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Role of GerD in Germination of *Bacillus subtilis* Spores[▽]

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Spores of a *Bacillus subtilis* strain with a *gerD* deletion mutation (Δ*gerD*) responded much slower than wild-type spores to nutrient germinants, although they did ultimately germinate, outgrow, and form colonies. Spores lacking GerD and nutrient germinant receptors also germinated slowly with nutrients, as did Δ*gerD* spores in which nutrient receptors were overexpressed. The germination defect of Δ*gerD* spores was not suppressed by many changes in the sporulation or germination conditions. Germination of Δ*gerD* spores was also slower than that of wild-type spores with a pressure of 150 MPa, which triggers spore germination through nutrient receptors. Ectopic expression of *gerD* suppressed the slow germination of Δ*gerD* spores with nutrients, but overexpression of GerD did not increase rates of spore germination. Loss of GerD had no effect on spore germination induced by agents that do not act through nutrient receptors, including a 1:1 chelate of Ca^{2+} and dipicolinic acid, dodecylamine, lysozyme in hypertonic medium, a pressure of 500 MPa, and spontaneous germination of spores that lack all nutrient receptors. Deletion of GerD's putative signal peptide or change of its likely diacylglycerylated cysteine residue to alanine reduced GerD function. The latter findings suggest that GerD is located in a spore membrane, most likely the inner membrane, where the nutrient receptors are located. All these data suggest that, while GerD is not essential for nutrient germination, this protein has an important role in spores' rapid response to nutrient germinants, by either direct interaction with nutrient receptors or some signal transduction essential for germination.

The germination of spores of *Bacillus* species initiates the conversion of metabolically dormant spores into growing cells. Germination of *Bacillus subtilis* spores normally begins with the binding of specific nutrient germinants, either L-alanine or a mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (AGFK), to specific receptors located in the spore's inner membrane (3, 18, 20, 30). Three functional nutrient receptors are found in *B. subtilis* spores, each encoded by the homologous tricistronic *gerA*, *gerB*, and *gerK* operons. The GerA nutrient receptor responds to L-alanine, while the GerB and GerK nutrient receptors cooperate in some fashion to respond to AGFK (18). Spores of *gerA* mutants are defective in germination with L-alanine, and spores of *gerB* or *gerK* mutants are defective in germination with AGFK. In addition, *gerD* and *gerF* mutants whose spores are affected in their germination with both L-alanine and AGFK have been identified (14, 18, 19, 20). Spores of *gerD* and *gerF* mutants have a defect early in germination, before the release of the spore's large depot of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]). GerF is a prelipoprotein diacylglycerol transferase necessary for nutrient receptor function (10, 11), but GerD function in spore germination is unknown.

GerD is an ~20-kDa protein with homologs encoded in all sequenced genomes from *Bacillus* species (37) (Fig. 1), although not in the more distantly related *Clostridium* species. The GerD sequence is not homologous to that of any of the subunits of the spore's nutrient receptors. Transcription of *gerD* is at the same time as that of the *gerA*, *gerB*, and *gerK* operons and is induced only in the developing forespore compartment of the sporulating cell under the control of the forespore-specific sigma factor for RNA polymerase, σ^G (15). The precise location of GerD in the spore remains unclear, although there is a preliminary report that this protein is in the spore's outer layers (20). The amino acid sequence of GerD (37) includes a short N-terminal hydrophobic region that is likely a signal peptide (SP), followed by a recognition sequence for diacylglycerol addition to a specific cysteine residue (Fig. 1). The presence of these sequence features suggests that GerD is a membrane protein, and its forespore-specific expression is most consistent with its location being the spore's inner membrane.

As noted above, previous work indicates that *gerD* mutant spores are slow to germinate with both L-alanine and AGFK, although this is reported to be to different extents (14, 18, 35). The *gerD* mutant spores do germinate in L-alanine, albeit slower than wild-type spores; however, the decrease in the rate of *gerD* mutant spore germination compared to that of wild-type spores is much greater in AGFK (14, 18, 35). This finding, as well as the much-improved germination with L-alanine of *gerD* mutant spores prepared in a resuspension medium (RM), a defined minimal medium (21, 32), has led to the suggestion that GerD may not be an essential component of the spore germination apparatus (35). However, previous work on *gerD* mutant spores has used strains with either *gerD* point mutations or with a Tn*917* insertion in the *gerD* coding sequence (35, 37). Since such mutants may retain partial GerD function, we have reexamined the *gerD* phenotype using strains in which a large portion of the *gerD* coding sequence has been deleted and replaced with an antibiotic resistance gene. We have also examined the importance of the likely SP and a putative site

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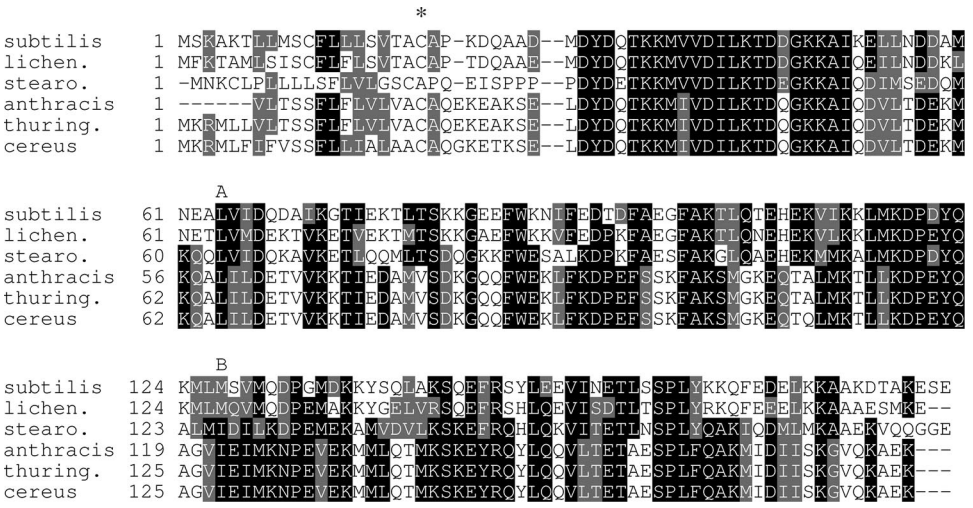

FIG. 1. Alignment of amino acid sequences of GerD from the various *Bacillus* species. The species compared were *B. subtilis*, *Bacillus licheniformis*, *Geobacillus stearothermophilis*, *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus cereus*. Gray shading indicates similar residues, and black shading indicates identical residues. The asterisk denotes the cysteine residue that is the putative site for diacylglycerol addition. The A and B over the sequences delineate the region of *gerD* coding sequence deleted and replaced with an antibiotic resistance gene in the Δ*gerD* strains used in this work.

for diacylglycerol addition in GerD for the function of this protein.

MATERIALS AND METHODS

***B. subtilis* strains and their construction.** *B. subtilis* strains used in this study are listed in Table 1. All are isogenic derivatives of strain PS832, a prototrophic derivative of strain 168. New strains generated in this work were made via transformation with chromosomal or plasmid DNA (1). Mutations in various *ger* genes are deletions, with much of the genes' coding sequences replaced by antibiotic resistance genes (2, 10, 26). In the case of the Δ*gerD* strain, 35% of the *gerD* coding sequence has been deleted (Fig. 1) and replaced with a spectinomycin resistance (Sp^r) gene (10). Plasmid pVK73 (8), for replacing an Sp^r gene with a kanamycin resistance (Km^r) marker, was obtained from the *B. subtilis* Genetic Stock Center and was used to replace the Sp^r gene in strain PS3502 with a gene encoding Km^r, giving strain PS3946.

Preparation of *B. subtilis* strains expressing modified forms of GerD. For construction of altered versions of *gerD*, a 1.2-kb fragment from 221 bp upstream to 421 bp downstream of the *gerD* translational start and stop codons, respectively, was PCR amplified (all primer sequences are available upon request) from *B. subtilis* PS832 genomic DNA. This fragment was digested with DraI (site upstream of *gerD*) and HindIII (site in extra nucleotides in the downstream primer) and cloned between the SmaI and HindIII sites in plasmid pUCΔ367 (12) (this plasmid is identical to pUC19 except that 367 bp between the SspI and NdeI sites has been deleted) in *Escherichia coli* JM109, giving plasmid p*gerD*. The 20th codon in *gerD* in this plasmid was then changed from TGC, encoding cysteine, to GCA, encoding alanine, by QuikChange II site-directed mutagenesis (Stratagene, Cedar Creek, TX), giving plasmid p$gerD^{A20}$. A 266-bp fragment from bp 173 to 421 downstream of the *gerD* translation stop codon was PCR amplified from PS832 DNA and digested with SalI and HindIII (sites in the 5′ and 3′ PCR primers, respectively), and the fragment was cloned between the SalI and HindIII sites in plasmid pUC19, giving plasmid pBack*gerD*. A fragment containing bp 192 to 81 upstream of the *gerD* translation start codon was PCR amplified from p*gerD*. This fragment was digested with SacI and BamHI (SacI site originally from the multiple cloning site in pUC19; BamHI site in 3′ primer) and cloned between the SacI and BamHI sites in plasmid pBack*gerD*, giving plasmid pF/B*gerD*. This plasmid was digested with SalI and BamHI, and the small fragment removed was replaced with a 1.3-kb BamHI-SalI fragment containing a chloramphenicol resistance (Cm^r) gene from plasmid pDG364 (9), giving plasmid pΔ*gerD*::Cm^r, in which the Cm^r gene is flanked by sequences upstream and downstream of *gerD* but with the *gerD* coding sequence, promoter, and transcription terminator deleted.

To remove the hydrophobic region near the N terminus of GerD (amino acids 7 to 15) that is likely an SP, a 261-bp fragment containing the first 16 bp of the

TABLE 1. *Bacillus subtilis* strains used

Strain	Genotype	Source[a] or reference
PS832	Wild type	Laboratory stock
FB10	*gerBB**	25
FB62	Δ*gerD*::*spc*	10
FB87	Δ*gerB*::*cat* Δ*gerK*::*erm*	26
PS3476	P_{sspD}-*gerA*	6
PS3502	P_{sspD}-*gerB**	6
PS3521	*gerBB** Δ*gerA*::*neo*	PS3609→FB10
PS3609	Δ*gerA*::*neo*	10
PS3651	Δ*gerA*::*neo*Δ*gerK*::*erm*	2
PS3665	*gerBB** Δ*gerA*::*neo* Δ*gerK*::*erm*	2
PS3926	Δ*gerA*::*neo*Δ*gerD*::*spc*	FB62→PS3609
PS3927	Δ*gerB*::*cat* Δ*gerK*::*erm* Δ*gerD*::*spc*	FB62→FB87
PS3928	Δ*gerA*::*neo* Δ*gerB*::*cat* Δ*gerK*::*erm* Δ*gerD*::*spc*	PS3609→PS3927
PS3929	*gerBB** Δ*gerD*::*spc*	FB62→FB10
PS3931	*gerBB** Δ*gerA*::*neo* Δ*gerD*::*spc*	FB62→PS3521
PS3932	*gerBB** Δ*gerA*::*neo* Δ*gerK*::*erm* Δ*gerD*::*spc*	PS3657→PS3931
PS3938	Δ*gerA*::*neo* Δ*gerK*::*erm* Δ*gerD*::*spc*	PS3657→PS3931
PS3940	P_{sspD}-*gerA* Δ*gerD*::*spc*	FB62→PS3476
PS3945	Δ*gerA*::*neo* Δ*gerB*::*cat* Δ*gerK*::*erm*	PS3609→FB87
PS3946	P_{sspD}-*gerB**	pVK73→PS3502
PS3947	P_{sspD}-*gerB** Δ*gerD*::*spc*	FB62→PS3946
PS3980	Δ*gerD*::*cam*	pΔ*gerD*::*cam*→PS832
PS3981	*gerD*-*spc*	p*gerD*Sp^r→PS3980
PS3982	$gerD^{C20A}$-*spc*	p$gerD^{A20}$$Sp^r$→PS3980
PS3983	$gerD^{\Delta SP}$-*spc*	p$gerD^{\Delta SP}$$Sp^r$→PS3980
PS3987	Δ*gerD amyE*::*gerD*	pΔ*amyE*::*gerD*→PS3980
PS3988	Δ*gerD amyE*::P_{sspB}-*gerD*	pΔ*amyE*::P_{sspB}-*gerD*→PS3980
PS3989	*amyE*::*gerD*	pΔ*amyE*::*gerD*→PS3981
PS3990	*amyE*::P_{sspB}-*gerD*	pΔ*amyE*::P_{sspB}-*gerD*→PS3981
PS3991	*amyE*::$gerD^{C20A}$	pΔ*amyE*::$gerD^{Ala20}$→PS3981
PS3992	*amyE*::$gerD^{\Delta SP}$	pΔ*amyE*::$gerD^{\Delta SP}$→PS3981
PS3993	$gerD^{C20A}$ *amyE*::*gerD*	pΔ*amyE*::*gerD*→PS3982
PS3994	$gerD^{C20A}$ *amyE*::P_{sspB}-*gerD*	pΔ*amyE*::P_{sspB}-*gerD*→PS3982
PS3995	$gerD^{\Delta SP}$ *amyE*::*gerD*	pΔ*amyE*::*gerD*→PS3983
PS3996	$gerD^{\Delta SP}$ *amyE*::P_{sspB}-*gerD*	pΔ*amyE*::P_{sspB}-*gerD*→PS3983

[a] Strains made in this work were constructed by transformation of the strain to the right of the arrow with chromosomal or plasmid DNA from the strain to the left of the arrow. The abbreviation ΔSP denotes a *gerD* gene lacking the region coding for amino acids 7 to 17, which is a likely SP.

gerD coding sequence plus 195 bp of upstream sequence was PCR amplified from plasmid p*gerD*. This fragment was digested with SacI and HindIII (SacI site originally from the multiple cloning site in pUC19; HindIII site in the 3′ primer) and cloned in SacI-HindIII-digested pUCΔ367, giving plasmid pFront^{SP}. A 949-bp fragment encompassing bp 45 of the *gerD* coding sequence to 421 bp downstream of the *gerD* translation stop codon as well as extra residues at both ends, including appropriate restriction sites, was PCR amplified from plasmid p*gerD*, and after digestion with BglII and HindIII this fragment was cloned between the same sites in pFront^{SP}, giving plasmid p*gerD*$^{\Delta SPR6}$, in which codons of *gerD* for 9 amino acids had been deleted while the sixth *gerD* codon had been mutagenized from a threonine codon to an arginine codon. Site-directed mutagenesis was then used to change this sixth codon to the original threonine codon, giving plasmid p*gerD*$^{\Delta SP}$.

Plasmid pBack*gerD* was digested with BamHI and SalI, and a 1.1-kb BamHI-SalI fragment containing an Sp^r gene from plasmid pJL74 (16) was inserted, giving pBack*gerD*Sp^r. Plasmids p*gerD*, p*gerD*^{A20}, and p*gerD*$^{\Delta SP}$ were used as substrates for PCR amplification of the *gerD* region from 192 bp upstream of the translation initiation codon to 144 bp downstream of the translation stop codon plus appropriate restriction sites in the PCR primers. These fragments were digested with SacI and BglII (SacI site originally from the multiple cloning site in pUC19; BglII site in the 3′ primer), and the resultant 0.9-kb fragments were cloned between the SacI and BamHI sites in plasmid pBack*gerD*Sp^r, giving plasmids p*gerD*Sp^r, p*gerD*^{A20}Sp^r, and p*gerD*$^{\Delta SP}$Sp^r, in which a 28-bp fragment beginning 75 bp downstream of *gerD*'s putative transcription terminator had been removed and replaced with an Sp^r gene. For generation of *B. subtilis* strains carrying various *gerD* alleles at the *gerD* locus, strain PS3980 was first generated by transformation of strain PS832 to Cm^r with ScaI-linearized plasmid pΔ*gerD*::Cm. This strain was then transformed with the ScaI-linearized plasmids p*gerD*Sp^r, p*gerD*^{A20}Sp^r, and p*gerD*$^{\Delta SP}$Sp^r with selection for Sp^r, giving strains PS3981, PS3982, and PS3983. Sp^r transformants were further screened for those that had lost Cm^r. In all cases, the appropriate chromosomal structure of the *gerD* locus in *B. subtilis* transformants was confirmed by PCR, and expected changes in the *gerD* sequence were confirmed by DNA sequencing.

Introduction of *gerD* and its variants at the *amyE* locus. DNA fragments of ~0.9 kb encompassing 203 bp upstream to 144 bp downstream of the *gerD* start and stop codons, respectively, were amplified by PCR from genomic DNA of *B. subtilis* strains PS832, PS3982, and PS3983. These fragments were digested with XhoI and BglII (sites within PCR primers), and the resultant ~0.9-kb fragments were inserted between the XhoI and BamHI sites in plasmid pTI8a (11), giving plasmids pΔ*amyE*::*gerD*, in which the *gerD* coding sequence with its promoter and putative transcription terminator sequences was inserted between the front and back regions of *amyE*, and plasmids pΔ*amyE*::*gerD*^{Ala20} and pΔ*amyE*::*gerD*$^{\Delta SP}$ (where ΔSP denotes a *gerD* gene lacking the region coding for amino acids 7 to 17, which is a likely SP) which contain *gerD*^{C20A} and *gerD*$^{\Delta SP}$ inserted in *amyE*, respectively. These plasmids were linearized with ScaI and used to transform *B. subtilis* strains PS3980 and PS3981 to Km^r, giving strains PS3987, PS3989, PS3991, PS3992, PS3993, and PS3995 (Table 1). For insertion of *gerD* at *amyE* but under the control of the strong forespore-specific promoter of the *sspB* gene (6), we utilized plasmid pTI8a (11), which contains the *sspB* promoter between segments of *amyE*. The full *gerD* coding sequence plus appropriate flanking restriction sites was PCR amplified from p*gerD*. This 724-bp fragment was digested with NdeI and BglII (sites in primers) and cloned between these sites in the 6.8-kb fragment from NdeI-BamHI-digested pTI8a, giving plasmid pΔ*amyE*::P$_{sspB}$-*gerD*. This plasmid was used to transform strains PS3980, PS3981, PS3982, and PS3983 to Km^r, giving strains PS3988, PS3990, PS3994, and PS3996, respectively. PCR and DNA sequencing confirmed the presence of the appropriate chromosomal structures at the *amyE* locus in these various transformants.

Spore preparation. Spores were routinely prepared at 37°C on 2× SG medium agar plates without antibiotics (21). Since the composition of the sporulation medium has been reported to affect the GerD requirement for rapid spore germination with nutrients (35), spores were also prepared at 37°C on Spizizen's minimal medium (SMM) plates with 0.1% Casamino Acids or in liquid 2× SG medium, SMM plus 0.1% Casamino Acids, or RM (21, 31, 32). Spores were harvested, cleaned and stored as described previously (21, 22, 28). All spore preparations were >98% free of vegetative or sporulating cells, as determined by observation in a phase-contrast microscope.

Spore germination. Prior to nutrient germination spores at an optical density at 600 nm (OD_{600}) of 10 in water were heat activated at 70°C for 30 min and cooled on ice. Unless noted otherwise, germination was routinely at 37°C with spores at an initial OD_{600} of 1 in either Luria-Bertani (LB) medium (26), SMM (31) with either 10 mM L-alanine or AGFK (see below), or 2× YT medium (26) or in 25 mM Tris-HCl (pH 8.4) with either 10 mM L-alanine or a mixture of L-asparagine (2.5 mM), D-glucose (5 mg/ml), D-fructose (5 mg/ml), and KCl (50 mM) (AGFK). Alternatively, in one experiment L-alanine germination was in 11 mM L-alanine, 7 mg/ml of both KH_2PO_4 and Na_2HPO_4 (pH 7.25), 100 mM NaCl, and 200 mM KCl and AGFK germination was in 3 mM asparagine, 0.5 mg/ml glucose, 0.5 mg/ml fructose, and 50 mM KPO_4 buffer (pH 7.4). Spore germination with L-alanine or AGFK was essentially identical under both the routine and alternative conditions. To examine the effect of pH on spore germination by L-alanine or AGFK the following buffers were used at 25 mM: succinate-NaOH (pH 6.0), Tris-HCl (pH 7.0 and 8.0), and glycine-NaOH (pH 9.0). In most cases spore germination was measured by monitoring the OD_{600} of spore cultures, which falls ~60% upon complete spore germination (2). The extent of spore germination was also confirmed by phase-contrast microscopy. Spore germination by L-alanine and AGFK was also assessed by measuring DPA release as described below. The rate of spore germination was determined from the maximum rate of the fall of the OD_{600} of cultures as determined from the maximum slope of a plot of OD_{600} versus time (2, 6). All values reported are the averages of two experiments performed on two independent spore preparations, and individual values varied by less than 20% from average values shown. Note that since the rate of spore germination is expressed as percent/hour, if germination occurs rapidly, this value may be over 100%.

For assessment of very low rates of spore germination, glass microscope slides were coated with a thin layer of agar containing LB medium plus 10 mM L-alanine. Heat-activated spores (10-μl aliquots [~10^5 spores]) containing kanamycin (50 μg/ml) to prevent cell growth were spotted on the slides, a glass coverslip was added and sealed with clear nail polish to prevent the slide from drying, and ~100 spores on the slide were examined by phase-contrast microscopy during incubation at 37°C. Control experiments demonstrated that the addition of kanamycin did not affect rates of spore germination. RM spores were germinated similarly, but with the addition of only L-alanine or AGFK to the agar.

In some cases colony formation assays were used to assess spore germination, since mutations in *gerD* have no effects on cell growth rates (37) (data not shown). Spores at an OD_{600} of 1 (~10^8 spores/ml) were heat activated as described above, and aliquots of various dilutions were spotted on LB medium agar plates, the plates were incubated at 37°C for 48 h, and colonies were counted. In another experiment, 200-μl aliquots of heat-activated spores containing ~1.5×10^3 spores/ml were spread on LB medium agar plates, the plates were incubated at 37°C, and colonies were counted at various times. Spores were also germinated without heat activation at 37°C at an OD_{600} of 1 with Ca^{2+}-DPA (60 mM $CaCl_2$, 60 mM DPA [pH 8.0]) or at 37°C at an OD_{600} of 2 with dodecylamine (1 mM dodecylamine–20 mM KPO_4 [pH 7.4]) as described previously (23, 28, 29). Spore germination with Ca^{2+}-DPA was assessed by flow cytometry after staining with the fluorescent nucleic acid stain Syto 16 (Molecular Probes, Eugene, OR) (4). Spore germination with dodecylamine was analyzed by monitoring DPA release by measuring the OD_{270} of the supernatant fluid from germination incubations (29). Aliquots (1 ml) of germinating cultures were removed and centrifuged for 2 min in a microcentrifuge to obtain the supernatant fluid. The total DPA content of spores was determined by boiling samples for 30 min, centrifuging, and measuring the OD_{270} of the supernatant fluid. Previous work has shown that DPA comprises ~85% of the material absorbing at 270 nm released from spores by boiling and >95% of the material absorbing at 270 nm released during spore germination (2, 6).

Germination was also performed on non-heat-activated spores at an OD_{600} of 1 in 50 mM Tris-HCl (pH 7.5) buffer either at 37°C with 150 MPa of pressure or at 50°C with 500 MPa of pressure (4, 5, 24, 36). The extent of spore germination due to pressure was analyzed by flow cytometry after staining with Syto 16 (4).

For germination with lysozyme, spores at an OD_{600} of 100 were first decoated for 30 min at 45°C in 0.1 M NaCl–0.1 M NaOH–0.5% sodium dodecyl sulfate–0.1 M dithiothreitol (34). The decoated spores were washed five to seven times with 0.15 M NaCl, resuspended in water at an OD_{600} of 10, and stored at 4°C. Washed decoated spores were resuspended at an OD_{600} of 1 in 1 ml hypertonic germination medium (0.3 M sucrose–10 mM Tris-HCl [pH 8.0]–10 mM $MgCl_2$–10 mM $CaCl_2$), germination was initiated by addition of 5 μl of 5 mg/ml lysozyme, and the suspension was incubated at 37°C (27). Germination was monitored by measuring the OD_{600} of the suspensions, as well as by phase-contrast microscopy.

RESULTS

Response of *gerD* spores to nutrients. As found previously with spores of strains with point or insertion mutations in *gerD*, rates of germination of spores with a deletion mutation in *gerD* were much lower than that of wild-type spores using either

TABLE 2. Germination of *gerD* spores with various genetic backgrounds[a]

Genetic background (strains[b])	Germinant	Rate of germination (%/h)	
		With *gerD*	Without *gerD*
Wild type (PS832/FB62)	Ala	110	<5
Wild type (PS832/FB62)	AGFK	60	<5
Wild type (PS832/FB62)	LB medium	87	<5
Δ*gerA* (PS3609/PS3926)	Ala	<5	<5
Δ*gerA* (PS3609/PS3926)	AGFK	61	6
Δ*gerA* (PS3609/PS3926)	LB medium	<12	<5
Δ*gerK* Δ*gerB* (FB87/PS3927)	Ala	105	6
Δ*gerK* Δ*gerB* (FB87/PS3927)	AGFK	6	<5
Δ*gerK* Δ*gerB* (FB87/PS3927)	LB medium	73	12
*gerBB** (FB10/PS3929)	Ala	239	15
*gerBB** (FB10/PS3929)	AGFK	219	10
*gerBB** Δ*gerA* (PS3521/PS3931)	Ala	83	5
*gerBB** Δ*gerA* (PS3521/PS3931)	AGFK	271	14
*gerBB** Δ*gerA* Δ*gerK* (PS3665/PS3932)	Ala	104	7
*gerBB** Δ*gerA* Δ*gerK* (PS3665/PS3932)	AGFK	78	7
P_{sspD}-*gerA* (PS3476/PS3940)	Ala	200	23
P_{sspD}-*gerA* (PS3476/PS3940)	AGFK	28	5
P_{sspD}-*gerB** (PS3946/PS3947)	Ala	247	6
P_{sspD}-*gerB** (PS3946/PS3947)	AGFK	257	<5

[a] Spore germination was induced by addition of l-alanine (Ala), AGFK, or LB medium; germination was measured by monitoring the OD_{600} of cultures, and rates of spore germination were calculated as described in Materials and Methods.

[b] The first strain listed contains *gerD*, and the second has the same genetic background but without *gerD*.

AGFK or L-alanine to trigger spore germination (Table 2) (14, 18, 19, 35). The rate of germination of *gerD* spores in LB medium was also low (Table 2). The slow germination of Δ*gerD* spores with L-alanine or AGFK was observed not only when monitoring spore germination by measuring the OD_{600} of the culture but also by measuring DPA release (Fig. 2). To examine spore germination over even longer times in LB medium plus alanine, we monitored spore germination by phase-contrast microscopy. Again, while wild-type spores germinated completely in a few hours under these conditions, Δ*gerD* spores germinated more slowly (Fig. 3).

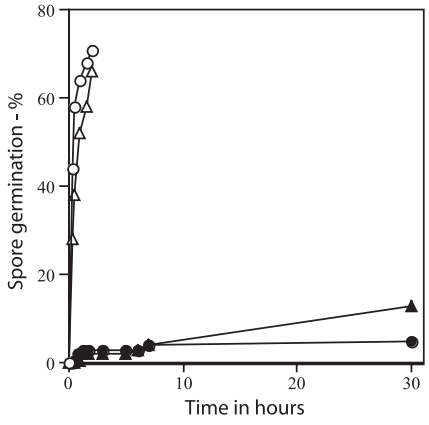

FIG. 2. Rates of germination of wild-type and Δ*gerD* spores. Wild-type (PS832) and Δ*gerD* (FB62) spores were germinated with either L-alanine or AGFK, and germination was assessed by measuring DPA release as described in Materials and Methods. ○, wild-type spores with L-alanine; △, wild-type spores with AGFK; ▲, Δ*gerD* spores with L-alanine; ●, Δ*gerD* spores with AGFK.

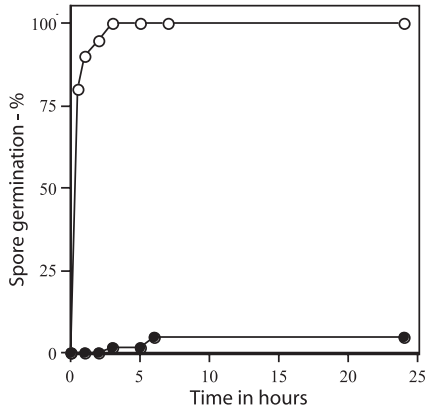

FIG. 3. Rates of germination of wild-type and *gerD* spores measured by microscopy. Wild-type (PS832) and Δ*gerD* (FB62) spores were spotted on LB medium agar slides with L-alanine and kanamycin, and spore germination was assessed by examining ~100 spores by phase-contrast microscopy as described in Materials and Methods. ○, wild-type spores; ●, Δ*gerD* spores.

Colony formation by Δ*gerD* spores. The slow germination of the Δ*gerD* spores noted above suggested that these spores might have a lower efficiency of colony formation than wild-type spores. However, this was not the case, as both wild-type and Δ*gerD* spores prepared in several different media had very similar efficiencies of colony formation, when colonies were counted after 48 h (Table 3). However, it was clear in this experiment that colonies from Δ*gerD* spores arose more slowly than from wild-type spores. The rate of appearance of colonies from heat-activated spores was determined by counting the colonies that appeared at various times from approximately identical numbers of spores, both Δ*gerD* and wild type, that were spread on agar plates and incubated at 37°C. While the number of colonies from wild-type spores was almost maximal after ≤16 h of incubation, the rate of appearance of colonies from Δ*gerD* spores was much lower (Fig. 4). The rates of appearance of colonies from Δ*gerD* spores were similar whether or not a heat activation step was applied, and this was also the case with wild-type spores (data not shown).

Germination of Δ*gerD* spores lacking or with elevated levels of nutrient germinant receptors. The *gerD* mutation reduced the rate of nutrient germination not only of spores with an

TABLE 3. Efficiency of colony formation by wild-type and Δ*gerD* spores[a]

Sporulation medium	Avg no. of colonies	
	Wild type	Δ*gerD*
2× SG	2.1×10^8	1.9×10^8
SMM plus 0.1% Casamino Acids	1.5×10^8	1.2×10^8
RM	6×10^7	6.9×10^7

[a] Spores of strains PS832 (wild type) and FB62 (Δ*gerD*) were prepared using various media. The spores were purified, and aliquots of various dilutions of spore suspensions with an OD_{600} of 1 (1×10^8 to 2×10^8 spores/ml) were spotted on LB medium agar plates. The plates were incubated for 48 h at 37°C, and colonies were counted. Values shown are the averages of duplicate determinations.

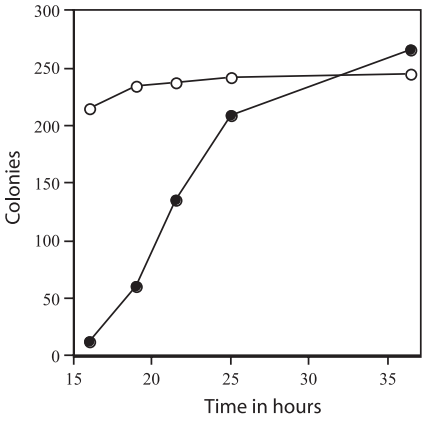

FIG. 4. Rates of appearance of colonies from wild-type and Δ*gerD* spores. Two hundred fifty to 300 spores of strains PS832 (wild type) or FB62 (Δ*gerD*) were spread on an LB medium agar plate without antibiotics, the plates were incubated at 37°C, and colonies were counted after various times of incubation. ○, wild-type spores; ●, Δ*gerD* spores.

otherwise wild-type background but also in spores lacking the GerA nutrient receptor, or both the GerB and GerK nutrient receptors (Table 2). The *gerD* mutation also reduced the rate of germination of spores carrying a point mutation in the *gerBB* cistron of the *gerB* operon, termed the *gerBB*∗ mutation. This mutation generates a GerB∗ nutrient receptor that functions even in the absence of the GerK nutrient receptor to trigger spore germination in response to either L-alanine or L-asparagine alone (2, 25).

Since a *gerD* mutation decreased spore germination triggered by any of the spore's nutrient receptors, a possible mechanism of GerD action is that this protein either positively regulates nutrient receptor formation or increases the function of individual nutrient receptors. If either of these explanations is correct, increasing the levels of nutrient receptors in spores might suppress the effects of a *gerD* mutation. In previous work we have constructed strains that have 10- to 20-fold-higher levels of GerA or GerB∗ receptors in spores by placing the *gerA* and *gerB*∗ operons under the control of the moderately strong forespore-specific promoter of the *sspD* gene (6). Although overexpression of these receptors increased the rates of spore germination with appropriate nutrients, as shown previously (6), this did not suppress the effects of the *gerD* mutation on spore germination (Table 2). Thus it is unlikely that GerD acts by increasing either the function or level of individual nutrient receptors.

Spores lacking all three functional nutrient receptors do not respond to nutrient germinants (26). However, the spores of such strains, termed *ger3* spores, exhibit a very low rate of spontaneous germination (0.01 to 0.1%/day), and this low rate of germination continues for at least a week (26). It was thus of interest to examine the effect of a *gerD* mutation on this spontaneous spore germination. Similar to results found previously (26), ~0.03% of the spores of a *ger3* strain (PS3945) germinated per day at 37°C, while approximately the same percentage (0.06%) of spores of an isogenic strain also lacking *gerD* (PS3928; *ger3* Δ*gerD*) germinated per day under the same conditions (data not shown). Thus a *gerD* mutation did not slow the rate of spontaneous germination of spores lacking all functional nutrient receptors.

TABLE 4. Germination of Δ*gerD* and wild-type spores prepared and germinated in various media[a]

Germinant	Rate of spore germination (%/h) for indicated strain[b] in:					
	2× SG		RM		SMM + CAA	
	PS832	FB62	PS832	FB62	PS832	FB62
LB medium	87	<5	ND[c]	ND	ND	ND
Ala	ND	ND	34	<5	ND	ND
AGFK	ND	ND	56	<5	ND	ND
SMM plus Ala	ND	ND	ND	ND	64	<5
SMM plus AGFK	ND	ND	ND	ND	90	<5

[a] Spores were prepared in 2× SG medium, RM, or SMM plus 0.1% Casamino Acids (CAA), and spore germination was carried out with either LB medium, L-alanine (Ala), AGFK, or SMM with 10 mM L-alanine or with AGFK. Spore germination was measured by monitoring the OD_{600} of cultures as described in Materials and Methods. Rates of germination are given as the percentages of spores that germinate in 1 hour as described in Materials and Methods.

[b] PS832 is the wild type, and FB62 lacks *gerD*.

[c] ND, not done.

Germination of Δ*gerD* spores prepared in different media or under different conditions. Previous studies have suggested that sporulation conditions may affect the ability of *gerD* mutant spores to germinate, as sporulation of *gerD* point mutants in RM yields spores that germinate with L-alanine at rates much closer to those of wild-type spores (35, 37). However, we observed a severe germination defect in L-alanine with Δ*gerD* spores that had been prepared in RM (Table 4). This was also true of Δ*gerD* spores prepared in SMM with 0.1% Casamino Acids, another more minimal medium (Table 4). While initial experiments with spores prepared in RM measured spore germination by monitoring the OD_{600} of cultures (Table 4), we also examined the nutrient germination of these spores over a longer time period using phase-contrast microscopy; again there was only slow germination of the Δ*gerD* spores prepared in RM (Fig. 5). In addition to modifications in the sporulation

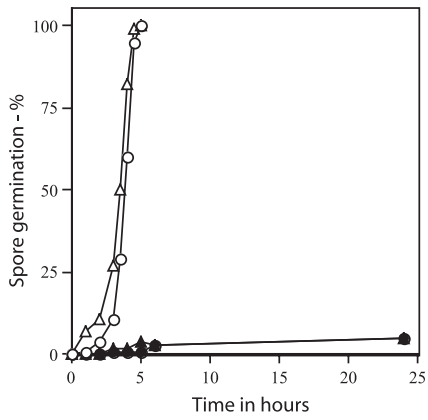

FIG. 5. Rates of germination of wild-type and Δ*gerD* spores prepared in RM. Wild-type (PS832) and Δ*gerD* (FB62) spores prepared in RM were spotted on agar slides with kanamycin and either L-alanine or AGFK, and spore germination was assessed by examining ~100 spores under phase-contrast microscopy as described in Materials and Methods. ○, wild-type spores with L-alanine; △, wild-type spores with AGFK; ●, Δ*gerD* spores with L-alanine; ▲, Δ*gerD* spores with AGFK.

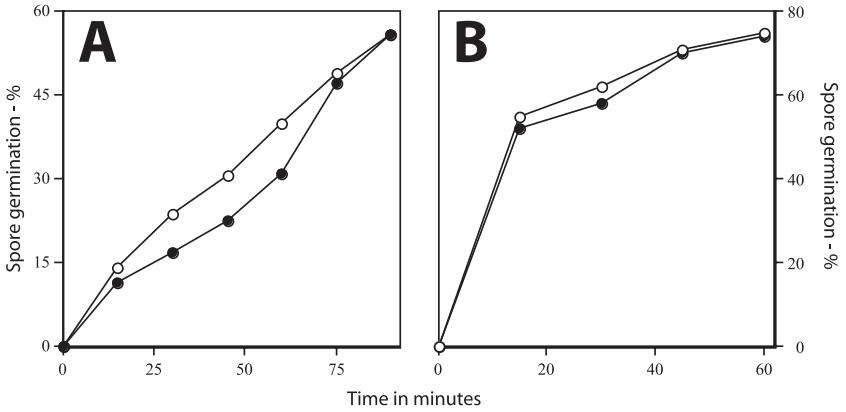

FIG. 6. Rates of germination of wild-type and Δ*gerD* spores with (A) dodecylamine or (B) Ca^{2+}-DPA. Wild-type (PS832) and Δ*gerD* (FB62) spores were germinated with 1 mM dodecylamine (A) or with Ca^{2+}-DPA (B), and spore germination was measured either by following DPA release (dodecylamine germination) or flow cytometry after staining with Syto 16 (Ca^{2+}-DPA germination), as described in Materials and Methods. ○, wild-type spores; ●, Δ*gerD* spores.

medium, we also examined the effects of preparation of Δ*gerD* spores in liquid 2× SG medium or SMM plus 0.1% Casamino Acids, as well as on solid 2× SG medium at temperatures from 23 to 44°C, as these changes have been found to affect spore properties, including rates of spore germination (17, 29). However, again these changes did not suppress the defect in the germination of Δ*gerD* spores with nutrients (data not shown).

Effects of monovalent cations, pH, and temperature on Δ*gerD* spore germination. One model for spore germination has suggested that spores require a pool of K^+ ions before germination can occur (35). Indeed work using spores with point mutations in *gerD* found that addition of monovalent cations to the germination medium suppresses the *gerD* mutant spore's germination defect (35, 37). However with Δ*gerD* spores addition of KCl, NaCl, LiCl, or NH_4Cl at concentrations up to 250 mM did not suppress the germination defect with either L-alanine or AGFK (data not shown). We also checked whether other changes in germination conditions might alter the GerD requirement for rapid spore germination with nutrients. However, varying the germination pH from 6 to 9 or the temperature from 23 to 44°C did not suppress the germination defect of the Δ*gerD* spores (data not shown). Increasing the L-alanine concentration from 10 to 100 mM to determine if Δ*gerD* spores have decreased sensitivity to nutrient germinants or removing the spore coats to determine if Δ*gerD* spores are defective in permeation of nutrient germinants (3) also did not suppress the nutrient germination defect of Δ*gerD* spores (data not shown). Under all of these conditions, the rates of germination of Δ*gerD* spores with either alanine or AGFK remained at ≤10% of those of wild-type spores (data not shown).

Germination of Δ*gerD* spores with nonnutrient germinants. While a *gerD* deletion mutation slowed spore germination triggered by nutrients, there are many nonnutrient agents that can trigger spore germination, including cationic surfactants such as dodecylamine, lysozyme, Ca^{2+}-DPA, and high pressures (4, 5, 23, 24, 26, 27, 29, 30). Many of these agents (dodecylamine, lysozyme, and Ca^{2+}-DPA) trigger spore germination by pathways different from that for nutrient germination. Examining the effects of this group of nonnutrient germinants showed that rates of spore germination with dodecylamine as measured by DPA release were nearly identical for both wild-type and Δ*gerD* spores (Fig. 6A). This was also the case for germination in hypertonic medium with lysozyme (data not shown) as well as with Ca^{2+}-DPA (Fig. 6B).

Previous work has established that different high pressures trigger spore germination via different pathways (4, 5, 24, 36). Pressures of about 150 MPa induce germination primarily via the nutrient receptor-mediated pathway, while pressures of about 500 MPa induce germination primarily via mechanisms independent of the nutrient receptors. Since Δ*gerD* spores are slowed in nutrient receptor-mediated germination, we would expect that germination of Δ*gerD* spores at 150 MPa of pressure would also be slow, which is what was found (Fig. 7). In contrast, since spore germination with 500 MPa of pressure does not proceed through the nutrient receptors, we would expect to see nearly wild-type spore

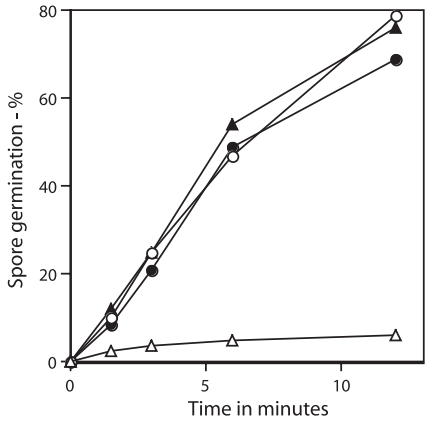

FIG. 7. Germination of wild-type and Δ*gerD* spores by high pressures. Wild-type (PS832) and Δ*gerD* (FB62) spores were germinated with pressure, and spore germination was assessed by measuring DPA release as described in Materials and Methods. ○, wild-type spores germinated with 150 MPa of pressure; ●, wild-type spores germinated with 500 MPa of pressure; △, Δ*gerD* spores germinated with 150 MPa of pressure; ▲, Δ*gerD* spores germinated with 500 MPa of pressure.

TABLE 5. Germination of spores of strains with different *gerD* genes in various locations[a]

Strain	Relevant genotype[b]	Relative rate of spore germination[c] with:	
		L-Alanine	AGFK
PS3980	Δ*gerD*	≤8	≤8
PS3981	Wild type	100	100
PS3982	$gerD^{C20A}$	≤8	≤4
PS3983	$gerD^{\Delta SP}$	≤8	≤4
PS3987	Δ*gerD amyE*::*gerD*	105	85
PS3988	Δ*gerD amyE*::P_{sspB}-*gerD*	105	85
PS3989	*amyE*::*gerD*	95	95
PS3990	*amyE*::P_{sspB}-*gerD*	100	95
PS3991	*amyE*::$gerD^{C20}$	105	110
PS3992	*amyE*::$gerD^{\Delta 7\text{-}15}$	100	110
PS3993	$gerD^{C20A}$ *amyE*::*gerD*	105	105
PS3994	$gerD^{C20A}$ *amyE*::P_{sspB}-*gerD*	100	110
PS3995	$gerD^{\Delta SP}$ *amyE*::*gerD*	100	95
PS3996	$gerD^{\Delta SP}$ *amyE*::P_{sspB}-*gerD*	105	80

[a] Spores prepared at 37°C on 2× SG plates were germinated using the alternative conditions with either L-alanine or AGFK, and the rates of spore germination were determined by measuring the rate of DPA release as described in Materials and Methods. All values reported are the averages of results with two preparations of spores and have been rounded off to the nearest 5, except for values less than 10.

[b] The abbreviation ΔSP denotes a *gerD* gene lacking the region coding for amino acids 7 to 17, which is a likely SP.

[c] All values are expressed relative to that for spores of strain PS3981, which was set at 100.

germination rates with Δ*gerD* spores under these conditions, and this is again what was observed (Fig. 7). These results are consistent with a previous pressure germination study with spores of a *gerD* point mutant (36).

Effects of modifications in the GerD level or amino acid sequence on spore germination. As noted above, GerD contains a likely SP followed by a putative recognition site for diacylglycerol addition, including the cysteine residue to which the diacylglycerol is added. One subunit of the spore's nutrient receptors also contains both of these sequence features, and the change of the diacylglycerylated cysteine to an alanine eliminates the function of the GerA nutrient receptor and greatly reduces GerB nutrient receptor function (10). Thus it was of obvious interest to determine the importance of these GerD sequence features. Perhaps not surprisingly, deletion of GerD's likely SP or the change of its putative diacylglycerylated cysteine to an alanine destroyed GerD function in spore germination with either L-alanine or AGFK (Table 5). However, when the latter mutant genes were at the *gerD* locus, expression of wild-type GerD from the *amyE* locus restored GerD function, as did expression of *gerD* from *amyE* in a strain with a *gerD* deletion at the *gerD* locus (Table 5). As expected, expression of the altered *gerD* genes from the *amyE* locus did not interfere with germination of spores that had wild-type *gerD* at the *gerD* locus (Table 5).

The fact that wild-type *gerD* expressed at *amyE* could restore GerD function indicated that functional GerD was expressed from *gerD* at *amyE*. Previous work has shown that overexpression of various nutrient receptors can have significant effects on both sporulation and spore germination (6, 11). However, when *gerD* was expressed at *amyE* under the control of either the *gerD* promoter or the very strong forespore-specific *sspB* promoter, the resultant strains sporulated normally (data not shown) and the spores of these strains exhibited rates of germination very similar to those of wild-type spores (Table 5). While we do not know the degree to which GerD was overexpressed in spores of the strain with *gerD* under P_{sspB} control, previous work has found that placing the *gerB* operon under P_{sspB} control results in a 200-fold increase in levels of GerBA (27).

DISCUSSION

The results with a Δ*gerD* mutation presented in this work revealed a greater effect of GerD on spore germination than observed previously, as our Δ*gerD* spores were very slow to germinate with all nutrients tested. This was the case irrespective of the medium used for spore preparation or the presence or absence of various cations in the germination solutions. The smaller effect(s) on spore germination of *gerD* mutations seen in earlier work, in particular with L-alanine as the germinant (18, 35, 37), is perhaps due to the retention of some function by the products of the mutant *gerD* genes used previously, as has been suggested for the *gerD* point mutants (37). The differences in the behavior of the different *gerD* mutations could also be due to differences in the genetic backgrounds of the *B. subtilis* 168 strains used in different laboratories.

It was somewhat surprising that the rate of germination of Δ*gerD* spores inferred from the rate of appearance of colonies from Δ*gerD* spores appeared to be significantly higher than the rate of Δ*gerD* spore germination seen either by monitoring DPA release or phase-contrast microscopy. While the reason for the differences between these different experiments is not clear, these experiments were carried out under significantly different conditions. One such difference is the much higher spore concentrations in experiments measuring germination by monitoring DPA release or phase-contrast microscopy compared to monitoring colony formation. It has been reported with spores of several species that the spore concentration can have a significant effect on the rate of germination, although there has usually been more rapid germination by spores at higher concentrations (7, 38). However, this phenomenon has not been studied with *B. subtilis* spores, and perhaps higher spore concentrations of *B. subtilis* Δ*gerD* spores result in inhibition of spore germination. Another significant difference between the various experiments is that in measuring spore germination by phase-contrast microscopy the spores were on only a thin layer of agar, and again this may have resulted in a lower rate of spore germination. Whatever the precise explanation for the differences in the rates of germination of Δ*gerD* spores in various experiments, it is clear that the rate of Δ*gerD* spore germination is significantly lower than that of wild-type spores, although within ~48 h Δ*gerD* spores germinate as well as wild-type spores.

Since GerD appears to play a significant, yet not absolutely essential role in spore germination, the important question is how GerD functions to stimulate the rate of spore germination. Knowledge of proteins involved in spore germination is currently confined largely to (i) the nutrient receptors that bind nutrient germinants, with this binding leading to subsequent germination events (30); (ii) the redundant enzymes that digest the spore's peptidoglycan cortex in response to some signal from the nutrient germinant receptors (30); (iii) the GerP

proteins that may be responsible for allowing nutrient germinants to pass through the spore's outer layers to reach their receptors (3); and (iv) the SpoVA proteins that may be involved in Ca^{2+}-DPA release upon activation of the spore's nutrient receptors (33). Since *gerD* mutants are blocked in or before Ca^{2+}-DPA release and cortex hydrolysis is not required for Ca^{2+}-DPA release (30), GerD must act before cortex hydrolysis and either on nutrient access to their receptors, on the activity of or the signal transduction from nutrient receptors, or on Ca^{2+}-DPA release. However, it seems unlikely that GerD is involved in allowing access of nutrients to their receptors, since L-alanine concentrations >100-fold higher than needed for maximal rates of spore germination did not suppress the effect of the *gerD* mutation. Similarly, decoating of spores, a process that suppresses effects of *gerP* mutations on spore germination (3), also did not suppress the effects of the *gerD* mutation.

It also seems unlikely that GerD functions directly in the release of Ca^{2+}-DPA that takes place early in germination, since Ca^{2+}-DPA release also takes place during germination induced by nonnutrient germinants such as dodecylamine, exogenous Ca^{2+}-DPA, and a pressure of 500 MPa, yet the rates of germination of Δ*gerD* spores with these agents were essentially equal to those of wild-type spores. We are left then with GerD function involving the spore's nutrient receptors in some fashion. That GerD does affect the nutrient receptors or their signaling in some way is consistent with the finding that germination with all agents that require these receptors is greatly slowed by the *gerD* mutation, while germination with all agents that do not require these receptors is not. How then may GerD function to increase the rate of germination caused by activation of nutrient receptors? As noted above, this seems unlikely to be due to allowing easier access of nutrient germinants to their receptors in the spore's inner membrane. Indeed, spore germination due to a pressure of 150 MPa, a process that requires no nutrient germinant yet proceeds through the nutrient receptors (4, 24, 36), is greatly slowed by the *gerD* mutation. It also seems unlikely that GerD increases the level of functional nutrient receptors, since overexpression of nutrient receptors did not suppress the effects of the *gerD* mutation. However, we cannot rule out the possibility that GerD somehow either modifies the nutrient receptors to make them more sensitive to their ligands or alters the sporulation process itself such that the responsiveness of the nutrient receptors is increased.

Analysis of the precise effects of GerD on nutrient germination strongly suggests that GerD is not essential for spore germination via the nutrient receptors, as Δ*gerD* spores germinate, outgrow, and eventually form colonies on plates; this process is just slower than that with wild-type spores. The difference between Δ*gerD* and wild-type spores in their germination with nutrients appears to be in the duration of the lag time after exposure of spores to nutrients and before initiation of obvious germination processes such as Ca^{2+}-DPA release, with this lag time being much longer with Δ*gerD* spores. The lag times for Δ*gerD* spores also are extremely variable for individual spores, as colonies from Δ*gerD* spores appeared over a long time period, much longer than that for wild-type spores. While the presence of a lag period after exposure of spores to nutrients and prior to measurable germination events has been seen with spores of a number of *Bacillus* species, the events taking place in this lag period are largely unknown and factors that determine the length of this lag period are not understood. However, one event determining the duration of this lag period could be the efficiency of transduction of signals from ligand-activated nutrient receptors to components acting later in the spore germination process, such as enzymes that degrade the peptidoglycan cortex. Thus it is attractive to adopt as at least a working model that GerD is involved in some fashion in the transduction of a signal of some sort between the nutrient receptors and some other component of the spore germination apparatus.

If GerD is involved in the latter signal transduction process, it seems likely that GerD would be located near the nutrient receptors in the spore's inner membrane. The presence of an essential SP and a cysteine that is probably diacylglycerylated suggests that GerD is likely a membrane protein, probably a peripheral membrane protein since most of the protein is hydrophilic (37). Since *gerD* is transcribed only in the developing forespore, GerD is most likely present in the spore's inner membrane, and attempts to definitively localize GerD in the spore are in progress. If, as seems likely, GerD is in the inner membrane, then a more intriguing question is what other proteins physically interact with GerD. The answer to this question may provide crucial information not only about GerD function but also about the overall signal transduction pathway in spore germination.

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