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Effects of Sporulation Conditions on the Germination and Germination Protein Levels of *Bacillus subtilis* Spores

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Bacillus subtilis **spores prepared in rich medium germinated faster with nutrient germinants than poor-medium spores as populations in liquid and multiple individual spores on a microscope slide. Poor-medium spores had longer average lag times between mixing of spores with nutrient germinants and initiation of Ca-dipicolinic acid (CaDPA) release. Rich-medium spores made at 37°C germinated slightly faster with nutrient germinants than 23°C spores in liquid, but not when spores germinated on a slide. The difference in germination characteristics of these spore populations in liquid was paralleled by changes in expression levels of a transcriptional *lacZ* fusion to the *gerA* operon, encoding a germinant receptor (GR). Levels of GR subunits were 3- to 8-fold lower in poor-medium spores than rich-medium spores and 1.6- to 2-fold lower in 23°C spores than 37°C spores, and levels of the auxiliary germination protein GerD were 3.5- to 4-fold lower in poor medium and 23°C spores. In contrast, levels of another likely germination protein, SpoVAD, were similar in all these spores. These different spores germinated similarly with CaDPA, and poor-medium and 23°C spores germinated faster than rich-medium and 37°C spores, respectively, with dodecylamine. Since spore germination with CaDPA and dodecylamine does not require GerD or GRs, these results indicate that determinants of rates of nutrient germination of spores prepared differently are primarily the levels of the GRs that bind nutrient germinants and trigger germination and secondarily the levels of GerD.**

Spores of *Bacillus* species are dormant and extremely resistant to a variety of harsh treatments, including extremes of heat and radiation as well as high levels of toxic chemicals (33, 34). As a consequence of their dormancy and resistance, such spores can survive for years in the absence of nutrients. However, given the proper stimulus, generally the presence of specific nutrient molecules termed germinants that are sensed by spores' specific germinant receptors (GRs), spores can rapidly return to active growth in the process of germination followed by outgrowth (26, 32, 33). GRs are synthesized only in the developing forespore late in sporulation and are located in spores' inner membrane. Generally spores of *Bacillus* species have multiple GRs, with three major ones in *Bacillus subtilis* spores termed GerA, GerB, and GerK, with each GR having three subunits, designated A, B, and C. Each of these GRs recognizes a different nutrient germinant or subset of germinants, with GerA alone responding to L-alanine or L-valine and with GerB and GerK both being required for germination with a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (termed AGFK). Catabolism of the nutrients plays no role in the triggering of spore germination. Rather, the binding of a nutrient germinant to its cognate GR or GRs triggers the release of the spore core's huge depot (~20% of core dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), although the mechanism of this process is not known. DPA release then triggers subsequent germination events leading to resumption of metabolism, macromolecular synthesis, and eventually vegetative growth. GR levels in spores are an important determinant of rates of nutrient germination, as the absence of a particular GR eliminates germination with the cognate nutrient germinant, while elevation of a GR's level increases rates of germination with the cognate germinant (1, 6, 32).

Spore germination has been of significant interest because spores' dormancy and in particular their extreme resistance properties are lost in the early minutes of germination. Since spores of a number of *Bacillus* species as well as their close relatives are responsible for much food spoilage and food-borne disease, there has long been interest in somehow triggering germination of spores in foodstuffs and subsequently killing the less-resistant germinated spores by relatively mild treatments with heat or other agents. Unfortunately, this strategy has proven difficult to apply successfully due to the significant heterogeneity in the germination of individual spores in populations, as not all spores in populations germinate rapidly, with some small percentage, termed superdormant spores, taking many hours or even days to germinate (10, 11, 35, 40, 42).

Spores that are used for model studies of germination are generally prepared in relatively rich sporulation media at optimal temperatures, as such conditions give high spore yields. However, the precise sporulation conditions, in particular sporulation temperature and medium composition, can significantly influence the properties of the spores produced (3, 5, 8, 14, 18, 19, 20, 29). In particular, there are a number of reports of alterations in spore germination properties as the sporulation medium is altered (8, 14, 29). In one such recent study, both the rates and efficiency of spore germination were greatly decreased when *Bacillus cereus* spores were prepared in a poor medium compared to a rich one (14). This is obviously of interest, since spores from environments that contaminate foodstuffs have most likely been formed under less-than-optimal conditions of temperature and medium composition. Thus, spores prepared under such suboptimal condi-

Received 17 December 2011 **Accepted** 30 January 2012
Published ahead of print 10 February 2012
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doi:10.1128/AEM.07908-11

tions might actually be better models for analysis of spore germination as it might be used in an applied setting. In addition, it would be of great interest to determine the reason(s) that sporulation temperature and medium composition alter the germination properties of the resultant spores, since such information could shed light on mechanisms determining rates of spore germination. Consequently, in this work we prepared spores of *Bacillus subtilis* strains in both rich and poor liquid media and on rich-medium plates at 23°C and 37°C and examined these spores' germination with a variety of germinants. The expression of GRs in these spores as well of two other proteins likely involved in spore germination, GerD and SpoVAD, was also measured, both by measuring β-galactosidase expression from a *lacZ* fusion to an operon encoding a GR and by Western blot analysis with specific antisera against GR subunits, GerD, and SpoVAD.

MATERIALS AND METHODS

Spore preparation and purification. The *B. subtilis* strains used in this work are the following isogenic derivatives of strain PS832, a prototrophic laboratory derivative of strain 168: (i) PS533 (31) (wild-type for spore germination), which contains plasmid pUB110, conferring resistance to kanamycin (10 μg/ml); (ii) FB10 (*gerBB**) (23), carrying a specific mutation in the *gerB* operon (encoding the GerB GR) such that the GerB variant, termed GerB*, triggers spore germination in response to L-asparagine alone; (iii) PS767 (*gerA-lacZ*) (9) (wild type for spore germination), which is PS832 transformed with plasmid AAM81, so that the resultant strain carries a transcriptional *lacZ* fusion to the promoter of the *gerA* operon (encoding the GerA GR) as well as the wild-type *gerA* operon and its wild-type promoter; (iv) PS4209 (*gerA-lacZ gerBB**), generated in the present work by transforming strain FB10 with plasmid AAM81 (9, 36); and (v) PS4217 (Δ*gerD gerD*-Flag) (28), which lacks the wild-type *gerD* gene but expresses a functional Flag-tagged GerD protein ([GerD-Flag]. Spores of these strains were prepared either (i) on rich 2× Schaeffer's medium-glucose (2× SG) medium agar plates at 23°C or 37°C, (ii) in 2× SG liquid medium (rich medium) at 37°C (21), or (iii) at 37°C in Spizizen's minimal liquid medium (poor medium), which contains only citrate, glucose, NH_4^+, and inorganic salts (21, 22, 36). Spores were harvested, purified, and stored as described previously (21), and all spore preparations used in this work were free (>98%) of growing or sporulating cells, germinated spores, and cell debris, as determined by phase-contrast microscopy.

Spore germination. *B. subtilis* spore germination was monitored by two methods, one for spore populations and another for simultaneous monitoring of multiple individual spores. Germination of spore populations was monitored by measurement of the release of DPA (present in spores as a 1:1 chelate with Ca^{2+} [CaDPA]) by its fluorescence with Tb^{3+} using a multiwell fluorescence plate reader as described previously (38–40). For nutrient germination, spore germination was preceded by a heat shock of 30 min at 70 or 75°C to maximally activate spores for germination (both temperatures gave identical results), followed by cooling on ice for ≥15 min so that subsequent spore germination was not at an elevated temperature. Germination conditions were as follows, and all incubation mixtures also contained 50 μM $TbCl_3$: (i) at 37°C with 10 mM L-valine in 25 mM K-HEPES buffer (pH 7.4); (ii) at 37°C with 10 mM L-asparagine–10 mM D-glucose–10 mM D-fructose–10 mM KCl (AGFK) in 25 mM K-HEPES buffer (pH 7.4); and (iii) at 37°C with 10 mM L-asparagine in 25 mM K-HEPES buffer (pH 7.4). In these experiments, aliquots were also examined on a slide by phase-contrast microscopy to distinguish phase-bright (dormant) and phase-dark (germinated) spores at the end of germination incubations to determine the percentage of spores that had germinated. In germination experiments in which rates of germination were determined by measuring rates of CaDPA release, the relative rates for different spore preparations were corrected for any slight differences in spore DPA content. Total spore DPA content was determined by boiling samples of dormant spores for 15 min, cooling on ice, centrifuging, and measuring DPA in the supernatant fluid by its fluorescence with Tb^{3+} (39). All rates of spore germination were determined at least in duplicate and on two independent spore preparations, and differences were analyzed for significance by a two-tailed Student's *t* test.

In addition to nutrient germinants, spore populations were also germinated with two nonnutrient germinants, CaDPA and dodecylamine, both of which trigger spore germination in a GR-independent manner (32). CaDPA germination was at 30°C with a 1:1 mixture of 60 mM $CaCl_2$ and 60 mM DPA made to pH 7.4 with dry Tris base and with spores at ~10^8/ml. The progress of CaDPA germination was monitored solely by phase-contrast microscopy. Germination with dodecylamine was at 45°C with 0.8 mM dodecylamine in 25 mM K-HEPES buffer (pH 7.4) plus 50 μM $TbCl_3$, and DPA release was monitored by Tb-DPA fluorescence in a multiwell fluorescence plate reader as described above (30, 40).

Measurement of the nutrient germination of multiple individual spores simultaneously also used heat-shocked spores and the nutrient germination conditions described above but without $TbCl_3$. However, the spores were adhered to a microscope slide, and the germination of hundreds of individual spores was monitored by differential interference contrast (DIC) microscopy as described previously (16, 42). This method allows determination of the time between germinant addition and the initiation of rapid CaDPA release (T_{lag}), the time between germinant addition and completion of rapid CaDPA release ($T_{release}$), and the time for the rapid release of >85% of a spore's CaDPA ($\Delta T_{release} = T_{release} - T_{lag}$), since release of CaDPA from spores during germination is accompanied by a parallel fall in DIC image intensity which accounts for ~70% of the total fall in an individual spore's image intensity that takes place in spore germination (16, 38, 42). The remaining 30% of the fall in intensity is due to hydrolysis of the spore cortex peptidoglycan (PG), accompanied by core water uptake and swelling, and takes place following CaDPA release. The precise period in which the latter fall in a spore's DIC image intensity takes place is defined as ΔT_{lysis} and is the time between $T_{release}$ and the end of the fall in a spore's DIC image intensity. Previous work has shown that by far the major difference in the germination of individual spores is variation in T_{lag} values (16, 38, 41), and this was also the case in the present work (see Results). Consequently, differences in rates of germination of spore populations measured by analysis of multiple individual spores were analyzed for significance by comparing average T_{lag} values by a two-tailed Student's *t* test.

β-Galactosidase assays. Assay of β-galactosidase in spores used 4-methylumbelliferyl-β-D-galactoside (MUG) as the substrate and measured generation of 4-methylumbelliferone fluorometrically essentially as described previously (9). In brief, purified spores were first decoated to render them sensitive to lysis by lysozyme and disrupted with lysozyme plus DNase I and brief sonication treatment to reduce the extract's viscosity. Following centrifugation, the supernatant fluid was assayed for β-galactosidase using MUG as described previously (9). β-Galactosidase specific activities are expressed in arbitrary fluorescence units, and these were calculated with respect to the amount of DPA in various spore preparations. Analyses have shown that DPA levels are essentially identical in *B. subtilis* spores made on 2× SG medium plates at 23 and 37°C (18) and in rich and poor liquid media (data not shown). Assays of β-galactosidase in spores were carried out in duplicate on two independent spore preparations, and differences were analyzed for significance by a two-tailed Student's *t* test.

Isolation of superdormant spores. Superdormant spores were isolated from spore preparations made in rich and poor sporulation media by germination of 125 mg (dry weight) of spores for 5 h at 37°C in L-valine (10 mM) or AGFK (10 mM concentrations of each component) but without $TbCl_3$ as described above and previously (10, 11). Germination was followed by centrifugation to concentrate the spores, removal of germinated spores by buoyant density gradient centrifugation, germination of the remaining spores again for 5 h, and removal of any germinated spores again by buoyant density gradient centrifugation.

Antibody production and purification. The preparation of rabbit antisera against the *B. subtilis* GerBC and SpoVAD proteins was described previously, as was the source of the anti-Flag antiserum and all secondary antisera (17, 28, 37). For production of antisera against the *B. subtilis* GerAA, GerAC, and GerKA proteins and the *Geobacillus stearothermophilus* GerD protein, truncated *gerAA*, *gerAC*, *gerKA*, and *gerD* genes were amplified by PCR using genomic DNA from *B. subtilis* strain PS832 or *G. stearothermophilus* strain ATCC 7953 as the template; the 5′ primers introduced a NotI site, and the 3′ primers introduced a KpnI site. The *gerAA* and *gerKA* PCR products were cloned into a modified pET15b vector containing a His_6 tag and a tobacco etch virus (TEV) protease cleavage site, and the *gerAC* and *gerD* PCR products were cloned into a modified pGEX plasmid containing a TEV protease cleavage site between an N-terminal glutathione *S*-transferase tag and the target gene. The GerAA protein (residues 2 to 239), GerAC protein (residues 20 to 373), GerKA protein (residues 39 to 276), and GerD protein (residues 60 to 180) were expressed in *Escherichia coli* BL21 Star (DE3) (Invitrogen, Grand Island, NY) by induction with 1 mM isopropyl-β-D-thiogalactoside at 21°C for 16 h. The GerAA protein was soluble and was purified by nickel-nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography under native conditions followed by TEV protease cleavage to remove the His_6 tag, and subsequent cation exchange and gel filtration (GE Healthcare, Piscataway, NJ) chromatography. The GerKA protein was insoluble and was purified by Ni^{2+}-NTA affinity chromatography under denaturing conditions using 8 M urea in 100 mM sodium phosphate, 10 mM Tris-HCl (pH 8.0) as the solubilization buffer, the same buffer but at pH 6.3 as wash buffer, and finally the same buffer at pH 4.5 as the elution buffer. The GerD and GerAC proteins were soluble and were purified by glutathione affinity chromatography followed by TEV protease cleavage and cation exchange and gel filtration chromatography.

The purified proteins were dialyzed against PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.2), adjusted to a concentration of 1 mg/ml in PBS, and submitted for polyclonal antibody production in rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA). The GerAA, GerAC, and GerD proteins were supplied in solution and the GerKA protein as a suspension. The antibodies were detected in a bleed 2 months after the initial injection, and the GerAC and GerD antisera were used without further treatment, while the GerAA and GerKA antisera were affinity purified using a Pierce AminoLink Plus immobilization kit (Thermo Fisher Scientific, Rockford, IL) as per the supplier's instructions. Briefly, 1 mg GerAA protein was added to an AminoLink Plus resin column in 0.1 M sodium citrate, 0.05 M sodium carbonate coupling buffer (pH 10), or 1 mg GerKA protein was added in the same buffer also containing 4 M urea. The columns were rocked at room temperature for 4 h resulting in the formation of semistable Schiff base bonds, washed with 4 ml PBS, reduced with sodium cyanoborohydride overnight at 4°C followed by quenching with 1 M Tris-HCl (pH 7.4), resulting in stable secondary amine bonds. The stable antigen columns were washed with 10 ml 1 M NaCl and equilibrated in PBS, and then 2 ml of antiserum was added. After mixing by rocking for 1 h at room temperature, the columns were washed with 8 ml PBS. Bound antibody was eluted with 2 ml 0.2 M glycine·HCl (pH 2.5) into a tube containing 100 μl of 1 M Tris-HCl (pH 8.9) neutralizing buffer. The purified antisera were finally dialyzed against PBS and used for Western blot analysis. The antiserum against *G. stearothermophilus* GerD was used for Western blot analysis of *B. subtilis* GerD, because the anti-*G. stearothermophilus* GerD serum had become available in the laboratory and cross-reacted reasonably well with *B. subtilis* GerD.

Determination of GR subunit, GerD and SpoVAD levels in spores. Levels of various GR subunits in spores were determined by Western blot analysis of equal aliquots of spores' inner membrane proteins, since GRs, GerD, and SpoVAD are located in the spore's inner membrane (15, 25, 26, 32). The binding of the antisera to proteins on Western blots was detected with horseradish peroxidase coupled to goat anti-rabbit IgG and binding of the secondary antibody was detected by chemiluminescence as described previously (16). To quantitate differences in GR levels between spores prepared under different conditions, the intensities given by different amounts of appropriate inner membrane samples in Western blot analysis were compared on the same blot (see below). The GerD-Flag, GerD, and SpoVAD proteins were also detected by Western blot analysis on inner membrane samples using antiserum against the Flag tag, GerD, or SpoVAD as described above and previously (28, 37). In a number of cases, blots were stripped and then reprobed with a different antiserum.

For all Western blot analyses, since recoveries of the inner membrane fraction could well vary between spore preparations, a graded series of aliquots from inner membrane fractions that were to be compared were first run on denaturing polyacrylamide gel electrophoresis, the gels were stained with Coomassie blue, and equivalent amounts of inner membrane protein were determined by inspection of the stained gels and further similar analyses if needed. This allowed comparison of levels of GR subunits, GerD-Flag, GerD, and SpoVAD in samples with Western blots prepared with equivalent amounts of inner membrane protein. All analyses of levels of various spore germination proteins were carried out in duplicate on two independent spore preparations, and the variation in protein levels between different determinations was ≤15%. Differences in germination protein levels in spores made in the rich and poor medium and at 37 and 23°C were analyzed for significance using a two-tailed Student's *t* test.

RESULTS

Nutrient germination of spores prepared under different conditions. To begin to analyze the germination and other properties of spores prepared under different conditions, we chose to use strains carrying a *gerA-lacZ* fusion, since this would allow an eventual estimation of the expression of the *gerA* operon encoding the GerA GR in developing spores by measuring spore β-galactosidase levels (see below). We used two strains, a wild-type strain carrying *gerA-lacZ* (strain PS767) and a similar strain with a modified GerB GR, termed GerB*, that responds to L-asparagine alone (strain PS4209). These strains were sporulated either on rich-medium plates at 23 or 37°C (23°C and 37°C spores, respectively) or at 37°C in either a rich or poor liquid medium (rich-medium and poor-medium spores, respectively). Sporulation efficiencies were similar (±20%) under all these conditions as determined by microscopic examination (data not shown), although sporulation took about twice as long at 23 as at 37°C and cell growth was ~3-fold lower in the poor medium, which thus gave lower yields of spores.

The germination of these spores was monitored with a number of different nutrient germinants, using levels of these germinants well above those needed to saturate the relevant GRs. L-Valine was used to activate the GerA GR; the AGFK mixture was used to activate the GerB and GerK GRs together (32), since both of these receptors are needed for AGFK germination of wild-type spores; and L-asparagine was used to activate the GerB* GR. When germination of spore populations in liquid with nutrient germinants was assessed by monitoring CaDPA release, the rich-medium spores germinated better than the poor-medium spores, in particular with AGFK (Tables 1 and 2). Nutrient germination of populations of 23°C spores was also poorer than that of 37°C spores (Tables 1 and 2).

It was clear from the results noted above that sporulation conditions, in particular the medium composition, had significant effects on the rate of germination of spore populations. However, it was not clear which step in the germination process was affected, whether it was GR triggering or the CaDPA release process itself. To attempt to answer this question, the germination of multiple

TABLE 1 Rates of spore germination and levels of β-galactosidase in *B. subtilis* PS767 (*gerA-lacZ*) spores prepared under different conditions[a]

Sporulation condition	β-Galactosidase sp act[b]	Germination rate (RFU/min)[c]		Germination in 2 h (%)[d]	
		AGFK	Val	AGFK	Val
Rich liquid medium at 37°C[e]	1.1×10^5	77	245	68	97
Poor liquid medium at 37°C[e]	3.8×10^4	2	21	2	27
Rich-medium plates at 37°C[f]	9.1×10^4	83	157	70	99
Rich-medium plates at 23°C[f]	5.2×10^4	57	89	52	36

[a] Spores of strain PS767 (*gerA-lacZ*) were prepared under various condition and, purified, spore β-galactosidase specific activity was measured, spores were germinated, and germination rates and extents were measured as described in Materials and Methods. All values reported are averages of at least duplicate measurements with two independent spore preparations. Standard deviations for all values were <15%.
[b] Values for β-galactosidase specific activity from PS533 spores that do not carry a *lacZ* fusion were ≤5% of those in spores made in rich liquid medium.
[c] Values are given as relative fluorescence units (RFU) of DPA released/min and were determined as described in Materials and Methods.
[d] Values were determined by phase-contrast microscopy after 2 h of germination as described in Materials and Methods.
[e] The differences in values for spores made in rich and poor medium are highly significant ($P \leq 0.001$).
[f] The differences in values for spores made at 37 and 23°C are highly significant ($P \leq 0.001$).

TABLE 2 Rates of spore germination and levels of β-galactosidase in *B. subtilis* PS4209 (*gerA-lacZ gerBB**) spores prepared under different conditions[a]

Sporulation condition	β-Galactosidase sp act[b]	Germination rate (RFU/min)[c]		% germination in 2 h[d]	
		Asn	Val	Asn	Val
Rich liquid medium at 37°C[e]	6.2×10^4	220	200	100	100
Poor liquid medium at 37°C[e]	1.4×10^4	50	63	31	26
Rich-medium plates at 37°C[f]	6.2×10^4	100	134	100	100
Rich-medium plates at 23°C[f]	5.5×10^4	67	83	86	95

[a] Spores of strain PS4209 (*gerA-lacZ gerBB**) were prepared under various conditions and purified, spore β-galactosidase specific activity was measured, spores were germinated, and germination rates and extents were measured as described in Materials and Methods. All values are averages of at least duplicate measurements with two independent spore preparations. Standard deviations for all values were <15%.
[b] Values of β-galactosidase specific activity from PS533 spores that do not carry a *lacZ* fusion were ≤5% of those in spores made in rich liquid medium.
[c] Values are given as relative fluorescence units (RFU) of DPA released/min and were determined as described in Materials and Methods.
[d] Values were determined by phase-contrast microscopy after 2 h of germination as described in Materials and Methods.
[e] The differences in values for spores made in rich and poor medium are highly significant ($P \leq 0.001$).
[f] The differences in values for spores made at 37 and 23°C are highly significant ($P \leq 0.001$) for L-asparagine germination by microscopy and the rates of valine germination, marginally significant ($P \leq 0.06$) for rates of L-asparagine germination and valine germination by microscopy), and not significant ($P = 0.11$) for β-galactosidase specific activities.

individual spores adhered to a microscope slide was monitored by DIC microscopy (16, 42). This method can determine the lag time following germinant addition and prior to fast CaDPA release (T_{lag}), the time for the fast release of the great majority of a spore's CaDPA ($\Delta T_{release}$), which begins at T_{lag} and ends at $T_{release}$, and the time for spore cortex PG hydrolysis following CaDPA release (ΔT_{lysis}) (16, 42). This analysis showed that poor-medium spores of either strain PS767 or FB10 had 3- to 9-fold-longer average T_{lag} with nutrient germinants than rich-medium spores (Tables 3 and 4). However, the average ΔT_{lysis} were at most only 2-fold longer for the poor-medium spores than for the rich-medium spores, while the average $\Delta T_{release}$ differed ≤1.4-fold (Tables 3 and 4). It was surprising that the AGFK germination of the poor-medium PS767 spores was more efficient with individual spores adhered to a microscope slide than with spores suspended in liquid (compare Tables 1 and 3). We do not know the reason for this difference, although differences in the germination of spores adhered to a slide and in liquid have been seen previously, with the spores adhered to the slide germinating more efficiently (42).

In contrast to the slower nutrient germination of individual poor-medium spores, the effects of sporulation temperature on the germination of individual spores prepared on rich-medium plates were much smaller (Tables 3 and 4). Not only were the differences in $\Delta T_{release}$ between 23 and 37°C spores minimal, but the differences in T_{lag} and ΔT_{lysis} were minimal also. However, the individual 23°C spores germinated as well as or even faster than the 37°C spores when adhered to a slide (compare Tables 1 and 2 with Tables 3 and 4). As noted above, the poor-medium PS767 spores also exhibited better AGFK germination when adhered to a slide than suspended in liquid.

Nonnutrient germination of spores prepared under different conditions. There are a variety of reasons that spores made under different conditions would exhibit different rates of germination with nutrient germinants. However, one likely possibility is that levels of proteins essential for nutrient germination, including GRs and GerD, vary significantly in spores prepared differently, since GR levels in particular are known to affect rates of spores' germination with nutrients and GerD is also required for efficient GR-dependent germination (1, 6, 24, 27). To test if it is differences in levels of GRs or GerD that are responsible for different rates of germination of spores prepared differently, we first examined the germination of these spores with two nonnutrient germinants, CaDPA and dodecylamine, neither of which requires GRs or GerD to trigger spore germination (30, 32). Strikingly, the CaDPA germination of poor-medium spores was only slightly slower than that of rich-medium spores, while 23 and 37°C spores had identical CaDPA germination rates (Table 5). In addition, the dodecylamine germination of 23°C and poor-medium spores was actually faster than that of 37°C and rich-medium spores, respectively (Table 5), the opposite of the results with nutrient germination. The faster dodecylamine germination of 23°C spores has been seen previously and has been ascribed to differences in the inner membrane structure or composition of spores made at different temperatures (18).

Levels of GerA-LacZ in spores prepared under different conditions. That rates of CaDPA germination of spores made at different temperatures and in rich or poor media were essentially identical, and that dodecylamine germination was faster with 23°C and poor-medium spores, was striking, since these results were in contrast to results obtained when nutrient germination was measured. Since CaDPA and dodecylamine germination do not require GRs or GerD, this suggests that differences in rates of nutrient germination of spores prepared under these different

TABLE 3 Kinetic parameters of L-valine and AGFK germination of multiple individual PS767 (*gerA-lacZ*) *B. subtilis* spores prepared under different conditions[a]

Spore prepn and germinant	Time (min)				No. of spores		Observation period (min)
	T_{lag}	$T_{release}$	$\Delta T_{release}$	ΔT_{lysis}	Examined (% germinated)	Counted	
Rich liquid medium at 37°C							
10 mM L-Val[b]	12.5 ± 15.3	15.6 ± 17.7	3.2 ± 4.1	7.1 ± 2.1	329 (95)	93	120
10 mM AGFK[b]	6.6 ± 6.2	9.7 ± 6.8	3.0 ± 1.5	5.0 ± 2.7	432 (99)	90	90
Poor liquid medium at 37°C							
10 mM L-Val[b]	37.4 ± 33.4	41.0 ± 34.0	3.5 ± 1.6	8.3 ± 6.0	613 (47)	86	120
10 mM AGFK[b]	26.6 ± 23.0	30.4 ± 23.2	3.8 ± 1.6	5.9 ± 1.0	488 (37)	51	90
Rich-medium plates at 37°C							
10 mM L-Val[c]	7.2 ± 7.5	11.0 ± 8.3	3.8 ± 1.8	4.6 ± 1.9	404 (99)	97	120
10 mM AGFK[d]	6.6 ± 5.0	11.1 ± 6.9	4.5 ± 2.3	3.6 ± 1.7	329 (99)	91	90
Rich-medium plates at 23°C							
10 mM L-Val[c]	7.5 ± 11.6	11.5 ± 12.1	4.0 ± 2.0	5.9 ± 3.2	285 (96)	103	120
10 mM AGFK[d]	4.9 ± 5.5	8.9 ± 6.4	4.0 ± 2.5	4.3 ± 2.3	273 (99)	93	90

[a] Spores of *B. subtilis* strain PS767 were prepared under different conditions and the kinetic parameters of their germination were determined as described in Materials and Methods. All values for T_{lag}, $T_{release}$, $\Delta T_{release}$, and ΔT_{lysis} are means ± standard deviations.
[b] The differences in T_{lag} values between spores made in rich and poor media and germinating with either L-valine or AGFK are highly significant ($P < 0.001$).
[c] The differences in T_{lag} values between spores made at 37 and 23°C and germinating with L-valine are not significant ($P > 0.8$).
[d] The differences in T_{lag} values between spores made at 37 and 23°C and germinating with AGFK are slightly significant ($P < 0.05$).

conditions are due to differences in spore levels of GRs or GerD. To begin to critically examine this suggestion, the levels of β-galactosidase from a transcriptional *gerA-lacZ* fusion were determined in spores made under different conditions. Strikingly, levels of GerA-LacZ were ~3-fold lower in poor-medium spores, and levels of GerA-LacZ were slightly lower in 23°C spores (Tables 1 and 2). These differences were in the direction expected if differences in the expression of genes encoding GRs are responsible for

TABLE 4 Kinetic parameters of the germination of multiple individual FB10 (*gerBB**) *B. subtilis* spores prepared under different conditions[a]

Spore prepn and germinant	Time (min)				No. of spores		Observation period (min)
	T_{lag}	$T_{release}$	$\Delta T_{release}$	ΔT_{lysis}	Examined (% germinated)	Counted	
Rich liquid medium at 37°C							
10 mM L-Asn[b]	7.2 ± 10.0	10.6 ± 10.4	3.3 ± 1.0	6.6 ± 2.3	316 (96)	94	60
10 mM L-Val[b]	6.3 ± 7.5	9.6 ± 7.8	3.3 ± 1.1	7.1 ± 2.6	653 (97)	114	60
Poor liquid medium at 37°C							
10 mM L-Asn[b]	56.6 ± 53.6	60.0 ± 53.3	3.5 ± 1.6	14.0 ± 5.9	624 (23)	62	180
10 mM L-Val[b]	34.5 ± 34.7	38.3 ± 34.4	3.7 ± 1.8	12.5 ± 9.0	673 (15)	54	180
Rich-medium plates at 37°C							
10 mM L-Asn[c]	4.8 ± 3.1	7.5 ± 3.4	2.7 ± 0.8	7.8 ± 1.7	344 (100)	104	60
10 mM L-Val[d]	6.0 ± 4.2	8.5 ± 4.7	2.5 ± 0.9	8.5 ± 4.7	374 (99)	109	60
Rich-medium plates at 23°C							
10 mM L-Asn[c]	3.8 ± 3.5	7.5 ± 3.8	3.8 ± 1.5	7.2 ± 1.7	320 (100)	96	60
10 mM L-Val[d]	4.2 ± 4.2	7.9 ± 4.5	3.7 ± 1.5	6.2 ± 1.5	447 (99)	105	60

[a] Spores of *B. subtilis* strain FB10 were prepared under different conditions and the kinetic parameters of their germination were determined as described in Materials and Methods. All values for T_{lag}, $T_{release}$, $\Delta T_{release}$, and ΔT_{lysis} are means ± standard deviations.
[b] The differences in T_{lag} values between spores made in rich and poor media and germinating with either L-asparagine or L-valine are highly significant ($P < 0.001$).
[c] The difference in T_{lag} values between spores made at 37 and 23°C and germinating with L-asparagine are not significant ($P > 0.1$).
[d] The difference in T_{lag} values between spores made at 37 and 23°C and germinating in L-valine are highly significant ($P < 0.002$).

TABLE 5 Rates of CaDPA and dodecylamine germination of spores prepared under different conditions[a]

Sporulation condition	Relative % germination as a function of time	
	CaDPA	Dodecylamine
Rich liquid medium at 37°C	100[b]	15
Poor liquid medium at 37°C	85	50
Rich-medium plates at 37°C	75	20
Rich-medium plates at 23°C	75	100[c]

[a] Spores of strain PS767 (*gerA-lacZ*) were prepared under different conditions, purified spores were germinated, and germination rates were measured as described in Materials and Methods. All values are averages of duplicate measurements with two independent spore preparations, and standard deviations for all values were <15%.
[b] This value was set at 100 and denotes 100% germination in 8 h.
[c] This value was set at 100 and denotes 100% germination in 10 min.

the lower germination of spores, in particular 23°C and poor-medium spore populations.

Levels of GRs in spores made under different conditions. The general correlation between levels of *gerA*-driven β-galactosidase and the L-valine germination rates of spores made under different conditions was certainly consistent with differences in levels of GRs being a major reason for the differences in rates of germination of these spores. However, levels of β-galactosidase from *gerA-lacZ* may not be a precise reflection of levels of the GerA GR in spores or levels of the GerB, GerB*, and GerK GRs. Consequently, we carried out Western blot analysis of spore inner membrane proteins using a variety of antisera against GR subunits. These antisera exhibited no detectable cross-reaction with subunits from other GRs (data not shown), which is perhaps not surprising since there is generally <40% sequence identity between comparable subunits from the *B. subtilis* GerA, GerB, and GerK GRs. In all cases, the identities of GR subunit bands on Western blots were determined by their appropriate molecular weight, and their absence from the inner membrane fraction of spores carrying a deletion of the gene encoding the appropriate GR (15, 25) (data not shown). This Western blot analysis showed that poor-medium PS767 spores (*gerA-lacZ*) had lower levels of GerAA, GerAC, GerBC, and GerKA than the rich-medium spores (Fig. 1). Similarly, 23°C spores had slightly lower levels of these GR subunits than 37°C spores (Fig. 2). Quantitation of these differences (Table 6), showed that levels of the four GR subunits were 3- to 8-fold lower in poor-medium spores while 23°C spores had 1.6- to 2-fold-lower levels of GR subunits, and all of these differences were significant, with *P* values being below 0.05 and most being much lower.

Levels of GerD and SpoVAD in spores made under different conditions. In addition to GR subunits, there are other proteins that are important in spore germination. These proteins include GerD and the proteins encoded by the *spoVA* operon, which are essential for DPA uptake in sporulation and probably CaDPA release during germination as well (26, 27, 32, 38). Since these proteins are also almost certainly in the spore's inner membrane (12, 15, 25, 28, 37), the levels of GerD as well as a functional Flag-tagged GerD protein (note that this is the only GerD protein present in these spores [28]) and SpoVAD were determined by Western blot analysis of inner membrane fractions from PS4217 spores (Δ*gerD gerD*-Flag) prepared under different conditions (Fig. 3 and Table 6; also data not shown). In contrast to results obtained when GR subunit levels were measured in inner membranes of

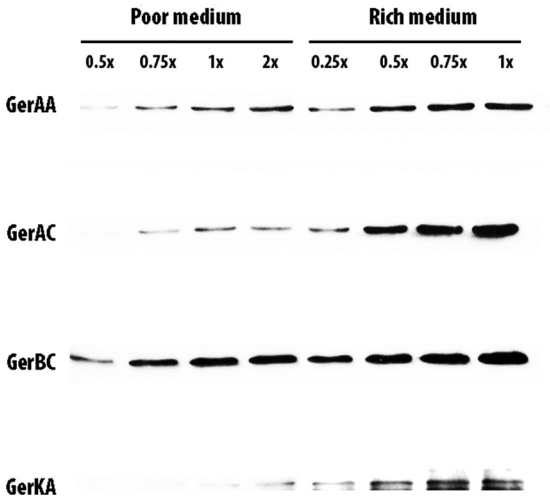

FIG 1 Levels of GR subunits in rich- and poor-medium spores. Rich- and poor-medium PS767 (*gerA-lacZ*) spores were prepared and purified, the inner membrane fractions were isolated, and aliquots were analyzed by Western blotting using various antisera as described in Materials and Methods. The values above the lanes refer to the amounts of inner membrane fractions run in the lanes. The 1× samples are from ~1 mg (dry weight) of spores, and the amounts of membrane protein in the 1× lanes from spores prepared differently were made essentially equal as described in Materials and Methods. The GerAA, GerAC, and GerBC lanes are all from the same blot, which was stripped and reprobed, while the GerKA lanes are from a separate blot.

spores prepared differently, levels of SpoVAD were at most very slightly higher in poor-medium and 23°C spores than in rich-medium and 37°C spores, respectively, and similar results were obtained with PS767 (*gerA-lacZ*) spores made under these different conditions; these slight differences were not significant (Table

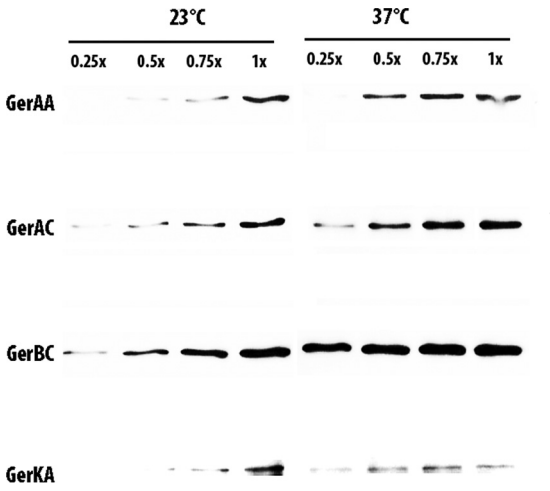

FIG 2 Levels of GR subunits in 23 and 37°C spores. The 23 and 37°C spores of strain PS767 (*gerA-lacZ*) were prepared and purified, the inner membrane fractions were isolated, and aliquots were analyzed by Western blotting using various antisera as described in Materials and Methods. The values above the lanes refer to the amounts of inner membrane fractions run in the lanes. The 1× samples are from ~1 mg (dry weight) of spores, and the amounts of membrane protein in the 1× lanes from spores prepared differently were made essentially equal as described in Materials and Methods. The GerAC, GerBC, and GerKA lanes are all from the same blot, which was stripped and reprobed, while the GerAA lanes are from a separate blot.

TABLE 6 Levels of GR subunits, GerD, and SpoVAD in spores prepared under different conditions[a]

Protein	Relative protein levels (arbitrary units) during sporulation			
	Rich medium[b]	Poor medium	37°C[c]	23°C
GerAA[d]	1.0	0.33	1.0	0.6
GerAC[e]	1.0	0.13	1.0	0.5
GerBC[e]	1.0	0.3	1.0	0.5
GerKA[e]	1.0	0.13	1.0	0.5
GerD[e,f]	1.0	0.3	1.0	0.25
SpoVAD[g]	1.0	1.2, 1.0	1.0	1.2, 1.0

[a] Spores of strains PS767 (*gerA-lacZ*) and PS4217 (Δ*gerD gerD*-Flag) were prepared in rich or poor liquid medium or on rich-medium plates at 23 or 37°C and purified, inner membrane fractions were isolated, and levels of various GR subunits, GerD, GerD-Flag, and SpoVAD were determined by Western blot analysis as described in Materials and Methods and shown in Fig. 1 to 3. All values are averages of at least duplicate determinations on two independent spore preparations, with differences between duplicates and different spore preparations being <15%. Values for GerAA, GerAC, GerBC, and GerKA are from PS767 spores. Values for GerD are from PS4217 spores, and the two values for SpoVAD levels in poor medium and 23°C spores are from PS4217 spores (first value) and PS767 spores (second value).
[b] Values for protein levels in spores made in the rich liquid medium were each set at 1.0.
[c] Values for protein levels in spores made at 37°C on plates were each set at 1.0.
[d] The difference between GerAA levels was significant ($P < 0.05$) for spores made in the rich and poor medium, but not significant ($P > 0.15$) for spores made at 37 and 23°C.
[e] The differences between this protein's level in spores made in rich and poor media or at 23 and 37°C are significant ($P < 0.05$) to highly significant ($P < 0.001$).
[f] Use of the anti-Flag serum to detect GerD-Flag gave essentially identical results.
[g] The differences between SpoVAD levels in spores made in rich and poor media or at 37 and 23°C were not significant.

6 and data not shown). However, levels of GerD and GerD-Flag were ~4-fold lower in poor-medium and 23°C spores than in rich-medium and 37°C spores, respectively (Fig. 3 and Table 6; also data not shown).

Amounts of superdormant spores in spore populations made in rich and poor media. The data above strongly suggested that the differences in rates of germination of spores made under different conditions, in particular in rich or poor media, were due largely to differences in GR or GerD levels, or levels of both proteins. Previous work has suggested that the amount of superdormant spores in spore populations is determined largely by that spore population's GR level, although effects of GerD levels on levels of superdormant spores have not been measured (10). The findings that have led to this suggestion are that spore populations with elevated average GR levels exhibit lower levels of superdormant spores, while spores lacking GRs exhibit minimal if any germination with all nutrient germinants (10, 24). However, spores with intermediate levels of GRs have not been examined for levels of superdormant forms. An obvious prediction from this previous work and the low levels of GRs in poor-medium spores is that these latter spores' populations would have an elevated percentage of superdormant spores. Consequently, we determined the levels of superdormant spores prepared using L-valine or AGFK in rich- and poor-medium PS533 spore populations. Similar to what has been found previously (10, 11), only a small percentage of rich-medium spores were superdormant following AGFK or L-valine germination (0.5% and 0.3% superdormant spores, respectively). However, 4- to 12-fold more of the poor-medium spores were superdormant for AGFK or L-valine germination (2.1% and 3.6% superdormant spores, respectively).

DISCUSSION

As seen in a number of previous studies of spores of *Bacillus* species (3, 8, 14, 18, 29), the present work also found that *B. subtilis* spores prepared under different conditions exhibited different germination kinetics with nutrient germinants. This was most pronounced for rich- and poor-medium spores, with the poor-medium spores germinating more slowly, and was seen with both spore populations and multiple individual spores. Germination of 23°C spore populations was also found to be slower than that of 37°C spore populations with nutrient germinants, but this difference was not seen when germination of multiple individual spores was examined. The reason for the difference in the germination of spores made at different temperatures with spore populations in liquid compared to multiple individual spores adhered to a microscope slide is not clear. However, poor-medium spores also germinated better adhered to a microscope slide with AGFK. Significant differences in the nutrient germination of spores germinated in liquid (populations) and adhered to a microscope slide (individual spores) have been seen previously (42), but the reason for this puzzling behavior is not clear.

Given that significant differences were seen between the nutrient germination of spores made under different conditions, the obvious question is that of what is responsible for these differences. Changes in levels of a number of proteins might be expected to alter spore germination significantly, including GRs, GerD, SpoVA proteins, and the redundant cortex-lytic enzymes (CLEs) CwlJ and SleB (25, 31). There were no significant differences in the levels of one SpoVA protein in spores prepared under different conditions, and thus the SpoVA proteins' levels seem unlikely to be responsible for the changes in spore germination with these spores. In addition, since CaDPA germination of rich-medium, poor-medium, 23°C, and 37°C spores was quite similar, it is likely that there is no notable change in CwlJ levels in these spores, since CaDPA triggers spore germination by activating CwlJ (32). That there is no major decrease in CwlJ levels in these different spores is further suggested by their very similar $\Delta T_{release}$ values for nutrient

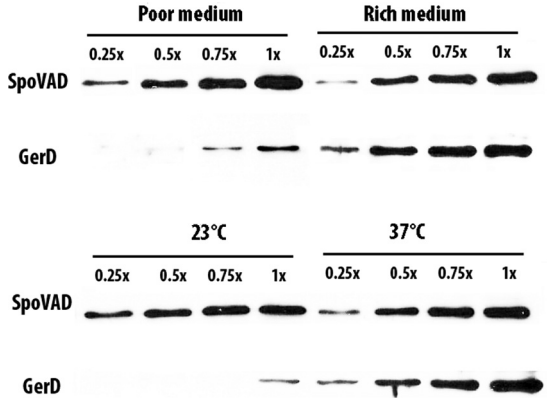

FIG 3 Levels of GerD and SpoVAD in spores prepared under different conditions. PS4217 spores (Δ*gerD gerD*-Flag) were prepared under different conditions, spores were purified, the inner membrane fractions were isolated, and aliquots were analyzed by Western blotting using various antisera as described in Materials and Methods. The values above the lanes refer to the amounts of inner membrane fractions run in the lanes. The 1× samples are from ~1 mg (dry weight) of spores, and the amounts of membrane protein in the 1× lanes from spores prepared differently were made essentially equal as described in Materials and Methods.

germination, since loss of CwlJ increases $\Delta T_{release}$ values ~10-fold (41, 42). There were, however, some small differences in average ΔT_{lysis} values for individual spores prepared under different conditions. While these differences could be due to changes in levels of CLEs, it is also possible that these spores have different cortex PG structures. Indeed, major alterations in cortex PG structure have significant effects on rates of nutrient germination, including both decreasing average T_{lag} and increasing $\Delta T_{release}$ (43). However, the effects of the different sporulation conditions used in the present work on average $\Delta T_{release}$ values were minimal (Tables 3 and 4). Indeed, while effects of sporulation medium richness on cortical PG structure have not been studied, at least different sporulation temperatures do not alter spore cortex PG structure notably (18).

While changes in levels of SpoVA proteins and CLEs seem unlikely to be responsible for the changes in nutrient germination of spores prepared under different conditions, the results in this work are certainly consistent with altered levels of GRs being a major factor in these changes in germination rates. Thus, spores prepared under different conditions that exhibited slower GR-dependent germination exhibited essentially identical germination with CaDPA, a GR-independent process, and faster germination with dodecylamine, again a GR-independent process (30, 32). More importantly, levels of a number of GR subunits and β-galactosidase levels from a *gerA-lacZ* fusion were significantly lower in spores that germinated more slowly, in particular in poor-medium spores. Given that all three of a GR's subunits are required for that GR's function (32), these data suggest that all GR function is greatly reduced in poor-medium spores. The present work is thus the first demonstration that significantly lower GR levels are correlated with lower rates of spores' nutrient germination. However, the correlation between rates of nutrient germination and GR levels is not linear, as an ~200-fold elevation in the GerBA level in *gerBB** spores gives only an ~3-fold elevation in the maximum spore germination rate with L-asparagine (1, 6), while 1.4- to 2-fold decreases in GR levels in 23°C spores compared to 37°C spores had only minimal effects on the rates of germination of multiple individual spores.

While it seems likely that lower GR levels play a significant role in determining lower rates of spore germination in spores prepared differently, in particular in rich or poor media, there are additional factors that could modulate rates of spore germination. One obvious possibility is the level of the GerD protein that is also essential for rapid GR-dependent spore germination (27) and is ~4-fold lower in 23°C and poor-medium spores. Indeed, the absence of GerD is known to increase average T_{lag} values for GR-dependent germination markedly and with no effect on average $\Delta T_{release}$ values (38). However, overexpression of GerD has no effect on rates of GR-dependent spore germination (27). In addition, the ~4-fold decrease in GerD levels in 23°C spores had less effect on the efficiency of spore germination than the 3.3-fold decrease in GerD levels in poor-medium spores. Given that GR levels were reduced much more in poor-medium spores than in 23°C spores, this suggests that GR levels are more important than GerD levels in determining overall rates and efficiency of spore germination with nutrients. Perhaps GerD is normally in excess in spores such that a 4-fold decrease in GerD level alone causes only a small decrease in GR-dependent germination. In order to critically examine this possibility, the effects on nutrient germination of decreasing GerD levels will need to be determined under conditions where there are no changes in GR levels.

Another possible factor in the differences in the altered germination of spores prepared differently could be levels of the spore coat GerP proteins that appear to be required for normal access of nutrient germinants to GRs in the spore's inner membrane (4, 7). Indeed, different sporulation temperatures result in altered profiles of spore coat proteins (18). However, the GerP proteins have also been reported to be essential for normal CaDPA germination, at least with *Bacillus anthracis* spores (7), and spores' CaDPA germination was reduced minimally if at all by the differences in sporulation used in the present work.

If, as seems most likely, changes in GR levels are a major factor determining changes in the germination of spores prepared under different conditions, a further obvious question is what causes changes in GR levels (and also GerD levels) as a function of sporulation conditions, in particular the richness of the sporulation medium. Since changes in the levels of β-galactosidase from a transcriptional *gerA-lacZ* fusion were generally similar to changes in levels of various GRs, this suggests that changes in transcription of operons encoding GRs as a function of sporulation conditions are responsible. Indeed, decreased levels of β-galactosidase from transcriptional fusions to promoters of operons encoding GRs in *B. cereus* spores prepared in a poor medium also generally reflected the slower germination of these poor-medium spores, although GR levels in these spores were not determined directly (14). The *spoVA* operon, *gerD*, and operons encoding GRs are transcribed by RNA polymerase containing the alternative sigma factor σ^G, and this transcription is further modulated by the transcription factor SpoVT, which has both positive and negative effects on σ^G-dependent gene expression (2, 32). There is, however, no information on how sporulation medium composition or temperature might modulate the transcription of these genes or their regulation by SpoVT. Analysis of the effects of sporulation conditions on *spoVT* expression could thus be informative.

There is also one report that a protein tyrosine phosphatase, PrpE, modulates expression of operons encoding GRs (13). Since how PrpE exerts this effect is not known, analysis of the effects of PrpE on GR levels would be worthwhile. It is also possible that there is translational modulation of GR levels. Indeed, the ~2.5-fold-higher relative level of GerAA (encoded by the first gene in the *gerA* operon) than GerAC (encoded by the third gene in the *gerA* operon) in poor-medium spores (Table 6) suggests that translational coupling of *gerA* mRNA may be less efficient during sporulation in a poor medium. Thus, analysis of the effects of sporulation conditions on expression of translational *gerA-lacZ* fusions could be worthwhile.

Given the slower nutrient germination of poor-medium than rich-medium spores, it was not surprising that the yields of superdormant spores were significantly higher from poor-medium spores. This finding as well as the lower GR levels in poor-medium spores is thus consistent with the suggestion that superdormant spores are likely those spores in a population that have the lowest GR levels (10). Since spores contaminating foodstuffs and medical products may well have formed in a nutrient-poor environment, it seems reasonable to suppose that levels of superdormant spores in such environmental spore populations will likely be significantly higher than if the spores were prepared in rich laboratory sporulation media. This also seems like a prediction

that would be well worth examining, given the importance of superdormant spores in a number of applied situations.

ACKNOWLEDGMENTS

This work was supported by a Multi-University Research Initiative award through the U.S. Army Research Laboratory and the Army Research Office under contract number W911NF-09-1-0286.

We are grateful to George Korza, Bing Hao, Yunfeng Li, and Barbara Setlow for preparation of protein antigens and purification of antisera to GR subunits, to Kerry Ann Stewart for advice on Western blot analyses, and to Sonali Ghosh for assistance with statistical analyses.

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