine})/(\text{maximum rate of the decrease in OD in 1 mM L-alanine only})]$. Germination was not seen in D-alanine in the absence of L-alanine (OD decrease of $\leq 5\%$ in 120 min).

approximately 90%. The *gerAA111* mutant is more sensitive to D-alanine inhibition, still showing some inhibition at an L-alanine/D-alanine ratio of 20:1. The affinity of this mutant for binding to D-alanine was probably not reduced, unlike the situation for L-alanine. In contrast, spores of the *gerAA110* mutant were more resistant to D-alanine inhibition; a 1:1 ratio of L- to D-alanine gave only 40% inhibition. This is consistent with the observation that these spores are more responsive than the wild type to L-alanine. Overall, the data for the *gerAA110* and *gerAA111* mutants suggest that although the responsiveness to L-alanine is altered, the sensitivity to the competitive inhibitor D-alanine remains unaffected.

Altering the conserved PFPP motif can affect spore maturation. A P326S change (mutant allele *gerAA113*) was introduced at the next residue in the PFPP motif. This mutant grows normally, but during sporulation, it lyses prematurely, so that cultures at the end of sporulation contain much cell debris and some free phase-dark spores. No free phase-bright spores are obtained. Only early, partially phase-gray forespores can be seen in mother cells, and these darken rather than becoming more phase bright before release. The forespore fails to mature properly in the mother cell, possibly as a consequence of spontaneous germination via a defective germinant receptor. This phenotype is entirely distinct from that caused by the absence of the receptor, which would not interfere with spore maturation.

Charged residues in a potential amphipathic region. Immediately after this likely PFPP hinge region is a sequence that could be within the membrane, as suggested in some topology predictions, and has some elements of an amphipathic helix. Two conserved glutamate residues in this region were altered to alanine—E330A and E335A in *gerAA114* and *gerAA115* alleles, respectively. Both of these changes resulted in loss of alanine germination. As discussed below, this was matched by the complete loss, or 95% reduction, of GerAC protein. This suggests that the region including these charged residues is likely to be of (at least) structural importance to the receptor complex.

Mutations define an important loop between membrane spans 4 and 5. Multiple isolates of mutants with a temperature-sensitive defect carry the G398S substitution (*gerA16* and other mutants [see above]) in a loop between potential membrane spans 4 and 5. The local sequence is not strongly conserved across GerAA homologs in sporeformers, but one of three adjacent residues in the loop is G, A, or P in a variety of homologs. This loop is also the site of a second point mutation, *gerA56*(S400F), that results in a severe loss of function of GerAA (group III). A site-directed mutation (L399N; *gerAA112*) introduced the amino acid present at the equivalent position in the loop in GerIA/GerHA, the inosine receptor of *B. cereus*/*B. anthracis*. This change resulted in an intermediate-level germination defect (group II); germination in L-alanine was reduced in both rate and extent, but germination was as fast as the wild type when glucose and fructose were also included (Fig. 2).

Conserved residues in the N-terminal domain of GerAA are important for germination. Altering either of two highly conserved amino acids at the extreme ends of a relatively conserved section within the N-terminal hydrophilic domain of

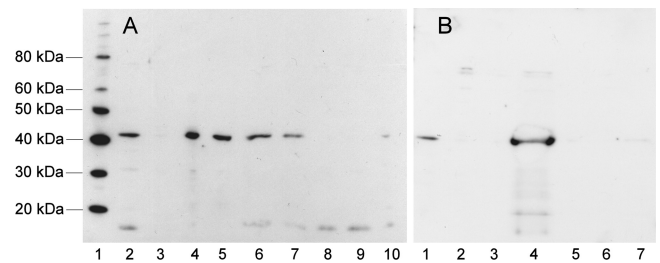


FIG. 5. Levels of GerAC protein in *gerAA* mutant spores. Western blot of *gerAA* mutant broken spore extracts were separated on a NuPAGE gel (Invitrogen), probed with anti-GerAC antibody, and detected using ECL Plus (GE Healthcare). The GerAC band runs at 42 kDa under the conditions used. (A) Lanes: 1, MagicMark protein markers (Invitrogen); 2, strain 1604; 3, *gerA84* mutant; 4, *gerA16* mutant; 5, *gerA80* mutant; 6, *gerAA105* mutant; 7, *gerAA106* mutant; 8, *gerAA109* mutant; 9, *gerAA114* mutant; 10, *gerAA115* mutant. (B) Lanes: 1, strain 160; 2, *gerAA108* mutant; 3, *gerAA109* mutant; 4, *gerA56* mutant; 5, *gerAA84* mutant; 6, *gerAA114* mutant; 7, *gerAA115* mutant.

GerAA (E129K in *gerAA80*; N146A in *gerAA108*) prevented germination in L-alanine (a group III phenotype).

Some changes to GerAA prevent assembly of GerAC in the spore, providing strong evidence for a germination receptor complex. If a *gerAA* mutant fails to germinate in L-alanine (group III), that phenotype could result from the loss of function of a crucial amino acid involved in the germination process, or in a more general sense a loss of receptor function because of a loss of stability of the GerAA protein, either individually or in a receptor complex. A GerAA antipeptide antibody (11) was no longer active enough to detect GerAA protein in spores, so no direct test of levels of GerAA in spores was possible. Instead, the more effective anti-GerAC polyclonal antibody raised against an overexpressed GerAC protein (11) was used to detect the GerAC protein in wild-type and *gerAA* mutant spores (Fig. 5). Some of the group III *gerAA* point mutants that show an extreme defect in alanine germination lack GerAC entirely, at least at this limit of detection.

The *gerA84* mutant, in which a frameshift mutation truncates the GerAA protein at residue 369, part way through the membrane-embedded domain, contains no GerAC protein. This could result from a failure of assembly of the entire complex as a result of a truncated GerAA protein or from some polar effect of the frameshift on downstream gene expression in the operon. However, a number of group III mutations that result in an amino acid substitution also lack GerAC protein; no polarity would be invoked in such a situation. The significance of this observation is considered in more detail and also in the context of GerAB mutant proteins in Cooper and Moir (6a), but briefly, these data demonstrate that some changes in the GerAA protein can result in a failure to assemble a receptor complex that includes GerAC; this provides strong evidence for protein interaction to form a receptor complex. Such changes in GerAA that result in the absence of GerAC from the spore are likely to have destabilized the overall structure of the GerAA protein, affecting receptor complex assembly, but changes in GerAA that allow normal assembly of GerAC into the complex are likely to have more specific effects. For example, consider the two amino acid substitutions in the N-termi-

nal hydrophilic domain that give group III type (severe) defects in alanine germination. The *gerAA108* mutant spores are likely to lack the GerA receptor on the basis of the absence of GerAC protein in Western blot analysis (Fig. 5); any failure to assemble the receptor would give a severe phenotype. In contrast, the spores with the E129K change in the N-terminal domain still contained approximately wild-type levels of GerAC, suggesting that the structure of the receptor may be intact, despite loss of its function. Substituting alanine for the conserved H304, E330, and E335 residues results in a severe depletion of GerAC, suggesting that they affect structural stability. In contrast, residue N146 discussed above and residues L373, G398, S400, and M409 do not destabilize complex assembly and are therefore likely to have a more specific, but unknown, functional role in the germination process.

DISCUSSION

It has so far proved impossible to overexpress the integral membrane proteins that form major components of the family of spore germinant receptors, of which the GerA receptor is the paradigm, and as a result, there is no structural information to guide functional analysis. One potential source of information relevant to receptor function, however, is an analysis of strains bearing point mutations. In the mutagenesis described in this work, the receptor operon was retained at its normal location, at single copy, but this meant that the time-consuming substitution approach adopted limited the number of mutations that could be made.

The data presented above are somewhat diverse but establish the usefulness of defining the consequences of individual amino acid substitutions on germinant receptor function. The major conclusions follow. (i) A region including the PFPP conserved motif beginning at residue 324, and including the following conserved E330 and E335 residues, is particularly important; changes in this region result in a variety of phenotypes, including increasing receptor sensitivity, a spore maturation failure, or a loss of receptor. This region might represent either an atypical membrane helix or an exposed loop between membrane helices, as there is currently no structural information on this protein. (ii) A loop between likely transmembrane spans 4 and 5 is also important for function; substitutions in this region can give a loss of function of the receptor or a reversible temperature-sensitive germination phenotype, suggesting the importance of conformation in this region (S400F or G398S, respectively). (iii) The loss of function in some *gerAA* mutants, including not just null but also some missense mutations, is accompanied by loss of the GerAC protein from the spore. This demonstrates that the incorporation of the GerAC protein is dependent on the GerAA protein (as well as on the GerAB protein, as described in the accompanying paper [6a]) and distinguishes GerAA amino acid substitutions resulting in a total functional defect, such as E129K and M409N, from those in which the overall receptor complex is destabilized, such as N146A or E330A.

The GerA proteins form a specialized receptor for L-alanine and its analogues, which bind to initiate germination, but the molecular details of the signal transduction process mediated by the membrane-associated receptor complex that results in germination-associated changes, including release of ions,

small molecules, and DPA, and activation of lytic events in cortex and coats (15, 23), remain largely unclear. Receptors may function as a single type or in concert with others of different specificity (2). It is not clear whether a single subunit of the receptor binds germinants or whether multiple subunits might. The GerAB component, a homolog of the amino acid-polyamine-organocation (APC) family of single-component membrane transporters, is most likely to bind germinant. Two mutant alleles (*gerAB38* and *gerAB44*) with amino acid changes in predicted membrane-spanning helices of GerAB alter dramatically the responsiveness of spores to L-alanine (17, 21), and tellingly, the alternate GerAB-related proteins GerVB and GerUB of *Bacillus megaterium* QM B1551 confer somewhat different germinant specificities on a single receptor (5, 6).

The range of phenotypes seen on changing amino acids in the local proline-rich hinge-like region emphasizes its importance to the function of the GerAA protein. Previously, the equivalent amino acid substitution to *gerAA110*(P324S) in the homologous GerBA protein of *B. subtilis* (2, 20) allowed a slow germination response to L-alanine as the sole germinant in the GerB receptor, which normally requires additional GerK receptor-mediated stimulation. This may also be the case in *gerAA110* for the GerA receptor and γ -aminobutyrate, although it is also possible that trace contamination with L-alanine, at <0.001%, could give this result, given the increased sensitivity of the mutant to L-alanine and its analogues. Overall, the increased sensitivity suggests that the conformation of this proline-rich bend region may influence the responsiveness of the overall receptor complex to germinant, perhaps by affecting germinant binding or by altering the likelihood of a conformational change following germinant binding. The absence of structural information makes this hard to interpret further, although one might speculate that changing this first proline in the PFPP motif renders the receptor more easily converted to a germinant-bound "activated" conformation, and that changing the next proline residue, as in the *gerAA113* mutant, results in a GerA receptor that is permanently activated, at least in the developing sporangium, leading to autogermination before spore maturation (7).

Receptors may function as a single type or in concert with others of different specificity (2). Mutants in group II have a significant defect in L-alanine germination; spores are much delayed in their response to L-alanine, and only one-third to one-half of the population germinate in L-alanine within 2 h. Nevertheless, a proportion of the spores germinate with a microlog well within the normal range. This fractional germination reflects some heterogeneity in the properties of individual spores (8). However, all the spores will respond at near-normal rates if both glucose and fructose are added, invoking GerK and GerB receptors (Fig. 2). The levels of GerAC protein have not been screened in all these mutants, so it is not known whether this slow and limited individual response reflects lower overall levels of GerA receptor or whether it is the direct consequence of a change in side chain interactions within the GerAA protein. Those group III mutations that cause a major germination defect but still contain GerAC protein in the spore at near-normal levels are easier to interpret; presumably, they identify changes that affect GerAA function rather than gross receptor structure. The next challenge is to define what such changes mean for the signal trans-

duction process and the role of the GerAA domains in the germination process. The current work highlights the need for topology analysis, and an appreciation of the requirements for formation of a stable germinant receptor complex in the spore, as well as for structural information on the receptor complex itself.

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