Amino Acid Substitutions in Transmembrane Domains 9 and 10 of GerVB That Affect the Germination Properties of *Bacillus megaterium* Spores[▽]

Graham Christie* and Christopher R. Lowe

Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, United Kingdom

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The molecular basis for differences in germinant recognition of *Bacillus megaterium* QM B1551 spores containing the GerVB and/or GerUB receptor proteins has been examined by site-directed mutagenesis and the construction of cross-homologue chimeras. Focusing on nonconserved residues predicted to reside in transmembrane domains 9 and 10, we demonstrate that GerVB residues Ser319 and Leu345 are of particular importance in defining the specificity and apparent affinity of the receptor for germinants. Kinetic analyses of mutants with different amino acid substitutions at these positions indicate that Ser319 and Leu345 are not involved directly in the binding of germinants, but probably reside in regions of the receptor where structural perturbations can affect the conformation of, or access to, germinant binding sites. Position 345 is also shown to be of importance in GerUB, where the F345A mutation severely impairs receptor function. Functionality is restored in the GerUB Ala345 background by substituting putative outer-loop residues adjacent to TM10 for the corresponding residues in GerVB, indicating that a degree of structural coordination between these regions is important to receptor function.

Dormant spores of the *Bacillus* and *Clostridium* genera, which can persist in the environment for considerable periods of time due to the protective properties of the spore structure, reenter the vegetative state via the process of germination (16, 25). The physiological route to germination is initiated by germinant molecules (typically an amino acid, nucleoside, or sugar) traversing the outer layers of the spore to interact with cognate receptors that reside in the inner membrane that surrounds the spore core (11, 19).

Orthologous proteins belonging to the GerA family form the receptors through which spores of *Bacillus* species sense their environment (9, 17, 20). Structural genes encoding the respective A, B, and C receptor subunits are arranged typically in tricistronic operons, the cotranscription of which suggests that the receptor is a complex of all three proteins. This view has been reinforced by molecular genetic (12, 18) and biochemical (30) approaches that suggest physical interaction between at least some of the receptor subunits. Genetic evidence that proteins from different receptors can physically interact has also been presented (3, 5). This may be significant, since a number of germinant receptors in different species appear to function cooperatively to initiate germination (3, 4, 10).

In an as yet poorly understood process, germinant-receptor interaction triggers changes to the permeability barrier presented by the inner membrane, resulting in the rapid efflux of select small molecules, including monovalent cations (26) and the spore's pool of Ca-dipicolinic acid (DPA) and free amino acids (glutamic acid and arginine) (15, 24), while permitting the influx of some water into the core. Precise mechanisms that

permit rapid movement of these molecules across the membrane have not been elucidated, although SpoVA proteins that are involved in uptake of DPA in the developing forespore during sporulation seem also to be involved in its release during germination, perhaps by forming a pore or channel in the spore inner membrane (27, 31, 32). Biochemical evidence for physical interaction between *B. subtilis* GerA receptor subunits and two different SpoVA proteins suggests a route to signal transduction between the receptor and putative DPA channel (30).

Evidence that the B protein of the receptor presents the site for ligand binding was first suggested following the observation that point mutations in the *B. subtilis* GerAB structural gene, located in regions predicted to encode membrane-spanning domains, led to an increased requirement for alanine to stimulate germination (23). Subsequent bioinformatic analyses have revealed a degree of homology between receptor B protein homologues and bacterial single-component membrane transporters (13, 17), and it may be that some functional characteristics, in addition to a common evolutionary lineage, are shared between these protein families.

More recently, the demonstration that two *Bacillus megaterium* receptor B protein homologues (GerUB and GerVB) each interact with the same A and C protein subunits to confer receptors with different specificities provided further evidence that the B protein is the site for germinant binding (7). Cross-homologue chimera constructs and site-directed mutagenesis studies have since been employed to probe the molecular basis for differences in germinant recognition between the two proteins (6). A number of residues predicted to reside in outer-loop (OL) regions facing the exterior of the spore were identified as being of structural or functional importance, particularly those in OL5, which connects transmembrane domain 9 (TM9) and TM10, positioned toward the C-ter-

* Corresponding author. Mailing address: Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge, United Kingdom CB2 1QT. Phone: 01223 764959. Fax: 01223 334162. E-mail: gc301@cam.ac.uk.

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TABLE 1. Strains and plasmids used in this study

<i>B. megaterium</i> strain or plasmid	Receptor genotype	Source or reference
Strains		
PV361	Nongerminative derivative of QM B1551	29
PV361 transformants ^a		
GC430	GerU (containing <i>gerUA</i> , <i>gerUC</i> , and <i>gerUB</i>)	7
GC431	GerU* (containing <i>gerUA</i> , <i>gerUC</i> , and <i>gerVB</i>)	7
GC432	GerU* with GerVB L306M	This study
GC433	GerU* with GerVB F312I	This study
GC434	GerU* with GerVB V313I	This study
GC435	GerU* with GerVB A318S	This study
GC436	GerU* with GerVB S319M	This study
GC437	GerU* with GerVB S319A	This study
GC438	GerU* with GerVB S319T	This study
GC439	GerU* with GerVB S319C	This study
GC440	GerU* with GerVB I339V	This study
GC441	GerU* with GerVB M342I	This study
GC442	GerU* with GerVB I344L	This study
GC443	GerU* with GerVB L345F	This study
GC444	GerU* with GerVB A346T	This study
GC445	GerU* with GerVB I347V	This study
GC446	GerU* with GerVB A356S	This study
GC447	GerU* with GerVB L345I	This study
GC448	GerU* with GerVB L345V	This study
GC449	GerU* with GerVB L345A	This study
GC450	GerU* with GerVB L345Y	This study
GC451	GerU with GerUB TM1 to -8 fused with GerVB TM9 and -10	6
GC452	GerU with GerUB TM1 to -9 fused with GerVB OL5 and TM10	This study
GC453	GerU with GerUB M319A	This study
GC454	GerU with GerUB F345A	This study
GC455	GerU with GerUB TM1 to -9 fused with GerVB OL5 and GerUB F345A	This study
GC456	<i>gerUA gerUC</i>	This study
Plasmids		
pGEM-3Z	<i>E. coli</i> cloning vector; Amp ^{rb}	Promega
pHT315	<i>B. megaterium</i> host plasmid; MLS ^r	2

^a Strains were transformed to MLS^r with plasmid pHT315 carrying the described GerU receptor variant.

^b Amp^r, ampicillin (β-lactam) resistance.

minimal part of the protein. Gain of function, however (in this case, the recognition of proline in a non-cognate receptor background), required residues predicted to reside in membrane-spanning domains (TM 9 and TM10) in addition to those identified in OL5 (6). This communication reports on the investigation to identify those residues and to ascertain their role as determinants of germinant recognition in the receptor.

MATERIALS AND METHODS

Bacterial strains and media. The *Bacillus megaterium* strains employed in this study (Table 1) are all derivatives of strain PV361 (29), a plasmidless variant of the wild-type QM B1551 strain that lacks the GerU operon and GerVB structural gene required to initiate germination in response to single trigger compounds. *Bacillus megaterium* strains were routinely cultured on LB agar or broth at 30°C, containing antibiotics where appropriate (1 μg/ml erythromycin and 25 μg/ml lincomycin for macrolide-lincosamide-streptogramin B resistance [MLS^r]). *Escherichia coli* strains used for site-directed mutagenesis (XL1-Blue [Stratagene]) or preparation of plasmids for transformation of *B. megaterium* (*E. coli* NovaBlue [Novagen]) were cultured in LB medium at 37°C supplemented with 75 μg/ml carbenicillin.

Protoplasting and transformation of *B. megaterium*. Protoplasts were prepared by inoculating 25 ml of RHAF medium (6) with 200 μl of an overnight suspension of *B. megaterium* cells and incubated (37°C at 225 rpm) until an optical density at 660 nm (OD₆₆₀) of ~0.6 was attained. Cells were harvested by

centrifugation (4,300 × *g* for 7 min) and resuspended in 2 ml RHAF medium. The wash procedure was repeated before resuspending the cells in 2 ml RHAF containing 1.2 mg/ml lysozyme and incubating without agitation at room temperature for 10 min. Efficient formation of protoplasts was confirmed by phase-contrast microscopy before centrifugation (1,250 × *g* for 7 min at 4°C) and gentle resuspension of the protoplasts in 2 ml RHAF medium. Following another round of centrifugation, protoplasts were resuspended in 1 ml RHAF medium and distributed into 200-μl aliquots. Transformation was conducted by adding 10 μl plasmid DNA (approximately 1 μg) to 200 μl protoplast suspension followed by the addition of 200 μl 30% (wt/vol) polyethylene glycol solubilized in RHAF medium. The suspension was mixed gently and incubated in a 37°C water bath for 4 min prior to the addition of 3 ml RHAF and centrifugation (1,250 × *g* for 7 min at 4°C). The protoplasts were resuspended in 1 ml RHAF and then incubated (30°C at 175 rpm) for 2 h before selecting for transformants on solid RHAF medium (30°C, overnight) containing appropriate antibiotics.

Spore preparation. *Bacillus megaterium* spores were prepared in 200-ml aliquots of supplemented nutrient broth (SNB) in 2-liter flasks (30°C at 200 rpm) supplemented with 1 μg/ml erythromycin to provide selective pressure for maintenance of the plasmid-borne receptor operon. Spores were harvested by centrifugation after 72 h of culture and then subjected to repeated rounds of centrifugation (4,300 × *g* for 7 min at 4°C), removal of the upper vegetative debris layer, and resuspension in ice-cold water. This wash procedure was repeated until spores were observed to be free of vegetative cells and debris, before storing on ice. SNB medium consists of the following (per liter): Difco nutrient broth, 8.0 g; glucose, 1.0 g; KCl, 1.0 g; MgSO₄ · 7H₂O, 246 mg; CaCl₂ · 2H₂O, 147 mg; MnCl₂ · 4H₂O, 4 mg; and FeSO₄ · 7H₂O, 0.3 mg. The pH was adjusted to 7.2 prior to autoclaving.

SDM. Site-directed mutagenesis (SDM) procedures were conducted using the Stratagene QuikChange II SDM kit, as directed by the manufacturer. Primers for SDM were designed using the QuikChange primer design program (Stratagene). All primer sequences are available upon request. The main parental plasmids subject to SDM comprised *gerU** (*gerUA*, *gerUC*, and *gerVB*) and *gerU* (*gerUA*, *gerUC*, and *gerUB*) receptor operons cloned into the BamHI site of pGEM-3Z. Receptor amplicons identified as carrying the correct mutation(s) by sequence analysis following SDM were digested with BamHI and ligated with plasmid pHT315 (2) as described previously (6). The fidelity of the entire receptor operon subcloned into pHT315 was assessed by sequence analysis prior to transformation of *B. megaterium* PV361 to MLS^r. The final *B. megaterium* constructs are detailed in Table 1.

Construction of GerUB-GerVB chimeras. Strain GC452, in which the receptor B protein has been engineered to comprise GerUB TM1 to -9 fused with GerVB OL5 and TM10, was constructed using an overlap PCR technique. A 4,307-bp amplicon encompassing the coding and putative regulatory sequences for GerUA, GerUC, and the first 332 codons of GerUB was prepared by PCR. A second fragment comprising a 327-bp amplicon encompassing codons 326 to 366 of GerVB and a potential rho-independent terminator sequence was prepared similarly. Both PCR fragments, which included 25 bp of overlapping sequence at the 3' end of fragment 1 and 5' end of fragment 2, were subject to agarose gel (1% [wt/vol]) electrophoresis and then purified using the QIAquick gel extraction procedure (Qiagen). The respective PCR products were mixed and then diluted 10-fold in sterile distilled water, and a 1- μ l aliquot was used as a template for a subsequent round of PCR using primers designed to generate an amplicon encompassing *gerUA*, *gerUC*, and the chimeric *gerUB/VB* gene arranged as a GerA-type receptor operon with appropriate regulatory sequences. This amplicon was purified by gel extraction, digested with BamHI, and then ligated with pHT315 restricted with the same enzyme. Recombinant plasmid purified from transformant *E. coli* was sequenced to confirm the fidelity of the fusion operon and then used to transform PV361 to MLS^r, giving strain GC452. Construction of strain GC451, in which the B protein comprises a fusion of GerUB TM1 to -8 with GerVB TM9 and -10, was described previously (6).

Germination assays. Spores at 5 to 10 mg/ml (dry weight) in water were heat shocked at 60°C for 10 min and then cooled on ice. Spore germination was monitored by recording the decrease in OD of spores suspended at an initial OD₆₀₀ of between 0.95 and 1.0 in germination buffer (5 mM Tris-HCl [pH 7.8] plus 10 mM of germinant [50 mM for KBr]) at 30°C for 40 min, using a 1-ml cuvette in a Hewlett-Packard 8452A diode array spectrophotometer. Reported values are the means of at least duplicate experiments utilizing independently prepared batches of spores. Where presented, maximum rates of spore germination are given relative to the absorbance loss observed for *B. megaterium* QM B1551 spores incubated with 10 mM glucose, where a 65% loss in OD₆₀₀ correlates with approximately 100% spore germination, as determined by loss of heat resistance.

Experiments designed for germination kinetic analyses were conducted in 96-well plates in a total volume of 300 μ l/well using a Tecan Infinite-200 series shaking incubating plate reader. Plates were sealed with transparent adhesive PCR film (Abgene) to reduce losses due to evaporation. Experiments were conducted in triplicate with at least two different spore preparations. Heat-activated spores (OD₆₀₀ of 0.4) were incubated at 30°C in 5 mM Tris-HCl (pH 7.8) with germinant concentrations ranging from 0.1 to 50 mM, and germination was monitored by measuring the decrease in OD₆₀₀ every minute for 60 min. Germination rates (v [OD units min⁻¹]) were determined from the slope of the linear segment of OD changes over time that follows the initial lag phase. The Michaelis-Menten function of SigmaPlot v.10 (Systat Software, Inc.) was employed to determine apparent K_m and V_{max} values. K_m in this context, therefore, is essentially a measure of the concentration of germinant that stimulates the half-maximal rate of decrease in OD of the spore suspension being tested. Additionally, the observation that the OD₆₀₀ decreases by ~60% in fully germinated spore suspensions under these conditions was used to estimate the proportion of spores that had germinated after incubation for 60 min during germinant titration experiments. Since plots of spore germination against germinant concentration yield hyperbolic curves, data were fitted by nonlinear regression using SigmaPlot v.10 (Systat Software, Inc.) and concentrations required to stimulate 50% spore germination, $K_{0.5}$ (germ), were determined.

In experiments designed to investigate cooperative germination in response to mixtures of germinants, heat-shocked spores (OD₆₀₀ of 0.4) were exposed to variable concentrations of primary germinant (typically 0.5, 1, 2.5, 5, 10, 25, 50, and 100 mM) in the presence of fixed concentrations of secondary germinant (typically 0.1, 0.5, 1.0, and 5.0 mM). Germination was monitored, and kinetic parameters were determined as described above.

FIG. 1. Predicted transmembrane topology of GerVB putative TM9 and TM10 based on the hydropathy plot of the primary amino acid sequence (28). TM domains are shown as boxes and labeled with Roman numerals. Circled residues represent nonconserved positions, identified by ClustalW alignment, which were substituted for the amino acid at the corresponding position in GerUB (Table 1).

DNA sequencing and bioinformatics analyses. DNA sequencing was performed by the Department of Biochemistry sequencing facility (University of Cambridge). DNA sequence analysis was performed using CLC combined workbench 3 (CLC bio). Protein topology and transmembrane helix predictions were made using the HMMTOP program (28), which is available on the ExPASy server (Swiss Institute of Bioinformatics) (<http://expasy.org/>). Helical wheel projections were made using Wheel, version 1.3 (33), which is freely available online (<http://r2lab.ucr.edu/scripts/wheel/wheel.cgi>).

RESULTS

Key residues in GerVB TM9 and TM10. The germinant specificity profile of the *B. megaterium* GerU receptor is defined, as far as we have established to date, by two close B protein homologues that interact with the GerUA and GerUC proteins (7). Thus, spores containing GerUB germinate in response to glucose and leucine, whereas spores with GerVB respond to proline and some inorganic salts (exemplified by KBr) in addition to glucose and leucine. Spores of strain GC451, whose receptor B protein is an engineered cross-homologue fusion comprising GerUB TM1 to -8 and GerVB TM9 and -10, show a gain-of-function germination response, responding to proline in a predominantly noncognate (GerUB) background (6). These data suggested that key GerVB residues predicted to reside in TM9 and/or TM10—in addition to those identified as being crucial in the connecting loop region (OL5)—might participate in the binding of proline. In order to identify these residues, a series of mutants were prepared in which nonconserved residues in TM9 and TM10 of GerVB, comprising 12 out of the 38 residues predicted to comprise these domains (Fig. 1), were substituted individually for those residues at the equivalent position in GerUB. Spores were prepared, and their responses to germinants were recorded (Table 2).

Of the five constructs containing mutations predicted to reside in TM9, only two (A318S and S319M) were observed to

TABLE 2. Rates of germination of spores carrying amino acid substitutions in GerVB TM9 and TM10^a

Strain	Substitution		Rate of spore germination with germinant ^b :					
	Domain	Residue	Buffer	GPLK	Glucose	Proline	Leucine	KBr
GC431 (wild type)	None	None	1	100	99	100	57	100
GC432	TM9	L306M	1	100	100	100	73	100
GC433	TM9	F312	1	100	100	100	88	100
GC434	TM9	V313I	1	100	100	100	76 ± 10	86 ± 15
GC435	TM9	A318S	1	100	93	96	17	70
GC436	TM9	S319M	<1	100	94	91	<1	49 ± 20
GC440	TM10	I339V	3	100	100	100	59	100
GC441	TM10	M342I	2	100	97	100	61 ± 10	92
GC442	TM10	I344L	1	100	100	100	84	100
GC443	TM10	L345F	3	100	65	2	2	8
GC444	TM10	A346T	1	100	100	100	84	100
GC445	TM10	I347V	1	100	98	100	45 ± 15	96
GC446	TM10	A356S	1	100	97	99	72	99

^a Spores were germinated in 5 mM Tris-HCl (pH 7.8) for 40 min with 10 mM germinant (50 mM KBr). GPLK is a mixture of 10 mM each glucose, proline, and leucine and 50 mM KBr. Spore germination was measured as described in Materials and Methods.

^b Rates of spore germination are given relative to the OD₆₀₀ loss (65%) for spores of the parental strain GC431 in 10 mM glucose, which was set at 100. This value is equivalent to 100% germination after a 40-min incubation. Values are the means of duplicate experiments employing two different spore preparations. Except where indicated, the standard deviation was ≤5% of the mean and is not shown.

impact spore germination properties, the most notable being the complete loss of response to leucine in strain GC436 (S319M). This observation was unexpected since both GerUB and GerVB germinate in response to leucine and probably would be expected to utilize conserved residues in germinant binding. None of the TM9 mutations appears to reduce significantly the ability of the spores to initiate germination in response to proline, suggesting that TM10 is important in this aspect. Indeed, germinative analysis of the seven constructs with substitutions in TM10 reveals that strain GC443, which contains the L345F substitution, has lost the ability to respond to proline. Germinability to other trigger compounds is also affected; leucine-mediated germination is completely lost, the germinative rate to KBr is reduced to below 10%, and the glucose response is also observed to be reduced significantly.

Cooperative germination. Having established the identity of two residues in particular that appear to be strong determinants of receptor function, and prompted by the observation that all mutant spores give 100% germination responses to a mixture of germinants (GPLK [10 mM each glucose, proline, and leucine and 50 mM KBr]), we sought to determine whether germinants that no longer act as single trigger compounds could still be utilized as cogerminants. Evidence derived from kinetic analyses suggests a sequential mechanism in

some spore receptors, including GerU, where binding of one germinant leads to a conformational change that may expose a binding site for a different germinant (1, 6). The ability to utilize leucine as a cogerminant, for example, by GerVB L345F and S319M spores, would indicate that the cognate binding site is still present, but either it is rendered inaccessible by structural perturbations introduced by the substituted residue or signal transduction upon binding is somehow impeded. To investigate this possibility, spores of strain GC443 (L345F) were germinated with glucose plus either leucine, proline, or KBr as a cogerminant, and kinetic parameters associated with germination were determined (Table 3). Similarly, strain GC436 (S319M) was germinated with either glucose or proline plus leucine as a cogerminant, and kinetic parameters were established (Table 4).

Since the GerVB L345F mutation reduces the apparent affinity of the receptor for all germinants, including glucose (K_m , 13.1 mM versus 4.05 mM for wild-type spores), it is evident from data presented in Table 3 that both leucine and KBr retain the ability to act as powerful cogerminants. Both cogerminants significantly reduce the apparent K_m for glucose even at the lowest concentration tested (0.5 mM), while the affinity of the spores for glucose continues to increase as the concentration of cogerminant increases. Since neither leucine nor

TABLE 3. Kinetic analysis of GC433 (GerVB L345F) spore germination in response to glucose plus cogerminants^a

Cogerminant concn (mM)	Result for cogerminant:					
	Leucine		Proline		KBr	
	K_m (glucose) (mM)	V_{max} (OD/min)	K_m (glucose) (mM)	V_{max} (OD/min)	K_m (glucose) (mM)	V_{max} (OD/min)
0.5	3.02 ± 0.22	0.10	11.19 ± 0.84	0.09	4.01 ± 0.20	0.07
1	2.77 ± 0.15	0.10	12.46 ± 0.67	0.09	3.04 ± 0.17	0.08
5	2.38 ± 0.21	0.10	11.87 ± 0.61	0.09	1.51 ± 0.08	0.08
10 ^b	2.26 ± 0.17	0.10	11.65 ± 0.60	0.09	0.92 ± 0.04	0.07

^a Spores were germinated in 5 mM Tris-HCl (pH 7.8), for 60 min with variable glucose (0.5, 1, 2.5, 5, 10, 25, 50, and 100 mM) concentrations and different fixed concentrations (0.5, 1, 5, and 10 mM) of cogerminant. Germination rates (v [OD units min⁻¹]) and apparent K_m and V_{max} values were determined as described in Materials and Methods. The standard deviations from the mean shown are from three independent experiments conducted with the same spore preparation.

^b The KBr concentration was 50 mM.

TABLE 4. Kinetic analysis of GC436 (GerVB S319M) spore germination in response to glucose and proline plus leucine as a cogerminant^a

Leucine concn (mM)	Result for germinant:			
	Glucose		Proline	
	K_m (mM)	V_{max} (OD/min)	K_m (mM)	V_{max} (OD/min)
0	10.50 ± 0.88	0.10	10.72 ± 0.87	0.07
0.1	5.40 ± 0.22	0.09	9.50 ± 0.73	0.05
0.5	5.31 ± 0.24	0.10	8.02 ± 0.83	0.06
1	4.28 ± 0.16	0.10	7.52 ± 0.74	0.06
5	3.45 ± 0.21	0.09	5.33 ± 0.49	0.06

^a Spores were germinated in 5 mM Tris-HCl (pH 7.8) for 60 min with variable glucose (0.5, 1, 2.5, 5, 10, 25, 50, and 100 mM) or variable proline (0.175, 0.25, 0.5, 1, 2.5, 5, 10, and 20 mM) concentrations and different fixed concentrations (0.1, 0.5, 1, and 5 mM) of leucine. Germination rates (v [OD units min⁻¹]) and apparent K_m and V_{max} values were determined as described in Materials and Methods. The standard deviations from the mean shown are from three independent experiments conducted with the same spore preparation.

KBr can initiate germination to appreciable levels on their own in L345F spores, it seems that binding sites, or a route to signal transduction, become available after a possible conformational change upon exposure to glucose, suggesting Leu345 is not involved directly in binding of these compounds. On the other hand, proline does not appear to be utilized as a cogerminant and the apparent affinity of the spores for glucose remains at approximately mutant receptor levels in the presence of increasing concentrations of proline. The loss of germinability to proline, either solely or cooperatively with glucose (observed in spores containing the parental receptor [data not shown]), suggests that Leu345 may have close proximity to, or be directly involved in, the proline binding site.

Spores of strain GC436 (S319M), which cannot utilize leucine as a sole germinant, demonstrate the capacity to utilize this compound as a cogerminant in the presence of glucose or proline, the apparent affinity of the receptor for the primary germinant increasing as the concentration of cogerminant increases (Table 4). It seems likely, therefore, that the introduced mutation masks access to the cognate binding site for leucine, which is revealed by a conformational change upon exposure to another germinant, as opposed to Ser319 being directly involved in binding of leucine. Alternatively, the mutation could disrupt the route to leucine-mediated signal transduction, which is restored by the presence of the primary germinant.

Amino acid substitution analysis. Following these initial findings, further Leu345 and Ser319 replacements were made and spores analyzed in an attempt to gain insights to the role of the amino acid side chains at these positions. The most notable observation from these experiments is the retention of proline recognition when Leu345 is substituted for any of the small nonpolar amino acids tested (isoleucine, valine, or alanine), whereas spores containing GerVB with tyrosine in this position only recognize glucose (Table 5). However, while substitution for isoleucine, valine, or alanine permits strong germinant responses to both glucose and proline, the response to other germinants is variable: e.g., L345I significantly reduces the germinative rates to leucine and KBr, whereas L345A spores show an increased germinative rate to leucine (99% germination) compared to the wild-type spores (57%).

TABLE 5. Rates of germination of spores with GerVB substituted at position 319 or 345^a

Strain	Mutation	Rate of spore germination with germinant ^b :					
		Buffer	GPLK	Glucose	Proline	Leucine	KBr
GC431 (wild type)	None	1	100	99	100	57	100
GC443	L345F	3	100	65	2	2	8
GC447	L345I	3	98	95	100	11	44
GC448	L345V	3	100	94	96	50	88
GC449	L345A	3	100	100	100	99	100
GC450	L345Y	<1	100	88	<1	<1	7
GC436	S319M	<1	100	94	91	<1	49 ± 20
GC437	S319A	2	100	84	74	13	82
GC438	S319T	2	100	77	33	3	28
GC439	S319C	2	100	98	95 ± 7	10	94

^a Spores were germinated in 5 mM Tris-HCl (pH 7.8) for 40 min with 10 mM germinant (50 mM KBr). GPLK is a mixture of 10 mM (each) glucose, proline, and leucine and 50 mM KBr. Spore germination was measured as described in Materials and Methods.

^b Rates of spore germination are given relative to the OD₆₀₀ loss (65%) for spores of the parental strain GC431 in 10 mM glucose, which was set at 100. This value is equivalent to 100% germination after a 40-min incubation. Values are the means of duplicate experiments employing two different spore preparations. Except where indicated, the standard deviation was ≤5% of the mean and is not shown.

More detailed kinetic analysis of Leu345 mutants reveals that the apparent affinity of the receptor for proline is comparable to that of the parental receptor only when Leu345 is substituted for alanine, whereas substitution for isoleucine or valine results in an approximate fourfold increase in apparent K_m (Table 6). As expected, increases in apparent K_m are also reflected in the concentration of germinant required to stimulate 50% germination. In contrast, only small effects are observed on apparent V_{max} values, where maximal reductions in OD₆₀₀ values remain at approximately 0.1 OD unit min⁻¹. Considered together, these data indicate that differences in apparent affinity and sensitivity to proline can be attributed primarily to alterations in germinant binding as opposed to downstream signal transduction events. However, tolerance for a range of small amino acids in this position, resulting in relatively modest differences in apparent K_m , suggests that Leu345 does not interact directly with proline, but probably influences the conformation of, or access to, the proline binding site.

A similar trend is observed for glucose-mediated germination, where enhanced K_m and $K_{0.5}$ (germ) values contrast with relatively unaffected V_{max} values in mutants where Leu345 is substituted for phenylalanine, isoleucine, or valine, indicating that the receptor binding site for glucose is impaired. Substitution of Leu345 for alanine, however, is observed to increase the apparent affinity of the receptor for glucose compared to the parental receptor. These spores (strain GC449) also show an improved germinative response to leucine compared to the wild type (Table 5), suggesting that possible increased conformational flexibility in this region of TM10 may help accommodate germinants in binding sites that are close in proximity in the receptor.

The importance of position 319 to GerVB function is also revealed further by amino acid substitution analysis, where, in addition to the loss of leucine-mediated germination in the original S319M mutant, substitution of serine for alanine, threonine, or cysteine is observed to have an impact on recep-

TABLE 6. Kinetic analysis of germination of spores containing amino acid substitutions in GerVB position 345 in response to proline and glucose^a

Strain	Mutation	Result for germinant:					
		Proline			Glucose		
		K_m (mM)	V_{max} (OD/min)	$K_{0.5}$ (germ) (mM)	K_m (mM)	V_{max} (OD/min)	$K_{0.5}$ (germ) (mM)
GC431 (wild type)	None	5.6 ± 0.3	0.10	0.26	4.05 ± 0.28	0.10	0.19
GC443	L345F	NA ^b	NA	NA	13.10 ± 0.98	0.10	2.56
GC447	L345I	20.3 ± 1.3	0.10	1.61	21.39 ± 1.53	0.11	1.99
GC448	L345V	22.9 ± 1.0	0.10	2.78	12.33 ± 0.90	0.10	2.00
GC449	L345A	6.3 ± 0.4	0.12	0.31	1.94 ± 0.12	0.12	0.10

^a Spores were germinated in 5 mM Tris-HCl (pH 7.8) for 60 min with variable glucose or proline (0.5, 1, 2.5, 5, 10, 25, 50, and 100 mM) concentrations, and germination was monitored as described in Materials and Methods. Variable glucose or proline concentrations for GC449 (L345A) spores, which show enhanced sensitivity to germinant, were 0.1, 0.175, 0.25, 0.5, 1, 2.5, 5, and 10 mM. Germination rates (v [OD units min⁻¹]) and apparent K_m and V_{max} values were determined as described in Materials and Methods. The standard deviations from the mean shown are from three independent experiments conducted with the same spore preparation.

^b NA, not applicable.

tor functionality to other trigger compounds (Table 5). Kinetic analysis of spore germinability of these strains to proline, where enhanced apparent K_m values (33.6 ± 2.0 for S319A and 36.1 ± 2.3 for S319C versus 5.6 ± 0.3 for the wild type) but only slightly altered V_{max} values (data not shown) indicate that these substitutions probably indirectly affect the conformation of binding sites for the various germinants via long-range structural effects.

Amino acid substitutions in GerUB. In an attempt to gain further information on the roles of residues at position 319 and 345 to receptor functionality, a series of mutants were made with substitutions at the same positions in GerUB, which mediates relatively strong germination responses to leucine and glucose. Strain GC453, which contains the M319A substitution, retains a strong germinative response to leucine (Table 7), indicating that this residue is not involved directly in the binding of germinant in this receptor. This observation, by extension, provides further evidence that Ser319 in GerVB, which is a major determinant in leucine-initiated germination, is involved in germinant binding only indirectly. These data are also consistent with the idea that both homologues are likely to employ conserved residues for binding of common germinants.

In contrast, spores of strain GC454, which contain the F345A mutation, show a considerable reduction in germinability to both glucose and leucine (Table 7). Despite being non-

conserved, this position is therefore of functional or structural importance to both GerUB and GerVB. Functionality is restored to parental levels in the GerUB F345A receptor background (strain GC455) by substituting OL5 residues for the corresponding residues in GerVB (Y334K, A335V, A336E, and K337M), which are predicted to be located immediately adjacent to TM10 in which position 345 putatively resides (Fig. 2). These spores do not, however, show an appreciable germinative response to proline. A cross-homologue chimera comprising GerUB TM1 to -9 and GerVB OL5/TM10 is also observed to be functional (strain GC452), but unlike GC451 spores, in which GerUB TM1 to -8 are fused with GerVB TM9 and -10, is not responsive to proline. Thus, while alanine can be tolerated at position 345 in GerUB when appropriate OL5 residues are present, further substitutions to TM9 residue(s) are required in order to confer significant proline recognition to this non-cognate receptor background. This distribution of functionally important residues hints at a degree of structural coordination or interaction between TM9, TM10, and OL5 residues, in determining the germinant profile for the GerU receptor.

Receptor abundance. The possibility that amino acid substitutions at structurally important regions of the protein might exert a deleterious effect on receptor abundance and/or ability to insert correctly into the membrane has to be considered

TABLE 7. Rates of germination of spores with substituted GerUB (position 319 or 345) or GerUB-VB fusions^a

Strain	GerUB modification	Rate of spore germination with germinant ^b :					
		Buffer	GPLK	Glucose	Proline	Leucine	KBr
GC430	None	3	100	80 ± 8	3	67 ± 15	8
GC453	GerUB M319A	2	100	80	1	64	9
GC454	GerUB F345A	3	43 ± 9	11	2	5	5
GC451 ^c	GerUB TM1 to -8, GerVB TM9 and -10	14	100	99	39	92	15
GC452	GerUB TM1 to -9, GerVB TM10	3	100	79 ± 6	6	92	5
GC455	GerUB Y334→K337 changed to KVEM and F345A	4	100	83	4	84	12

^a Spores were germinated in 5 mM Tris-HCl (pH 7.8) for 40 min with 10 mM germinant (50 mM KBr). GPLK is a mixture of 10 mM each glucose, proline, and leucine and 50 mM KBr. Spore germination was measured as described in Materials and Methods.

^b Rates of spore germination are given relative to the OD₆₀₀ loss (65%) for spores of the parental strain GC431 in 10 mM glucose, which was set at 100. This value is equivalent to 100% germination after a 40-min incubation. Values are the means of duplicate experiments employing two different spore preparations. Except where indicated, the standard deviation was ≤5% of the mean and is not shown.

^c Data from reference 6.

FIG. 3. Helical wheel projections of GerVB putative TM9 and TM10, assuming a periodicity of 3.6 residues per turn. Hydrophobic residues are represented by diamonds and hydrophilic residues by circles, as adjudged by Wheel software (33). Residues of functional importance identified in this study are shaded.

nine in GerUB suggest that this position influences germinant recognition via indirect structural effects. In contrast to TM10, helical wheel analysis of TM9 indicates a degree of clustering of relatively hydrophilic residues that could potentially be solvent exposed (Fig. 3), and it may be significant for germinant interaction that residues that demonstrably affect receptor function (Ser319 and Ala318) are predicted to reside on the same relatively hydrophilic aspect of the helix.

The ability of some germinants to act only as cogerminants in both the GerVB L345F and S319M mutants provides further evidence that the receptor B protein undergoes a conformational change upon binding of a primary germinant, since these data indicate that binding sites for cogerminants only become available upon binding of the first germinant. Whether this putative conformational change permits concomitant or sequential binding of the cogerminant to the same primary germinant-activated B protein subunit, or leads to exposure of binding sites on adjacent receptors, remains to be established. Our current model predicts that different germinant binding sites are close in spatial proximity in the receptor, which could encourage coordinated binding between different ligands in the same receptor subunit, as observed in prokaryotic secondary amino acid transporters (14). Equally, steric hindrance might prevent concomitant binding of two or more germinants to the same subunit. Clearly, structural information at sufficient resolution will be required to provide insights into this aspect of receptor function.

Additionally, the possibility also exists that observed cooperativity between germinants may involve activation of receptor proteins and germinant pathways that have yet to be characterized in *B. megaterium*. Evidence that other receptor proteins might be involved is provided by the observation that spores carrying only GerUA and GerUC proteins exhibit a moderate germinative response to GPLK, indicating that an as yet unidentified B protein may be capable of interacting with these subunits to create a functional receptor. The recently sequenced *B. megaterium* QM B1551 genome should facilitate the identification of candidate receptor proteins.

A final objective of this study was to define further the minimum requirements in terms of conferring proline recog-

nition to a non-cognate receptor background. Information derived from cross-homologue chimeras demonstrate that introduced residues identified as being crucial to proline recognition in GerVB are insufficient to confer significant proline recognition to the GerUB receptor background. These differences presumably reflect subtle differences in structure between the two proteins, and since key residues are distributed throughout the putative TM9-TM10 region it appears that a degree of structural coordination between these domains is required for efficient receptor function.

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REFERENCES

1. Akoachere, M., R. C. Squires, A. M. Nour, L. Angelov, J. Brojatsch, and E. Abel-Santos. 2007. Identification of an *in vivo* inhibitor of *Bacillus anthracis* spore germination. *J. Biol. Chem.* **282**:12112–12118.
2. Arantes, O., and D. Lereclus. 1991. Construction of cloning vectors for *Bacillus thuringiensis*. *Gene* **108**:115–119.
3. Athuri, S., K. Ragkousi, D. E. Cortezo, and P. Setlow. 2006. Cooperativity between different nutrient receptors in germination of spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB receptor. *J. Bacteriol.* **188**:28–36.
4. Barlass, P. J., C. W. Houston, M. O. Clements, and A. Moir. 2002. Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of *gerL* and *gerQ* operons. *Microbiology* **148**:2089–2095.
5. Cabrera-Martinez, R.-M., F. Tovar-Rojas, V. R. Vepachedu, and P. Setlow. 2003. Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis*. *J. Bacteriol.* **185**:2457–2464.
6. Christie, G., M. Lazarevska, and C. R. Lowe. 2008. Functional consequences of amino acid substitutions to GerVB, a component of the *Bacillus megaterium* spore germinant receptor. *J. Bacteriol.* **190**:2014–2022.
7. Christie, G., and C. R. Lowe. 2007. Role of chromosomal and plasmid-borne receptor homologues in the response of *Bacillus megaterium* QM B1551 spores to germinants. *J. Bacteriol.* **189**:4375–4383.
8. Dogovski, C., J. Pi, and A. J. Pittard. 2003. Putative interhelical interactions within the PheP protein revealed by second-site suppressor analysis. *J. Bacteriol.* **185**:6225–6232.
9. Feavers, I. M., J. Foulkes, B. Setlow, D. Sun, W. Nicholson, P. Setlow, and A. Moir. 1990. The regulation of transcription of the *gerA* spore germination operon of *Bacillus subtilis*. *Mol. Microbiol.* **4**:275–282.
10. Fisher, N., and P. Hanna. 2005. Characterization of *Bacillus anthracis* germinant receptors in vitro. *J. Bacteriol.* **187**:8055–8062.
11. Hudson, K. D., B. M. Corfe, E. H. Kemp, I. M. Feavers, P. J. Coote, and A. Moir. 2001. Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. *J. Bacteriol.* **183**:4317–4322.
12. Igarashi, T., and P. Setlow. 2005. Interaction between individual protein

- components of the GerA and GerB nutrient receptors that trigger germination of *Bacillus subtilis* spores. *J. Bacteriol.* **187**:2513–2518.
13. Jack, D. L., I. T. Paulsen, and M. H. Saier. 2000. The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. *Microbiology* **146**:1797–1814.
  14. Jung, H., T. Pirch, and D. Hilger. 2006. Secondary transport of amino acids in prokaryotes. *J. Membr. Biol.* **213**:119–133.
  15. Loshon, C. A., P. Kraus, B. Setlow, and P. Setlow. 1997. Effects of inactivation or overexpression of the *sspF* gene on properties of *Bacillus subtilis* spores. *J. Bacteriol.* **179**:272–275.
  16. Moir, A., B. M. Corfe, and J. Behravan. 2002. Spore germination. *Cell Mol. Life Sci.* **59**:403–409.
  17. Moir, A., E. H. Kemp, C. Robinson, and B. M. Corfe. 1994. The genetic analysis of bacterial spore germination. *J. Appl. Bacteriol.* **77**:9S–16S.
  18. Paidhungat, M., and P. Setlow. 1999. Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. *J. Bacteriol.* **181**:3341–3350.
  19. Paidhungat, M., and P. Setlow. 2001. Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. *J. Bacteriol.* **183**:3982–3990.
  20. Paidhungat, M., and P. Setlow. 2000. Role of Ger proteins in nutrient and nonnutrient triggering of spore germination in *Bacillus subtilis*. *J. Bacteriol.* **182**:2513–2519.
  21. Pirch, T., M. Quick, M. Nietschke, M. Langkamp, and H. Jung. 2002. Sites important for Na⁺ and substrate binding in the Na⁺/proline transporter of *Escherichia coli*, a member of the Na⁺/solute symporter family. *J. Biol. Chem.* **277**:8790–8796.
  22. Quick, M., S. Tebbe, and H. Jung. 1996. Ser57 in the Na⁺/proline permease of *Escherichia coli* is critical for high-affinity proline uptake. *Eur. J. Biochem.* **239**:732–736.
  23. Sammons, R. L., A. Moir, and D. A. Smith. 1981. Isolation and properties of spore germination mutants of *Bacillus subtilis*-168 deficient in the initiation of germination. *J. Gen. Microbiol.* **124**:229–241.
  24. Setlow, B., E. Melly, and P. Setlow. 2001. Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. *J. Bacteriol.* **183**:4894–4899.
  25. Setlow, P. 2003. Spore germination. *Curr. Opin. Microbiol.* **6**:550–556.
  26. Swerdlow, B. M., B. Setlow, and P. Setlow. 1981. Levels of H⁺ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. *J. Bacteriol.* **148**:20–29.
  27. Tovar-Rojo, F., M. Chander, B. Setlow, and P. Setlow. 2002. The products of the *spoVA* operon are involved in dipicolinic acid uptake into developing spores of *Bacillus subtilis*. *J. Bacteriol.* **184**:584–587.
  28. Tusnady, G. E., and I. Simon. 2001. The HMMTOP transmembrane topology prediction server. *Bioinformatics* **17**:849–850.
  29. Vary, P. S., and Y. P. Tao. 1988. Development of genetic methods in *Bacillus megaterium*, p. 403–407. In A. T. Ganesan and J. A. Hoch (ed.), *Genetics and biotechnology of bacilli*, vol. 2. Academic Press, New York, NY.
  30. Vepachedu, V. R., and P. Setlow. 2007. Analysis of interactions between nutrient germinant receptors and SpoVA proteins of *Bacillus subtilis* spores. *FEMS Microbiol. Lett.* **274**:42–47.
  31. Vepachedu, V. R., and P. Setlow. 2004. Analysis of the germination of spores of *Bacillus subtilis* with temperature sensitive *spo* mutations in the *spoVA* operon. *FEMS Microbiol. Lett.* **239**:71–77.
  32. Vepachedu, V. R., and P. Setlow. 2007. Role of SpoVA proteins in release of dipicolinic acid during germination of *Bacillus subtilis* spores triggered by dodecylamine or lysozyme. *J. Bacteriol.* **189**:1565–1572.
  33. Zidovetzki, R., B. Rost, D. L. Armstrong, and I. Pecht. 2003. Transmembrane domains in the functions of Fc receptors. *Biophys. Chem.* **100**:555–575.