JOURNAL OF BACTERIOLOGY, Apr. 2003, p. 2457–2464 Vol. 185, No. 8
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.8.2457–2464.2003

Effects of Overexpression of Nutrient Receptors on Germination of Spores of *Bacillus subtilis*

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Received 4 November 2002/Accepted 22 January 2003

The rates of germination of *Bacillus subtilis* spores with L-alanine were increased markedly, in particular at low L-alanine concentrations, by overexpression of the tricistronic *gerA* operon that encodes the spore's germinant receptor for L-alanine but not by overexpression of *gerA* operon homologs encoding receptors for other germinants. However, spores with elevated levels of the GerA proteins did not germinate more rapidly in a mixture of asparagine, glucose, fructose, and K^+ (AGFK), a germinant combination that requires the participation of at least the germinant receptors encoded by the tricistronic *gerB* and *gerK* operons. Overexpression of the *gerB* or *gerK* operon or both the *gerB* and *gerK* operons also did not stimulate spore germination in AGFK. Overexpression of a mutant *gerB* operon, termed *gerB, that encodes a receptor allowing spore germination in response to either D-alanine or L-asparagine also caused faster spore germination with these germinants, again with the largest enhancement of spore germination rates at lower germinant concentrations. However, the magnitudes of the increases in the germination rates with D-alanine or L-asparagine in spores overexpressing *gerB** were well below the increases in the spore's levels of the GerBA protein. Germination of *gerB** spores with D-alanine or L-asparagine did not require participation of the products of the *gerK* operon, but germination with these agents was decreased markedly in spores also overexpressing *gerA*. These findings suggest that (i) increases in the levels of germinant receptors that respond to single germinants can increase spore germination rates significantly; (ii) there is some maximum rate of spore germination above which stimulation of GerA operon receptors alone will not further increase the rate of spore germination, as action of some protein other than the germinant receptors can become rate limiting; (iii) while previous work has shown that the wild-type GerB and GerK receptors interact in some fashion to cause spore germination in AGFK, there also appears to be an additional component required for AGFK-triggered spore germination; (iv) activation of the GerB receptor with D-alanine or L-asparagine can trigger spore germination independently of the GerK receptor; and (v) it is likely that the different germinant receptors interact directly and/or compete with each other for some additional component needed for initiation of spore germination. We also found that very high levels of overexpression of the *gerA* or *gerK* operon (but not the *gerB* or *gerB** operon) in the forespore blocked sporulation shortly after the engulfment stage, although sporulation appeared normal with the lower levels of *gerA* or *gerK* overexpression that were used to generate spores for analysis of rates of germination.**

Spores of various *Bacillus* species can remain dormant for long periods of time but can rapidly "return to life" through the process of spore germination (18, 19, 20, 28). Spore germination is normally triggered by the stereospecific binding of specific low-molecular-weight nutrients to receptors located in the spore's inner membrane (12, 27, 28). These receptors are comprised of three separate proteins, at least two of which appear to be integral membrane proteins (18, 19, 20, 28). In *Bacillus subtilis* the functional germinant receptors are encoded by three homologous tricistronic operons, *gerA*, *gerB*, and *gerK* (termed *gerA* operon homologs), in addition to two other homologous operons that are not expressed under normal laboratory sporulation conditions (18, 19, 20, 26, 28). There are multiple similar tricistronic operons in other *Bacillus* species, as well as in *Clostridium* species (5, 6, 13, 18, 26, 28). The individual *gerA* operon homologs encode a receptor for a particular germinant or group of germinants; where this operon has been studied, mutations in any cistron of a *gerA* operon homolog can destroy that receptor's function (19, 20, 28). In *B. subtilis* the *gerA* operon encodes a receptor for L-alanine, while the products of the *gerB* and *gerK* operons interact or collaborate in some fashion to provide the germination response to a mixture of asparagine, glucose, fructose, and potassium ions (AGFK). However, any of several specific mutations in the *gerB* operon allow spores to germinate in D-alanine alone (25). As yet, the structure of these various germinant receptors is not known, but there is evidence that the three proteins encoded by the various *gerA* operon homologs interact, at least with other proteins encoded by the same *gerA* operon homolog (19, 20, 26).

Binding of a germinant to its receptor triggers subsequent events in spore germination, including the release of the spore core's large depot (~10% of the spore's dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) and the re-

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TABLE 1. *B. subtilis* strains used

Strain	Genotype and/or phenotype	Reference, source, or construction[a]
FB10	*gerB**	25
FB20	Δ*gerA*::*spc* Sp^r	26
FB58	P*sspB*::*gerB* Sp^r	27
FB60	Δ*gerB*::*spc* Sp^r	26
FB68	Δ*gerK*::*ermC* MLS^r	26
PS832	Wild-type	Laboratory stock
PS3415	P*sspB*::*gerB** Sp^r	pFE135A (26)→FB10
PS3441	P*sspB*::*gerA* MLS^r	pPS3439→PS832
PS3461	P*sspB*::*gerK* MLS^r	pPS3456→PS832
PS3468	*sspE-lacZ* Cm^r	pPS918 (9)→PS832
PS3469	P*sspB*::*gerB sspE-lacZ* Cm^r Sp^r	pPS918 (9)→FB58
PS3471	P*sspB*::*gerB sspE-lacZ* Cm^r MLS^r	pPS918 (9)→PS3441
PS3472	P*sspB*::*gerK sspE-lacZ* Cm^r MLS^r	pPS918 (9)→PS3461
PS3476	P*sspD*::*gerA* MLS^r	pPS3473→PS832
PS3477	P*sspD*::*gerB* Sp^r	pPS3474→PS832
PS3478	P*sspD*::*gerK* MLS^r	pPS3475→PS832
PS3498	*gerB* Δ*gerK*::*ermC* MLS^r	FB68→FB8
PS3499	*gerB** Δ*gerK*::*ermC* MLS^r	FB68→FB10
PS3501	P*sspD*::*gerA gerB** MLS^r	PS3476→FB10
PS3502	P*sspD*::*gerB** Sp^r	pPS3474→FB10
PS3503	P*sspB*::*gerB** Δ*gerK*::*ermC* MLS^r Sp^r	FB68→PS3415
PS3521	*gerB** Δ*gerA*::*spc* Sp^r	FB20→FB10
PS3556	P*sspB*::*gerB* P*sspD*::*gerK* MLS^r Sp^r	PS3431→PS3478
PS3557	P*sspD*::*gerB* P*sspD*::*gerK* MLS^r Sp^r	PS3477→PS3478

[a] Strains constructed by transformation of plasmid or chromosomal DNA from the strain to the left of the arrow into the strain to the right of the arrow.

lease of other ions, including H^+, Na^+, K^+, and Ca^{2+}, with the concomitant uptake of some water (26, 28, 34). DPA release is followed by degradation of the spore's peptidoglycan cortex, and cortex hydrolysis is stimulated either indirectly or directly by DPA release (23, 28, 29, 32). While proteins that are likely candidates for DPA channels in the spore's membranes have been identified (35) (these are not the germinant receptors themselves [26]), there is as yet no understanding of how germinant-receptor interaction triggers the earliest events in spore germination, including the release of DPA and cations. Cortex hydrolysis is followed or accompanied by the swelling of the spore core via further water uptake, and this allows the initiation of metabolism (28, 32).

Some information that might bear on the questions of the precise role that the germinant receptors play in spore germination and how they play this role might be obtained if the concentration of specific germinant receptors in spores was increased by a large amount. Analysis of the rates of germination of spores carrying elevated levels of germinant receptors might, for example, then suggest whether these receptors act alone to trigger initial events in spore germination or require direct interaction with other proteins to do this. Such an analysis of spores overexpressing the GerA, GerB, or GerK receptor is the subject of this communication.

MATERIALS AND METHODS

Bacterial strains used. All *B. subtilis* strains used are listed in Table 1 and are isogenic except for the differences noted. The parental strain is PS832, which was derived from strain 168. Transformations of *Escherichia coli* and *B. subtilis* were as described previously (2, 24, 25). *E. coli* strains were routinely grown in LB medium (31) supplemented with ampicillin (50 μg/ml). *B. subtilis* strains were grown in LB medium with antibiotics added as appropriate (100 μg of spectinomycin per ml for spectinomycin resistance [Sp^r]; 5 μg of erythromycin per ml plus 25 μg of lincomycin per ml for macrolide-lincosamide-streptogramin B resistance [MLS^r]; and 5 μg of chloramphenicol per ml for chloramphenicol resistance [Cm^r]).

Spore preparation. Spores were routinely prepared at 37°C on 2×SG medium agar plates without antibiotics as described previously (21, 24); in one experiment sporulation was in 2×SG liquid medium. Spores were harvested, stored in a cold room, and cleaned by repeated rounds of centrifugation and washing with water over a period of 1 to 2 weeks (21). Cleaned spores were stored protected from light at ~6°C in water. All spore preparations used in this work were free (>98%) of growing or sporulating cells or cell debris.

Construction of strains overexpressing the GerA homolog receptors. The strain overexpressing the wild-type *gerB* operon under the control of the strong forespore-specific *sspB* promoter has been described previously (27). The plasmid used to place *gerB* under P*sspB* control, pFE135A (27), was also used to transform strain FB10 to Sp^r. *B. subtilis* strain FB10 carries a mutant *gerB* operon, termed *gerB**, whose product allows spores to germinate in D-alanine alone (25), and plasmid pFE135A does not carry any *gerB* sequence that overlaps with the point mutation in the *gerB** operon, as this mutation is in the *gerBB* cistron (25). An Sp^r transformant was termed PS3415, and its correct construction was confirmed by Southern blot analysis.

To place the *gerA* operon under P*sspB* control, a 300-bp region of *gerAA* (from bp 1 to 300 in the *gerAA* coding sequence) was amplified by PCR (all primer sequences are available on request); the primers also contained an *Nde*I site in the upstream primer and a *Pst*I site in the downstream primer. The PCR product was ligated into plasmid pCR2.1 (Invitrogen) and transformed into *E. coli* to give plasmid pPS3435. The *gerAA* fragment was excised from pPS3435 by digestion with *Nde*I and *Pst*I, ligated between the *Nde*I and *Pst*I sites in plasmid pPS3396 (see below), and cloned in *E. coli* to give a plasmid, pPS3439, in which P*sspB* is just upstream of the 5′ end of *gerAA*. In this construct an additional ATG codon has been added at the 5′ end of *gerAA* to generate the *Nde*I site, as *gerAA* translation normally starts with TTG; the new translation-initiating ATG codon is positioned optimally downstream of the strong ribosome-binding site of *sspB*. This plasmid was used to transform strain PS832 to MLS^r by a single-crossover event in *gerAA*, giving strain PS3441 in which the *gerA* operon is under the control of P*sspB*. The correct construction of this strain was confirmed by Southern blot analysis. Plasmid pPS3396 noted above was constructed by insertion of the *ermC* gene from plasmid pFE140 (26) on a 1.2-kb *Kpn*I-*Xba*I fragment between the *Xba*I and *Kpn*I sites of plasmid pFE133 (27), which carries the P*sspB* promoter in a pUC19 derivative.

The *gerK* operon was placed under the control of P*sspB* by first amplifying the region from bp 1 to 378 of the *gerKA* coding sequence with an *Nde*I site on the upstream primer and a *Pst*I site on the downstream primer. The PCR fragment was cloned in *E. coli* as described above, giving plasmid pPS3454, and the *gerKA* fragment was excised with *Nde*I and *Pst*I and cloned between the *Nde*I and *Pst*I sites of plasmid pPS3396, giving a plasmid, pPS3456, in which *gerKA* is just downstream of P*sspB* and is preceded by the strong *sspB* ribosome-binding site. Plasmid pPS3456 was used to transform PS832 to MLS^r, giving strain PS3461, and the correct construction of this strain was verified by Southern blot analysis.

The plasmid to place *gerA* operon homologs under the control of the promoter of the *sspD* gene was constructed as follows. A region encompassing 289 bp upstream of *sspD* (bp −4 to −292 relative to the first nucleotide of the translational start codon [7, 22]) was amplified by PCR with the primers also providing *Hin*dIII and *Nde*I sites at the farthest upstream and less upstream ends, respectively. This fragment encompasses the *sspD* promoter and at the end adjacent to the *sspD* coding sequence contains the *sspD* ribosome-binding site. The primer-provided *Nde*I site is appropriately spaced relative to this ribosome-binding site such that translation of coding sequences beginning at the ATG in this *Nde*I site will be efficient. The PCR product was cloned in *E. coli* as described above, giving plasmid pPS3467. For placement of the *gerA* operon under P*sspD* control, the P*sspD*-containing fragment from pPS3467 was excised as a *Hin*dIII-*Nde*I fragment. This fragment was ligated with plasmid pPS3439 (described above) that had been digested with *Hin*dIII and *Nde*I to remove the P*sspB* promoter fragment, giving plasmid pPS3473. This plasmid was used to transform *B. subtilis* strain PS832 to MLS^r, giving strain PS3476 in which *gerA* expression is under the control of P*sspD*; the correct genomic structure of this strain was confirmed by Southern blot analysis. For placement of *gerK* under P*sspD* control, the *Hin*dIII-*Nde*I fragment from plasmid pPS3467 was used to replace the small *Hin*dIII-*Nde*I fragment of plasmid pPS3456, giving pPS3475, and this plasmid was used to transform *B. subtilis* strain PS832 to MLS^r, giving strain PS3478. The correct construction of this strain was confirmed by Southern blot analysis. For placement of *gerB* under P*sspD* control, the ~320-bp *Xho*I-*Nde*I fragment from plasmid pPS3467 that contains the *sspD* promoter and ribosome-binding site was used to replace the *Xho*I-*Nde*I fragment containing the *sspB* promoter in plasmid

pFE135A (27), giving plasmid pPS3474. This plasmid was used to transform *B. subtilis* strain PS832 to Sp^r, and the correct construction of this strain was again confirmed by Southern blot analysis.

Spore germination. Prior to spore germination, spores at 2 to 3 mg (dry weight) per ml in water were heat shocked (30 min, 70°C) and then cooled in ice. Spores were routinely germinated at 37°C and optical densities at 600 nm (OD_{600}s) of 0.8 to 1 in 50 mM KPO_4 (pH 7.4) with L-alanine or AGFK and in 25 mM Tris-HCl (pH 8.2) with D-alanine (with or without 10 mM D-glucose) or L-asparagine (25, 26). Control incubations with heat-shocked spores incubated at 37°C in the appropriate buffer alone were included for each germination experiment. Spore germination was usually monitored by measurement of the OD_{600} of the culture (30) over a period of 4 h in a 1-ml cuvette in a Spectronic Genesys 5 spectrophotometer (Milton Roy, Rochester, N.Y.). The maximum germination rate of spores germinating with any given nutrient concentration was calculated from the maximum rate in the fall of the OD_{600} versus time as determined graphically from the maximum slope of the plot of OD_{600} values versus time. All values reported are the averages of at least duplicate determinations, generally from two independent spore preparations, and individual values varied by less than 20% from values shown. In all experiments, the maximum rate of germination of spores incubated in buffer alone was only 0.5 to 1% of the maximum rate found with the highest level of various germinants tested (data not shown). Some of the fall in the OD_{600}s of germinating cultures (≤30% monitored by the spectrophotometer noted above [B. Setlow and P. Setlow, unpublished data]) requires the hydrolysis of the spore cortex (14), and this is not the earliest event in the spore germination process (28, 29, 32). The earliest easily measured event in spore germination is the release of various ions, prominent among these being the spore's large depot of the 1:1 chelate of Ca^{2+}-DPA, and DPA release is accompanied by the loss of ~70% of the total amount of the OD_{600} that is lost upon spore germination (24, 29, 32; Setlow and Setlow, unpublished). Consequently, measurement of the OD_{600}s of germinating spores, in particular, the maximum rate of the fall of the OD_{600} at a given germinant concentration, is a simple and reliable method for quantitating and comparing rates of spore germination. However, in some experiments we also monitored spore germination by measuring DPA release. Aliquots of germinating cultures (1 ml) were centrifuged (1 min in a microcentrifuge), and the DPA released into the supernatant fluid was measured by its absorption at 270 nm. In one experiment the spontaneous germination of spores in the absence of germinants was determined by dialysis of spores (OD = 1) at 37°C in the germination buffers; at various times, 150 spores were examined in a phase-contrast microscope to determine the percentage of spores that had germinated. Spores were incubated in dialysis tubing to minimize the possibility that small molecules released from a few spores that germinated would trigger the germination of other spores in the incubation.

Analytical methods. β-Galactosidase from strains carrying an *sspE-lacZ* fusion was assayed after cells were made permeable by lysozyme treatment as described previously (17). All β-galactosidase specific activities are expressed in Miller units as described previously (17). Membranes were isolated from dormant spores, GerB and GerA were detected, and their relative levels in spore membranes were determined by Western blot analysis as described previously (27).

RESULTS

Overexpression of germinant receptors and levels of overexpression. Previous work has shown that the GerB receptor can be overexpressed ~500-fold in spores when the *gerB* operon is under the control of the strong forespore-specific *sspB* promoter (17, 22, 27). This strategy was also applied to the *gerB**-encoded receptor, as well as the receptors encoded by the *gerA* and *gerK* operons. While the strain overexpressing the *gerB** receptor sporulated well and overexpressed GerB* (see below), <0.1% of cells of strains with the *gerA* and *gerK* operons under P*sspB* control completed sporulation (data not shown). Microscopic examination of the *gerA* and *gerK* operon strains indicated that the block in their sporulation was after the engulfment stage (data not shown, but we have not studied the precise nature of the sporulation block further). In order to demonstrate the block in sporulation conclusively, we examined the expression of β-galactosidase from an *sspE-lacZ* fusion, as *sspE* is expressed in parallel with *sspB*, as well as the *gerA* operon and its homologs, in the developing forespore (8, 10, 17, 22). Expression of *sspE-lacZ* was initially normal in strains expressing *gerA* and *gerK* under P*sspB* control (Fig. 1), indicating that there is no defect in the sporulation of these strains up to and including the time that the synthesis of GerA and GerK begins. However, after reaching levels ~70% of those in strains carrying *gerB* or *gerB** under P*sspB* control or with no overexpressed *gerA* operon homolog, levels of β-galactosidase in strains with *gerA* or *gerK* under P*sspB* control fell rapidly (Fig. 1). In addition, at this time the OD_{600} of the last-named cultures leveled off and microscopic analyses indicated that the developing forespores were lysing (Fig. 1 and data not shown). In contrast, *sspE-lacZ* expression and the OD_{600} continued to increase in cultures of strains containing only *sspE-lacZ* with or without P*sspB-gerB* or P*sspB-gerB** (Fig. 1 and data not shown). Thus, extremely high levels of GerA or GerK receptors do not allow completion of sporulation, in contrast to the situation with either the GerB or GerB* receptor. Note that levels of β-galactosidase also fell in the strain carrying *gerB* under P*sspB* control (Fig. 1), but this was due to the acquisition of lysozyme resistance by the developing forespore (data not shown), as was seen previously (17).

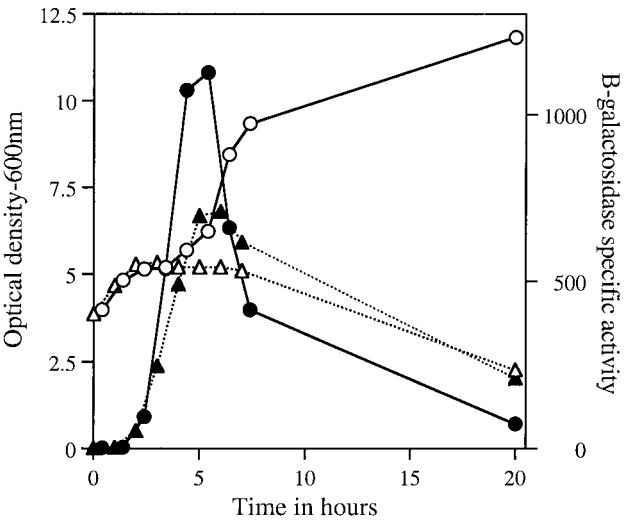

FIG. 1. OD_{600} values and levels of β-galactosidase from *sspE-lacZ* in sporulating cultures of various *B. subtilis* strains. *B. subtilis* strains were grown and sporulated in liquid 2×SG medium without antibiotics, the OD_{600}s were measured, and β-galactosidase was extracted and assayed as described in Materials and Methods. Open symbols indicate OD_{600} values and filled symbols indicate β-galactosidase specific activities for strain PS3471 (P*sspB*::*gerA sspE-lacZ*) (triangles) and strain PS3469 (P*sspB*::*gerB sspE-lacZ*) (circles). The curves for strains carrying only *sspE-lacZ* (PS3468) or P*sspB*::*gerK sspE-lacZ* (PS3472) were essentially identical (within 20%) to those for the strain with P*sspB*::*gerB sspE-lacZ* or P*sspB*::*gerA sspE-lacZ*, respectively (data not shown). Values for the strain carrying P*sspB*::*gerB* sspE-lacZ* (PS3415) were essentially identical to those for strains PS3468 and PS3469 (data not shown).

Since expression of *gerA* and *gerK* under the control of the strong *sspB* promoter was not compatible with sporulation, we turned to the use of the *sspD* promoter (22). This promoter is also forespore specific but is only ~10% as strong as the *sspB* promoter (4, 17, 22). Strains with the *sspD* promoter driving expression of the *gerA*, *gerB*, *gerB**, or *gerK* operon sporulated

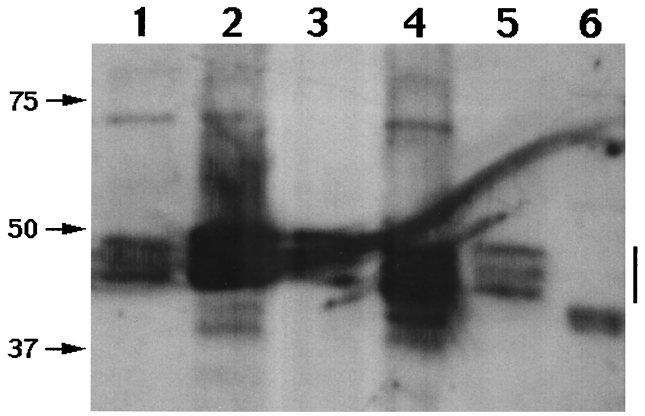

FIG. 2. Level of GerBA in spores of various *B. subtilis* strains. The inner membranes from spores of various strains were isolated, aliquots of membrane protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and GerBA was detected by Western blot analysis as described in Materials and Methods. The amounts and the identities of the membrane samples in the various lanes are 1 mg of wild-type spores (lane 1), 100 μg of P*sspB*::*gerB** spores (strain PS3415) (lane 2), 10 μg of P*sspB*::*gerB** spores (strain PS3415) (lane 3), 1 mg of P*sspD*::*gerB** spores (strain PS3502) (lane 4), 40 μg of P*sspD*::*gerB** spores (strain PS3502) (lane 5), and 1 mg of Δ*gerB* spores (strain FB60) (lane 6). The numbers and arrows to the left of the figure show the migration positions of molecular mass markers in kilodaltons, and the vertical bar on the right shows the position of the GerBA bands. Note that GerBA gives multiple bands, as seen previously (27).

normally (data not shown). We were unable to assess the level of overexpression of the *gerA* and *gerK* operons with the *sspD* promoter due to a lack of antisera against the encoded proteins. However, Western blot analyses of proteins from spore inner membranes showed that the *sspB* promoter led to an ~200-fold overexpression of GerBA from both the *gerB* and *gerB** operons, similar to what was seen previously (27), but that the *sspD* promoter led to an ~20-fold overexpression (Fig. 2 and data not shown). Previous work has also shown that at least some of the GerB receptor overexpressed from P*sspB* is still functional (27). Note that the overexpressed GerBA in spores was not in inclusion bodies (data not shown), as was also found previously (27). We also found that deletion of the *gerA* or *gerK* operon had no effect on levels of GerBA in spores, whether GerBA was overexpressed or expressed only at the low wild-type level (data not shown).

Effect of elevated levels of the GerA or GerK receptor on spore germination. With spores overexpressing the various germinant receptors in hand, we then examined the effects of high levels of germinant receptors on spore germination. Notably, the germination of spores overexpressing GerA was significantly faster in L-alanine than was germination of wild-type spores, particularly at low L-alanine concentrations (Table 2; Fig. 3). This faster germination was seen when spore germination was measured either by the fall in OD_{600} or by the release of DPA and was particularly obvious when spore germination was carried out at ~7°C (Fig. 3b). Note that DPA release precedes the full loss in OD_{600} of germinating cultures, since only ~70% of the total decrease in OD_{600} of germinating spores accompanies DPA release (14, 29, 32).

One obvious explanation for the more rapid germination of spores overexpressing the GerA receptor, in particular at low

TABLE 2. Maximum germination rates of spores of various strains as a function of the L-alanine concentration[a]

Spore genotype (strain)	Germination rate (% maximum loss in OD_{600}/h) at indicated L-alanine concn						
	1 μM	5 μM	10 μM	33 μM	100 μM	330 μM	1 mM
Wild-type (PS832)	1	14	48	210	276	302	307
P*sspD*::*gerA* (PS3476)	15	87	286	575	630	680	750
P*sspD*::*gerB* (PS3477)			51	218	293	310	300
P*sspB*::*gerB* (FB58)			40	210	240	270	275
P*sspD*::*gerK* (PS3478)			55	192	255	270	275

[a] Heat-shocked spores of various *B. subtilis* strains were germinated at 37°C in 50 mM KPO_4 (pH 7.4) and various concentrations of L-alanine, the OD_{600}s were measured, and maximum germination rates were calculated as described in Materials and Methods. Use of concentrations of L-alanine up to 10 mM gave no increase in the maximum germination rate over that observed with 1 mM L-alanine.

L-alanine concentrations, is that these spores spontaneously germinate much more readily than wild-type spores. However, this was not the case, as <1% of spores of the strain overexpressing GerA exhibited germination when they were incubated for 24 h at 37°C in dialysis tubing in either 50 mM KPO_4 (pH 7.4) or 25 mM Tris-HCl (pH 8.2) as described in Materials and Methods; similar results were obtained with spores overexpressing the *gerB* or *gerB** operon from P*sspB* or overexpressing *gerK* from P*sspD* (data not shown).

In contrast to the effects of elevated GerA levels on L-alanine germination, high levels of GerA actually inhibited spore germination in AGFK approximately twofold (Table 3). Spore germination in AGFK requires both the GerB and GerK receptors but not the GerA receptor (18, 19, 20, 28). However, spores in which either GerB or GerK had been overexpressed germinated as did wild-type spores in both L-alanine and AGFK (Tables 2 and 3). Similarly, spores in which GerB and GerK had been overexpressed together (strains PS3556 and PS3557) also exhibited no increase in the rate of AGFK germination when spore germination was measured by either the loss in OD_{600} or the release of DPA (data not shown). Spores of strains PS3556 and PS3557 also germinated like wild-type spores in L-alanine (data not shown).

Effect of elevated levels of the GerB or GerB* receptors on spore germination. As was found with spores with elevated levels of the GerK receptor, spores with elevated levels of the GerB receptor driven by either the *sspD* or *sspB* promoter germinated like wild-type spores in both L-alanine and AGFK (Tables 2 and 3). The lack of effect of elevated levels of the GerK or GerB receptor on spore germination, in particular with AGFK, is undoubtedly because both the GerB and GerK receptors (and probably at least one other component [see Discussion]) are needed for germination with AGFK (18, 19, 20).

In order to assess the effects of elevated levels of another single germinant receptor on spore germination, we turned to the GerB* receptor, which is the result of a mutation in the *gerB* operon that allows this receptor to respond to D-alanine alone (25). Strikingly, spores with higher levels of the GerB* receptor germinated more rapidly in D-alanine than did spores with the wild-type levels of GerB* (Fig. 2; Table 4). Previous work has shown that D-glucose markedly stimulates the germi-

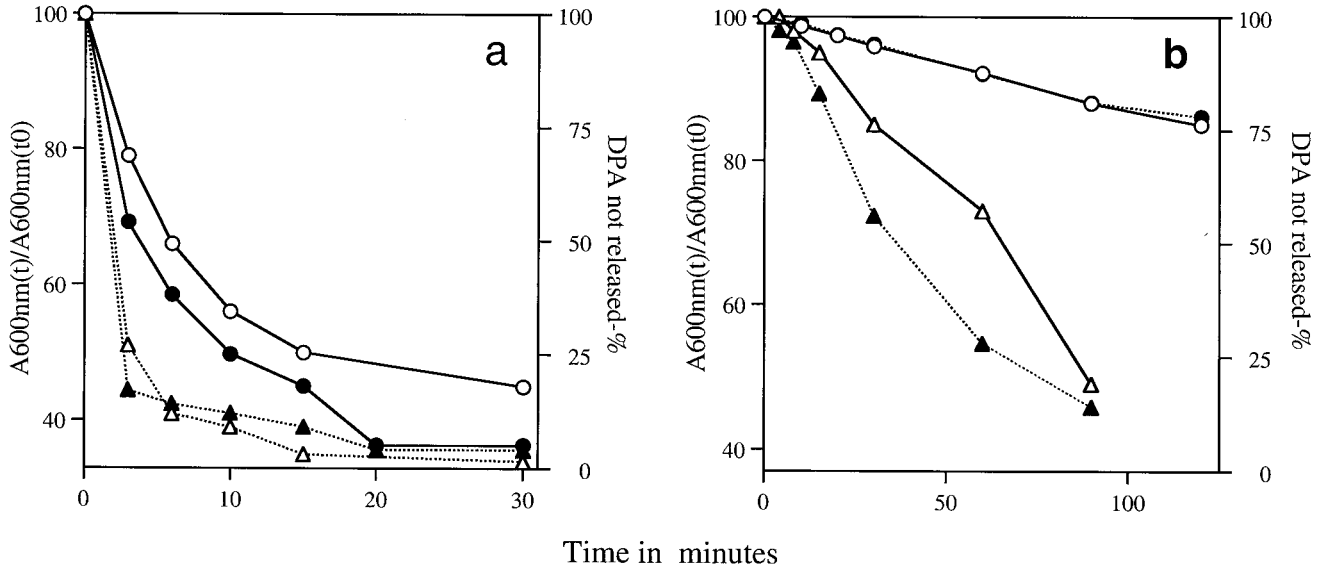

FIG. 3. Rate of loss in OD_{600} values and DPA release during germination of spores with or without elevated levels of the GerA receptor. Spores of strain PS832 (wild type) (○, ●) or PS3476 (P*sspD*::*gerA*) (△, ▲) were germinated in 50 mM KPO_4 (pH 7.4) and 1 mM L-alanine at either 37°C (a) or 7°C (b), and the OD_{600} values (○, △) and the DPA released (●, ▲) were measured as described in Materials and Methods. Note that not all of the data points taken in the experiment whose results are indicated in panel a are shown. t, time; t0, time zero.

nation of spores with the GerB* receptor (25), and germination with D-alanine plus D-glucose was also increased in spores with increased levels of GerB* (Fig. 2; Table 4). The gerB* mutation also allows spore germination in L-asparagine alone (25), and again germination in L-asparagine was more rapid in spores with elevated levels of GerB* than in spores with normal levels (Table 5). The elevated levels of the GerB* receptor caused the greatest increases in rates of spore germination with the lower concentrations of L-asparagine or with the lower concentrations of D-alanine with or without glucose (Tables 4 and 5), similar to what was found with L-alanine germination of spores overexpressing the gerA operon (Table 2).

Previous work has indicated that the GerB and GerK receptors can cooperate in some fashion, since germination in AGFK is abolished by mutations in either *gerB* or *gerK* (18, 19, 20). Thus, it was of interest to analyze the effect of a *gerK* mutation on the germination of spores with the *gerB** mutation in D-alanine. Loss of the *gerK* operon did not abolish spore germination in L-asparagine or D-alanine (with or without glucose) but did slightly decrease spore germination rates in these germinants compared to the germination rates of spores carrying either normal or elevated levels of GerB* (Tables 6 and 7). We also found that overexpression of *gerA* significantly decreased (up to fivefold) the germination rate of *gerB** spores with either L-asparagine or D-alanine (with or without glucose) (Tables 6 and 7). In contrast, previous work (24) has shown that the deletion of *gerA* has no effect on the germination of *gerB** spores, and this finding was confirmed in the present work (data not shown).

TABLE 3. Maximum germination rates of spores of various strains as a function of the concentration of AGFK[a]

Spore genotype (strain)	Germination rate (% maximum loss in OD_{600}/h) at indicated AGFK concn[b]				
	1*n*	3*n*	9*n*	27*n*	81*n*
Wild-type (PS832)	3	16	90	162	205
P*sspD*::*gerA* (PS3476)	3	7	45	74	105
P*sspD*::*gerB* (PS3477)	3	12	105	180	232
P*sspB*::*gerB* (FB58)	2	14	89	155	210
P*sspD*::*gerK* (PS3478)	3	12	78	160	200

[a] Spores of various *B. subtilis* strains were germinated at 37°C in 50 mM KPO_4 (pH 7.4) with various concentrations of asparagine, glucose, and fructose (note that K^+ levels were constant), the OD_{600}s were measured, and the maximum germination rates were determined as described in Materials and Methods.

[b] The concentrations of asparagine, glucose, and fructose represented by *n* are 31 μM, 6 μg/ml, and 6 μg/ml, respectively.

DISCUSSION

In this work we set out to assess the effects of increasing levels of nutrient germinant receptors on rates of spore germination. Previous work has shown that the GerBA protein is present in the spore's inner membrane at a level of ~25 molecules per spore and that the GerBA level was increased ~500-

TABLE 4. Maximum germination rates of spores of various *gerB** strains with different concentrations of D-alanine with or without D-glucose[a]

Spore genotype (strain)	Germination rate (% maximum loss in OD_{600}/h) at indicated D-alanine concn[b]				
	123 μM	370 μM	1.1 mM	3.3 mM	10 mM
*gerB** (FB10)	1 (11)	2 (19)	6 (36)	15 (60)	19 (100)
P*sspD*::*gerB** (PS3502)	1 (18)	7 (48)	28 (76)	38 (108)	51 (139)
P*sspB*::*gerB** (PS3415)	3 (60)	14 (84)	47 (120)	64 (164)	95 (209)

[a] Spores of various *B. subtilis* strains were germinated at 37°C in 25 mM Tris-HCl (pH 8.2) and various concentrations of D-alanine with or without 10 mM D-glucose, the OD_{600}s were measured, and the maximum germination rates were determined as described in Materials and Methods.

[b] Values in parentheses are with 10 mM D-glucose.

TABLE 5. Maximum germination rates of spores of various *gerB** strains at different concentrations of L-asparagine[a]

Spore genotype (strain)	Germination rate (% maximum loss in OD_{600}/h) at indicated L-asparagine concn				
	33 μM	100 μM	330 μM	1 mM	3 mM
*gerB** (FB10)	3	12	35	78	86
P*sspD*::*gerB** (PS3502)	5	21	57	116	130
P*sspB*::*gerB** (PS3415)	18	68	125	240	259

[a] Spores of various *B. subtilis* strains were germinated at 37°C in 25 mM Tris-HCl (pH 8.2) and various concentrations of L-asparagine, the OD_{600}s were measured, and maximum germination rates were determined as described in Materials and Methods. Wild-type spores (strain PS832) do not germinate in L-asparagine alone.

TABLE 7. Effect of deletion of *gerK* or overexpression of *gerA* on spore germination due to the GerB* receptor[a]

Spore genotype (strain)	Germination rate (% maximum loss in OD_{600}/h) at indicated D-alanine concn[b]		
	400 μM	2 mM	10 mM
*gerB** (FB10)	1 (17)	6 (64)	15 (126)
*gerB** Δ*gerK* (PS3498)	<1 (12)	4 (44)	9 (84)
P*sspB*::*gerB** (PS3415)	17 (103)	44 (240)	78 (300)
P*sspB*::*gerB** Δ*gerK* (PS3503)	17 (52)	43 (100)	84 (165)
P*sspD*::*gerA gerB** (PS3501)			9 (27)

[a] Spores of various *B. subtilis* strains were germinated at 37°C in 25 mM Tris-HCl (pH 8.2) and various concentrations of D-alanine (with or without 10 mM D-glucose), and maximum germination rates were determined as described in Materials and Methods.

[b] Values in parentheses are with 10 mM D-glucose.

fold (27). We presume that the other two proteins encoded by the *gerB* operon, GerBB and GerBC, are also overexpressed and assembled properly in the spore's inner membrane, as at least some of the overexpressed GerB receptor is functional and there is evidence that at least the GerBA and GerBB proteins physically interact (27). In the present work, we found that putting either *gerB* or *gerB** under the control of P*sspB* led to an ~200-fold overexpression of GerBA, while our use of the *sspD* promoter to drive the expression of *gerB* or *gerB** led to an ~20-fold overexpression of GerBA. This last number is reasonable, since levels of SspD in spores (and hence presumably *sspD* expression) are 10 to 15% of those of SspB (4).

The GerA receptor proteins GerAA and GerAC are also in the spore's inner membrane (12), and it is presumed that GerAB is there as well. Levels of *gerA* expression are ~15-fold higher than levels of *gerB* expression based on measurements of the expression levels of similar transcriptional *lacZ* proteins fused to both genes (8, 10), although there is no knowledge of the level of *gerK* expression. In contrast to our knowledge of the levels of GerBA in spores of strains overexpressing *gerB* or *gerB**, we have no data on the levels of the GerA or GerK receptor in spores of strains overexpressing *gerA* or *gerK*. However, since sporulation was aborted due to forespore lysis with strains expressing *gerA* or *gerK* from P*sspB* and spores from the strain expressing *gerA* from P*sspD* germinate more rapidly in L-alanine than do wild-type spores, it seems most likely that the *gerA* and *gerK* operons are significantly overexpressed from either P*sspD* or P*sspB*.

The reason for the sporulation defect of strains overexpressing *gerA* or *gerK* from P*sspB* is not clear. These strains appear to initiate sporulation normally and progress through forespore engulfment. However, midway through the period when P*sspB* is active, further development stops and the engulfed forespores lyse within the sporulating cells. The reason for this lysis is also not clear. One possibility is that it is the incorporation of high levels of these membrane proteins into the forespores' inner membranes alone that causes forespore lysis. This is certainly possible, although high levels of some (albeit not all) functional bacterial membrane proteins have been overexpressed in bacteria with no obvious deleterious effects (3, 15, 36). A second possibility is that high levels of the GerA or GerK receptor in the developing forespore somehow trigger premature forespore germination that in turn leads to forespore lysis, much as does the lack of accumulation of DPA by the developing forespore (24). Although expression of *gerB* or *gerB** from P*sspB* does not cause these effects, perhaps the absolute levels of GerA or GerK accumulated under P*sspB* control are much higher than levels of GerB accumulated under the control of this promoter. Alternatively, perhaps the GerA and GerK receptors much more readily trigger spore germination either spontaneously or in response to small molecules within the sporulating cell than does the GerB receptor. Indeed, spores containing only the GerA or the GerK receptor germinate quite well in a complex mixture of nutrients, while germination of spores containing only the GerB receptor is reduced up to 100-fold (26). It is also worth noting that expression from P*sspB* of the two additional *B. subtilis gerA* operon homologs, *yndDEF* and *yfkQRT*, which encode receptors for which no germinant has yet been identified (26), also did not cause any sporulation defect (E. Opoku-Serebuoh, M. Paidhungat, and P. Setlow, unpublished data).

TABLE 6. Effect of deletion of *gerK* or overexpression of *gerA* on spore germination due to the GerB* receptor[a]

Spore genotype (strain)	Germination rate (% maximum loss in OD_{600}/h) at the indicated L-asparagine concn		
	330 μM	1 mM	3 mM
*gerB** (FB10)	30	57	78
*gerB** Δ*gerK* (PS3498)	19	42	52
P*sspB*::*gerB** (PS3415)	145	270	294
P*sspB*::*gerK** Δ*gerK* (PS3503)	67	153	205
P*sspD*::*gerA gerB** (PS3501)			19

[a] Spores of various *B. subtilis* strains were germinated at 37°C in 25 mM Tris-HCl (pH 8.2) and various concentrations of L-asparagine, and maximum germination rates were determined as described in Materials and Methods.

The increases in the germination rates with L-alanine of spores overexpressing *gerA* or in the germination rate with L-asparagine or with D-alanine (with or without glucose) of spores overexpressing *gerB** are further evidence for the crucial role the proteins encoded by these operons play in spore germination and are consistent with their role as receptors whose ligand binding somehow triggers further steps in the spore germination process. It seems very unlikely that the effects on germination rates of spores of strains overexpressing the various GerA homolog receptors are due to general alterations in spore properties as a result of alterations in levels of gene expression during sporulation, since no effects were seen with spores overexpressing GerB. In addition, the effects on

spore germination rates were specific to germination with the germinants expected. It is notable that the degree of stimulation in spore germination at concentrations of germinants giving the maximum response was not great: ~2.5-fold for spores overexpressing *gerA* and germinating with L-alanine and 2-, 3-, or 5-fold for spores overexpressing *gerB** and germinating with D-alanine plus glucose, L-asparagine, or D-alanine, respectively. These increases at maximum germinant concentrations for spores overexpressing *gerB** are far below the increases in the levels of GerBA in the inner membranes of spores with *gerB** under P*sspD* or P*sspB* control. One obvious explanation is of course that much of the overexpressed GerB receptor is not functional, and we cannot eliminate this possibility but reiterate that overexpressed GerBA is localized properly in the spore and that at least some of the overexpressed GerB receptor is functional (27). A second explanation is that much, if not all, of the overexpressed GerA or GerB* receptor is functional but that as germinant receptor concentrations rise, something other than the concentration of these receptors becomes rate limiting for the initial events in spore germination. That this conclusion may indeed be at least partially correct is suggested by analysis of spore germination at germinant concentrations well below those needed to give maximum germination rates. At these lower concentrations, overexpression of either *gerA* or *gerB** gives significantly larger (6- to 15-fold) increases in rates of spore germination. This is the behavior that is expected if initiation of spore germination requires that only a certain number of receptors be occupied by their germinant ligand. At germinant concentrations well below receptor saturation, the absolute number of receptor-germinant complexes should increase as the total number of receptors in the inner membrane increases, assuming that the affinity of the receptors for their ligands is the same for all receptor molecules.

Despite the increases in germination rates of spores overexpressing the GerA or GerB* receptors, the degree of stimulation of spore germination by overexpression of GerB* in particular was well below the increases in levels of GerBA. One explanation for this anomaly is that much (although not all!) of the overexpressed GerB* and other GerA operon homologs are nonfunctional. The covalent addition of diacylglycerol near the N termini of the C proteins of at least the GerA and GerB receptors appears necessary for the function of these receptors (18, 19, 20; M. Paidhungat, B. Setlow, and P. Setlow, unpublished data). Diacylglycerol addition is catalyzed by the *lgt* (also termed *gerF*) gene product, and perhaps sufficient Lgt to covalently modify a high percentage of overexpressed GerA homolog receptors is lacking in the developing forespore. If this is the case, it would explain the decreases in the germination rates of *gerB** spores in D-alanine or L-asparagine upon overexpression of *gerA* and the decreases in AGFK germination in otherwise wild-type spores overexpressing *gerA*. However, if Lgt is limiting for assembly of high levels of functional GerA receptor homologs, then overexpression of either *gerB*, *gerB**, or *gerK* would also have been expected to significantly reduce spore germination in L-alanine by decreasing the amount of functional GerAC, and this was clearly not the case.

The small increases in the spore germination rates at maximal concentrations of germinants relative to the apparent increases in the levels of overexpressed GerA and GerB* receptors may be because some or even most of the overexpressed receptors are nonfunctional. However, it seems equally likely that as the concentration of GerA homolog receptors is increased, some other component of the initiating event in spore germination becomes rate limiting. Unfortunately, this other component and the initial step triggered or carried out by the germinant-receptor complex are not known. One early event in spore germination is DPA release, a process that will likely require proteins in the inner spore membrane (35), and prior to DPA release there is also the release of monovalent ions, including H^+, K^+, and Na^+ (34). One monovalent ion antiporter has been identified as a protein involved in the germination of *B. cereus* spores in some fashion (33), but the role of this protein in *B. subtilis* spore germination has not yet been established. Somehow the various GerA homolog receptors must interact with one or more of these additional inner membrane proteins, with the germinant binding to a GerA homolog receptor triggering the germination cascade. Complicating our understanding of protein-protein interactions in the inner spore membrane is the likely extremely slow movement of proteins in this membrane, as has recently been shown for lipids (A. E. Cowan, D. E. Koppel, B. Setlow, E. Melly, and P. Setlow, unpublished data).

In addition to the evidence for some sort of interaction between different GerA homolog receptors in *B. subtilis* spores, there is also evidence with spores of other *Bacillus* species that different GerA homolog receptors must interact, in particular to facilitate spore germination in mixtures of germinants (5, 13). However, precisely what is involved in the interaction between GerA homolog receptors is not clear. For example, do GerA homolog receptors interact to form heterooligomers or homooligomers? Clearly, the GerB receptor can function on its own, when it has acquired the *gerB** mutation. However, the decrease in GerB* function in strains carrying a *gerK* mutation suggests that some type of GerB*-GerK complex may be more efficient in stimulating spore germination with D-alanine or L-asparagine than is the GerB* receptor alone. That there may be some type of interaction between GerA homolog receptors is also consistent with the decrease in the rates of germination with D-alanine or L-asparagine of spores carrying the *gerB** mutation and also overexpressing *gerA*. Perhaps the high level of the GerA receptor in these spores binds the GerB* receptor in some fashion, decreasing the ability of the complexed GerB* to trigger spore germination with D-alanine or L-asparagine. Increased levels of the GerA receptor also decreased AGFK germination of spores with the wild-type GerB receptor, again possibly through the binding of the GerB or GerK receptor by the GerA receptor, thus decreasing the amount of the GerB-GerK receptor complex available for spore germination with AGFK. While the precise interaction of these various GerA homolog receptors is certainly unclear, it seems reasonable to call upon such possible interactions to explain both our and previous data. Indeed, recent work has shown that another type of membrane-localized receptor in bacteria can also form complexes and/or act collaboratively (1, 11, 16). Perhaps the GerA homolog receptors are another example of this phenomenon.

A final point concerns the somewhat surprising result that spores overexpressing both *gerB* and *gerK* exhibited no increase in their germination with AGFK. One trivial explanation is of course that functional GerK is actually not significantly over-

expressed in our strains, and we currently have no way of ruling out this possibility, although as noted above, this seems unlikely. A second possibility is that none of the overexpressed GerK receptor is functional; again, we cannot rule this out, but as noted above, this is not the case for the overexpressed GerA, GerB, and GerB* receptors. A third possibility is that the rate of spore germination with AGFK is already maximal in wild-type spores and thus that overexpression of GerB and/or GerK cannot increase this rate. While this is certainly possible, spores can germinate faster than the maximum rate seen with AGFK (e.g., with L-alanine [Table 2]). In addition, spores overexpressing the GerB and/or the GerK receptor also exhibited no increase in germination at submaximum AGFK concentrations. Consequently, we favor a fourth possibility, that some component in addition to the GerK and GerB receptors is essential for the triggering of spore germination by AGFK. This idea is consistent with the lack of increase in AGFK germination of spores overexpressing either GerB or GerK, since if either of these receptors is present in an amount smaller than that of the other and only GerB and GerK are needed for germination with AGFK, then overexpression of the normally low-level receptor should have increased the rate of spore germination with this mixture of germinants. The identity of this other component of the AGFK germination pathway is currently unclear, although one possibility is the *gerD* gene product that appears to be needed primarily for germination in AGFK (20, 28). Clearly, there is still much to learn about the early steps in the germination of spores of *Bacillus* species.

ACKNOWLEDGMENT

This work was supported by a grant from the National Institutes of Health (GM 19698 to P.S.).

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