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Characterization of Spores of *Bacillus subtilis* Which Lack Dipicolinic Acid

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Spores of *Bacillus subtilis* with a mutation in *spoVF* cannot synthesize dipicolinic acid (DPA) and are too unstable to be purified and studied in detail. However, the spores of a strain lacking the three major germinant receptors (termed Δ*ger3*), as well as *spoVF*, can be isolated, although they spontaneously germinate much more readily than Δ*ger3* spores. The Δ*ger3 spoVF* spores lack DPA and have higher levels of core water than Δ*ger3* spores, although sporulation with DPA restores close to normal levels of DPA and core water to Δ*ger3 spoVF* spores. The DPA-less spores have normal cortical and coat layers, as observed with an electron microscope, but their core region appears to be more hydrated than that of spores with DPA. The Δ*ger3 spoVF* spores also contain minimal levels of the processed active form (termed P_{41}) of the germination protease, GPR, a finding consistent with the known requirement for DPA and dehydration for GPR autoprocessing. However, any P_{41} formed in Δ*ger3 spoVF* spores may be at least transiently active on one of this protease's small acid-soluble spore protein (SASP) substrates, SASP-γ. Analysis of the resistance of wild-type, Δ*ger3*, and Δ*ger3 spoVF* spores to various agents led to the following conclusions: (i) DPA and core water content play no role in spore resistance to dry heat, dessication, or glutaraldehyde; (ii) an elevated core water content is associated with decreased spore resistance to wet heat, hydrogen peroxide, formaldehyde, and the iodine-based disinfectant Betadine; (iii) the absence of DPA increases spore resistance to UV radiation; and (iv) wild-type spores are more resistant than Δ*ger3* spores to Betadine and glutaraldehyde. These results are discussed in view of current models of spore resistance and spore germination.

Spores of *Bacillus* and *Clostridium* species normally contain ≥10% of their dry weight as pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (21, 22, 39). This compound is synthesized late in sporulation in the mother cell compartment of the sporulating cell but accumulates only in the developing forespore (6, 36). The great majority of the spore's DPA is in the spore core, where it is most likely chelated with divalent cations, predominantly Ca^{2+}, although there are also significant amounts of Mg^{2+} and Mn^{2+}, with smaller amounts of other divalent cations (21, 22, 37, 39). In the first minutes of spore germination the DPA is excreted, along with the associated divalent cations (36, 37).

Since DPA is found only in dormant spores of *Bacillus* and *Clostridium* species and since these spores differ in a number of properties from vegetative cells, in particular in their dormancy and heat resistance, it is not surprising that DPA and divalent cations have been suggested to be involved in some of the spore's unique properties. There is some evidence in support of this suggestion, since mutants whose spores do not accumulate DPA have been isolated in several *Bacillus* species, and often these DPA-less spores are heat sensitive (1, 4, 25, 42, 43). Unfortunately, for some of these latter mutants the specific genetic lesion(s) giving rise to the DPA-less spore phenotype is not known. DPA is synthesized from an intermediate in the lysine pathway, and the enzyme that catalyzes DPA synthesis is termed DPA synthetase (6). In *B. subtilis* this enzyme is encoded by the two cistrons of the *spoVF* operon, which is expressed only in the mother cell compartment of the sporulating cell, the site of DPA synthesis. Mutants of *B. subtilis* likely to be in or known to be in *spoVF* result in lack of DPA synthesis during sporulation, and the spores produced never attain the wet heat resistance of wild-type spores (1, 4, 6, 25). Unfortunately, it has been impossible to isolate and purify free spores from these *spoVF* mutants of *B. subtilis*, since the spores are extremely unstable and germinate and lyse during purification (B. Setlow and P. Setlow, unpublished results). This observation suggests that, at least in *B. subtilis*, DPA is needed in some fashion to maintain spore dormancy (7, 15), although the specific mechanism whereby this is achieved is not clear.

In addition to its possible roles in spore dormancy and resistance, DPA complexed with a divalent cation, usually Ca^{2+}, is an effective germinant of spores of almost all *Bacillus* and *Clostridium* species (15). These and other data have led to the suggestion that DPA may activate, possibly allosterically, some enzyme involved in spore germination (15). To date, this spore enzyme involved in spore germination has not been identified. However, DPA does allosterically modulate the activity of the germination protease (GPR) that initiates the degradation of the spore's depot of small, acid-soluble spore proteins (SASPs) during spore germination (14, 32). GPR is synthesized as an inactive zymogen (termed P_{46}) during sporulation, and P_{46} autoprocesses to a smaller active form (termed P_{41}) approximately 2 h later in sporulation. This conversion of P_{46} to P_{41} is stimulated allosterically by DPA, and only the physiological DPA isomer is effective (14, 32). The activation of this zymogen is also stimulated by the acidification and dehydration of the spore core, and together these conditions ensure that P_{41} is generated only late in sporulation, when the conditions in the spore core preclude enzyme action (14, 32). As a result, GPR's SASP substrates, which are synthesized in parallel with P_{46}, are stable in the developing and dormant spore. This is important for spore survival, as some major SASP (the α/β-type) are essential for the protection of spore DNA from a variety of

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types of damage, while degradation of both the α/β-type SASP and the other major SASP (γ) provides amino acids for protein synthesis early in spore germination (38, 39, 40).

We recently described a mutant strain of *B. subtilis* that lacks the three operons encoding the proteins responsible for sensing and triggering spore germination in response to nutrient germinants (24). This strain sporulates normally but its spores germinate extremely poorly in response to nutrient germinants; however, the spores germinate normally in response to a mixture of Ca^{2+} and DPA (24). These observations suggested that introduction of a *spoVF* mutation into this strain lacking nutrient receptors for spore germination might result in the production of DPA-less spores that were stable enough to be isolated and purified, yet which could be recovered by spore germination in Ca^{2+} and DPA. This was indeed the case, and in this study we describe the properties of these stable DPA-less spores.

MATERIALS AND METHODS

Strains used and production and purification of spores. The *B. subtilis* strains used in this work are all derivatives of strain 168 and are derived from PS832, a *trp*$^+$ revertant of 168. The strains are: PS533, containing plasmid pUB110 carrying a gene for kanamycin resistance (Km^r); FB72 Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*erm* (24) (this strain will be referred to here as Δ*ger3*); and FB108 Δ*gerA*::*spc* Δ*gerB*::*cat* Δ*gerK*::*erm* Δ*spoVF*::*tet* (this strain will be termed here Δ*ger3 spoVF* [see below]). Unless otherwise noted, the spores of these various strains were prepared on 2×SG medium (23) agar plates without or with DPA (100 μg/ml). The plates were spread with 0.1 ml of a suspension (~10^5 cells/ml) of growing cells of the appropriate strain and incubated at 37°C for ~48 h. Spores and sporulating cells were scraped from the plates, and the spores were purified as described previously (20); all spore preparations used in this work were free (>95%) of growing or sporulating cells or germinated spores and were initially stored in water at 12°C, the temperature of our cold room. Spores stored in this manner were stable for several weeks but are even more stable if stored at 4°C.

Construction of the Δ*spoVF* strain. The Δ*spoVF*::*tet* plasmid pFE229 was derived from plasmid pECE98 (*Bacillus* Genetic Stock Center) as follows. The 3′ end of the *spoVF* operon, spanning nucleotides (nt) 932 to 1278 relative to the first codon of the *spoVFA* translation start site (defined as +1), was amplified by PCR from PS832 chromosomal DNA with primers ΔspoVFC5 and ΔspoVFC3 (primer sequences will be provided on request) and the PCR fragment cloned in the TA vector pCR2.1 (Invitrogen), yielding plasmid pFE226. The insert was excised from pFE226 with *Hin*dIII (site present in primer ΔspoVFC5) and *Eco*RI (site present in primer ΔspoVFC3) and cloned between the *Hin*dIII and *Eco*RI sites in pECE98, yielding plasmid pFE228. The 5′ end of the *spoVF* operon spanning nt 21 to 386 relative to the *spoVFA* translation start site was PCR amplified as described above, but with primers ΔspoVFN5 and ΔspoVFN3, and cloned into pCR2.1, yielding plasmid pFE227. The insert in plasmid pFE227 was excised with *Bam*HI and *Pst*I (sites in primers ΔspoVFN5 and ΔspoVFN3, respectively) and cloned between the *Bam*HI and *Pst*I sites in plasmid pFE228, yielding the Δ*spoVF*::*tet* plasmid pFE229. The Δ*spoVF*::*tet* derivative of strain FB72 was constructed by transforming (5) this strain with plasmid pFE229 and using Southern blot analysis to identify tetracycline-resistant transformants that had arisen by a double-crossover event. One of these transformants was called FB108.

Analyses of spore resistance, spore proteins, and spores. Resistance of spores to treatment with wet and dry heat, dessication, hydrogen peroxide, glutaraldehyde, formaldehyde, UV radiation, and the iodine-based disinfectant Betadine (Purdue-Frederick Company, Norwalk, Conn.) was tested as described previously (17, 23, 34, 35, 41). However, because Δ*ger3* spores do not germinate in response to nutrient germinants, after spore treatment the spores of all strains were germinated in 60 mM CaDPA as described elsewhere (24) prior to dilution and plating on Luria-Bertani (LB) medium agar plates (41) to determine the number of survivors.

For analysis of GPR, 15 to 25 mg (dry weight) of spores of various strains was dry ruptured with 100 mg of glass beads in 8×1-min bursts in a dental amalgamator (23). The resultant dry powder was extracted with 0.5 ml of cold 25 mM Tris-HCl (pH 7.5)–5 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride and, after 30 min on ice, the mix was centrifuged in a microcentrifuge. After determination of the protein concentration in the supernatant fluid by the Lowry method (18), aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, the proteins were transferred to polyvinylidine difluoride paper (Immobilon), and GPR was detected with anti-GPR serum as described earlier (27, 31, 32).

For analysis of SASP, 7 to 12 mg of dry spores or the dry pellet from 10 ml of sporulating cells was disrupted as described above and extracted twice with 0.5 ml of cold 3% acetic acid, and the supernatant fluids were combined, dialyzed overnight in Spectrapor 3 tubing (molecular mass cutoff, 3,500 kDa) against cold 1% acetic acid, and lyophilized (23). The dry residue was dissolved in a small volume of 8 M urea, aliquots were subjected to PAGE at low pH, and the gels were stained with Coomassie blue (23).

DPA was analyzed after extraction of spores with boiling water as described elsewhere (23, 29). For determination of spore core wet densities, spore coats were removed by extraction with SDS, dithiothreitol, and urea, and the decoated spores were centrifuged in Nycodenz or metrizoic acid density gradients as described earlier (16, 28). For the determination of spore germination, spores were diluted in water, and appropriate dilutions were spread onto LB medium plates; colonies were counted after incubation for 24 h at 37°C. Spores were prepared for electron microscopy as described previously (19).

TABLE 1. DPA, core water content, and colony formation of various spores[a]

Strain	Spore properties			
	DPA content (μg/OD_{600})	Core wet density (g/ml)	Germination	
			Colonies formed/OD_{600} in 24 h[b]	Phase dark spores in 36 h (%)[c]
PS533 (wild type)	14.8	1.365	6.5×10^7	ND[d]
FB72	15.5	1.370	4×10^4	<0.5
FB108	<0.7	1.297	1.3×10^7	40
FB108 (sporulated with DPA)	9.7	1.345	4.5×10^5	ND

[a] Spores were prepared, and the DPA content, core wet densities, and colonies formed/OD_{600} on LB medium plates were determined as described in Materials and Methods.

[b] Values are averages of data from two determinations on one spore preparation. These values differed by <2-fold between three different spore preparations. After germination with CaDPA prior to spreading onto LB medium plates, the values for all four strains were 10^8 colonies formed/OD_{600} in 24 h.

[c] Spores (10^7/ml) were incubated at 37°C in 10 mM Tris-HCl (pH 7.4) for 36 h, and then 120 to 200 spores were examined in the phase-contrast microscope.

[d] ND, not determined.

RESULTS

Preparation and characterization of Δ*ger3 spoVF* spores. Initial analyses showed that the Δ*ger3 spoVF* strain sporulated to a similar degree as the Δ*ger3* parent. However, liquid cultures of spores produced by the Δ*ger3 spoVF* strain had a higher percentage of germinated spores than the Δ*ger3* parent. This was much less evident with spore preparations made on plates, and the spores prepared on plates from both the Δ*ger3 spoVF* and Δ*ger3* strains were also easy to purify and readily gave clean spore preparations containing >95% spores which appeared bright in the phase-contrast microscope. Consequently, Δ*ger3 spoVF* and Δ*ger3* spores were prepared on plates unless noted otherwise. Wild-type (PS533) and Δ*ger3* spores had similar levels of DPA, while the Δ*ger3 spoVF* spores had <5% of this level (Table 1). Sporulation of the Δ*ger3* strains with DPA (100 μg/ml) had no effect on the level of DPA in the Δ*ger3* spores, but raised the DPA level in the Δ*ger3 spoVF* spores to 65% of that in Δ*ger3* spores (Table 1 and data not shown).

While the DPA-less spores did appear bright in the phase-contrast microscope, suggesting at least partial core dehydration, their core wet density was significantly lower than that of the wild-type and Δ*ger3* spores; however, sporulation with DPA raised the core wet density of Δ*ger3 spoVF* spores significantly (Table 1). These differences in core wet densities indicate that the core of the Δ*ger3 spoVF* spores prepared without DPA contains significantly more water per gram (dry weight) than the core of the Δ*ger3* spores. However, the core wet density of Δ*ger3 spoVF* spores is still significantly greater than that of germinated spores (1.228 g/ml) (26).

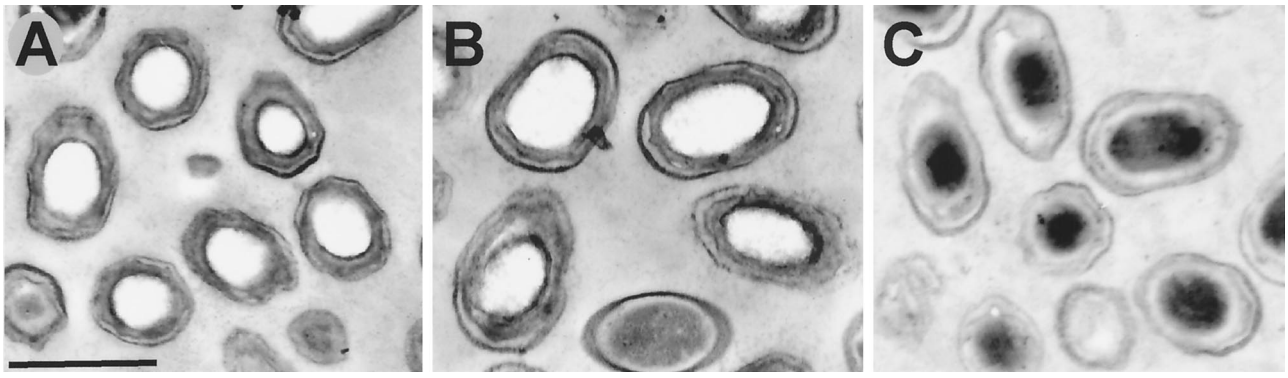

FIG. 1. Thin-section electron micrographs of spores with or without DPA. Spores were prepared for electron microscopy as described in Materials and Methods. The spores shown are PS832 (wild-type) (A), FB72 (Δ*ger3*) (B), and FB108 (Δ*ger3 spoVF*) (C). The bar in panel A denotes 1 μm, and the other two panels are at the same magnification as panel A.

Examination of spores of the Δ*ger3* and Δ*ger3 spoVF* strains by electron microscopy indicated that spores of both strains had normal-looking coats and cortex layers (Fig. 1). The core of Δ*ger3* spores showed no evidence of significant structure and appeared white, as is typical of the dehydrated wild-type spore core; in contrast, the core region of Δ*ger3 spoVF* spores showed the dark, often punctate staining pattern associated with the presence of ribosomes and a significant amount of water in the spore core (10). This is consistent with the higher level of core hydration in Δ*ger3 spoVF* spores noted above, and these results are similar to those obtained previously in one study of spores of a *B. subtilis spoVF* mutant (1). However, an earlier electron microscopic analysis of sporulating *spoVF* cells suggested that the cortex of the spores produced was incompletely developed (4). Perhaps this was due to some early germination-like changes in the spores within these *spoVF* sporangia.

Previous work has shown that Δ*ger3* spores exhibit very low levels of colony formation when plated on rich medium plates because of the infrequent, albeit spontaneous, germination of these spores (24). This was also observed here, as three different preparations of Δ*ger3* spores gave <0.1% of the colonies/optical density at 600 nm (OD_{600}), as did the wild-type spores (Table 1). However, these spores can be fully recovered by germination with CaDPA prior to plating on rich medium plates (24). While the colony-forming ability of Δ*ger3 spoVF* spores on rich medium plates was not as high as that of wild-type spores, it was 100-fold higher than that of Δ*ger3* spores; as expected, the Δ*ger3 spoVF* spores were also fully recovered by prior germination with CaDPA (Table 1). Restoration of significant DPA levels to Δ*ger3 spoVF* spores suppressed much of their spontaneous colony-forming ability on rich medium plates, but not their colony-forming ability after CaDPA treatment (Table 1). As found previously (24), the Δ*ger3* spores also did not undergo germination in dilute buffer, when spore germination was measured by the conversion of a bright spore to a dark one as seen in a phase-contrast microscope (Table 1). However, a large fraction of the Δ*ger3 spoVF* spores underwent spore germination when dilute spores were incubated at 37°C in dilute buffer (Table 1).

Levels of GPR forms and SASP in spores. The lack of DPA and the elevated core hydration in Δ*ger3 spoVF* spores suggested that the SASP-specific protease, GPR, might be poorly processed in these spores, since both DPA accumulation and core dehydration stimulate conversion of P_{46} to P_{41} (14, 32). Analysis of the extent of GPR processing in various spores revealed that ~75% of the P_{46} was converted to P_{41} in Δ*ger3* spores (Fig. 2, lane 1), a value similar to that found previously in wild-type spores (27, 32). However, in Δ*ger3 spoVF* spores ≥90% of the GPR was present as P_{46} (Fig. 2, lane 2), while in the spores of the latter strain prepared with DPA about 40% of the GPR had been processed to P_{41} (Fig. 2, lane 3).

Although very little P_{46} is processed to P_{41} in Δ*ger3 spoVF* spores, any P_{41} produced in these spores might be expected to be able to act on its SASP substrates because of the greater spore core hydration at the time of P_{41} generation (27, 39). This P_{41} action would most likely be on SASP-γ since this protein is not protected from proteolysis by binding to some spore macromolecule, unlike SASP-α and -β which are bound to spore DNA (39, 40). Indeed, previous work has shown that increased spore core hydration during the period of P_{41} production does lead to significantly reduced levels of SASP-γ in spores (27). Consequently, it was not surprising to find that SASP-γ was almost completely absent in Δ*ger3 spoVF* spores, while levels of SASP-α and -β were similar to those in Δ*ger3* spores (Fig. 3, compare lanes 1 and 2, and note that more protein was run on lane 1). As expected, Δ*ger3 spoVF* spores

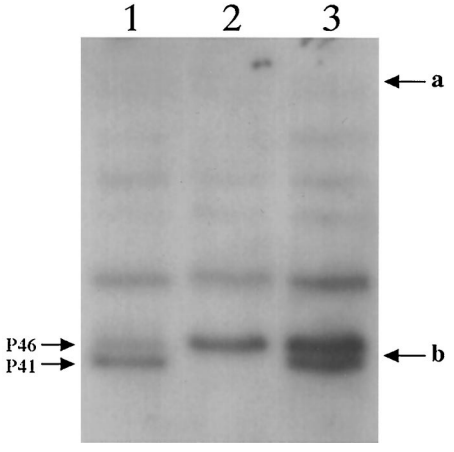

FIG. 2. Levels of the P_{46} and P_{41} forms of GPR in spores. Soluble proteins from spores of various strains were isolated and 10 μg of soluble protein were subjected to SDS-PAGE and Western blot analysis using anti-GPR antiserum as described in Materials and Methods. The samples run on the various lanes are as follows: lane 1, Δ*ger3* spores; lane 2, Δ*ger3 spoVF* spores; and lane 3, Δ*ger3 spoVF* spores sporulated with DPA. The migration positions of the P_{46} and P_{41} forms of GPR are given on the left of the figure. The migration positions of molecular mass markers of 84 and 41 kDa are denoted by arrows a and b, respectively. The bands above P_{46} and P_{41} reacted nonspecifically with the antiserum used in this experiment.

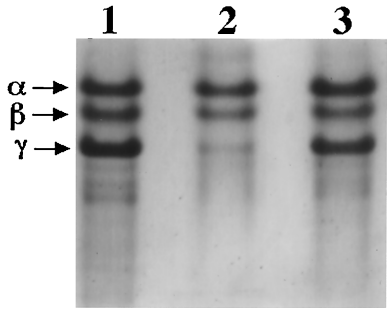

FIG. 3. Levels of SASP-α, -β, and -γ in spores. SASPs were extracted from purified spores of various strains, dialyzed, and lyophilized; aliquots were subjected to PAGE at low pH, and the gels were stained as described in Materials and Methods. The samples run on the various lanes (and the dry weight of the spores in the samples run) were as follows: lane 1, Δ*ger3* spores (1 mg); lane 2, Δ*ger3 spoVF* (0.6 mg); and lane 3, Δ*ger3 spoVF* spores prepared with DPA (1 mg).

prepared with DPA did contain a significant level of SASP-γ, although this level was a bit lower than in Δ*ger3* spores (Fig. 3, lanes 1 and 3).

Since the spores analyzed for SASP levels as described above remained for ~2 weeks at 12°C during preparation, it was of obvious interest to determine if developing spores of the Δ*ger3 spoVF* strain had never contained SASP-γ or had accumulated and then degraded this protein and, if the latter was the case, when the protein was degraded. Consequently, we analyzed SASP levels in sporulating cells during incubation at 37°C or subsequent incubation at 12°C (Fig. 4). Levels of SASP-α and -β were relatively constant once these proteins had accumulated in developing Δ*ger3 spoVF* spores, and SASP-γ was also present at high levels shortly after completion of SASP synthesis (Fig. 4, lane 1). However, levels of SASP-γ then began to decrease, and ~9 h later levels of this protein had fallen significantly and possibly fell even more after the culture had been harvested, washed with cold water, and incubated at 12°C. These data indicate that relatively normal levels of SASP-γ are accumulated by developing Δ*ger3 spoVF* spores but that the SASP-γ then disappears, presumably by degradation as sporulation and spore incubation proceeds.

Resistance of Δ*ger3* and Δ*ger3 spoVF* spores. The normal levels of SASP-α and -β in Δ*ger3 spoVF* spores suggested that the protection of spore DNA from damage by these proteins should be normal in Δ*ger3 spoVF* spores, and thus some aspects of spore resistance should be normal in these spores (39, 40). Indeed, previous work has shown that the spores formed by a *B. subtilis* strain with a mutation that is probably in *spoVF* are fully resistant to some chemical agents, including octanol and chloroform (1). However, these same spores were sensitive to a number of other chemicals and were also sensitive to wet heat (1). Given the known relationships between spore resistance and spore core hydration and mineral levels (11, 20, 40), it was of obvious interest to test the resistance of Δ*ger3* and Δ*ger3 spoVF* spores to a variety of agents. As seen previously and as expected based on the elevated level of core water in Δ*ger3* spores (1, 11, 28), the Δ*ger3 spoVF* spores were much less resistant to wet heat than were Δ*ger3* spores, while the latter spores had identical wet heat resistance to wild-type spores (Fig. 5A and data not shown). Spores of the Δ*ger3 spoVF* strain prepared with DPA exhibited an intermediate level of wet heat resistance (Fig. 5A). Although the Δ*ger3 spoVF* spores were significantly more sensitive to wet heat than were the wild-type spores, they were much more resistant than germinated spores or growing cells (<0.01% survival after 5 min at 70°C; data not shown). In contrast to the differences observed in the wet heat resistance of Δ*ger3 spoVF* and Δ*ger3* spores, both of these spores exhibited identical resistance to dry heat and were fully resistant to dessication (Table 2 and data not shown). The resistance of these spores to dry heat and dessication was identical to that of wild-type spores (data not shown) and much greater than that of growing cells (9, 35).

Analysis of resistance to hydrogen peroxide and formaldehyde gave results which were qualitatively similar to those with wet heat. The Δ*ger3* and wild-type spores exhibited identical resistance to formaldehyde and hydrogen peroxide (data not shown), while Δ*ger3 spoVF* spores were more sensitive and Δ*ger3 spoVF* spores prepared with DPA had intermediate levels of resistance (Fig. 5B and C). The UV resistance of Δ*ger3* spores was also identical to that of wild-type spores (data not shown), but Δ*ger3 spoVF* spores were more UV resistant than were Δ*ger3* spores, while Δ*ger3 spoVF* spores prepared with DPA had an intermediate level of resistance (Fig. 5D).

In contrast to wet heat, dry heat, UV, hydrogen peroxide, and formaldehyde, which had essentially identical efficiencies of killing of wild-type and Δ*ger3* spores, Δ*ger3* spores were significantly more sensitive to both the iodine-based disinfectant Betadine and to glutaraldehyde than were the wild-type spores (Fig. 6). The Δ*ger3 spoVF* spores exhibited decreased resistance to Betadine compared to that of Δ*ger3* spores, with Δ*ger3 spoVF* spores prepared with DPA exhibiting intermediate resistance (Fig. 6A). However, Δ*ger3* and Δ*ger3 spoVF* spores (with or without DPA) had identical resistance to glutaraldehyde (Fig. 6B).

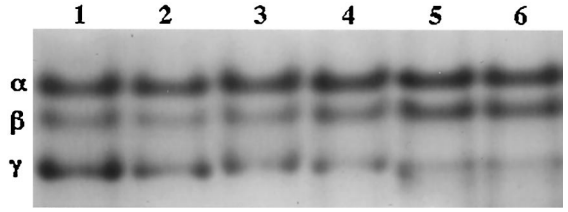

FIG. 4. Levels of SASP-α, -β, and -γ in sporulating cultures. Samples (10 ml) of strain FB108 (Δ*ger3 spoVF*) sporulating in liquid 2×SG medium at 37°C were harvested, frozen, and lyophilized. After 36 h of growth, the remaining culture was harvested, washed several times with cold water, and resuspended in cold water. Again, aliquots equal to 10 ml of original culture were harvested, frozen, and lyophilized. Dry samples were disrupted; SASP was extracted; extracts were dialyzed, lyophilized, and redissolved; equal aliquots were subjected to PAGE at low pH, and the gel stained as described in Materials and Methods. The times (t) (in hours) in sporulation that the samples run in the various lanes were harvested were as follows: lane 1, t_4; lane 2, t_6; lane 3, $t_{7.5}$; lane 4, $t_{13.5}$; lane 5, t_{28}; and lane 6, t_{160}. Note that the last sample was incubated at 12°C for ~145 h. The migration positions of SASP-α, -β, and -γ are given on the left of the figure.

DISCUSSION

Although DPA was discovered in spores of *Bacillus* species over 40 years ago, its specific function in spores has remained somewhat obscure. Correlations have been noted between spore wet heat resistance and DPA content (11, 22), but there are a number of observations indicating that DPA need not be essential for spore heat resistance. Thus, DPA plus associated divalent cations can be removed from the mature spores of several species by appropriate treatments, yielding spores with <1% of untreated spore DPA levels; these DPA-less spores retain a high level of wet heat resistance which is often similar to that of untreated spores (2, 11). Strikingly, these DPA-less spores of *Bacillus stearothermophilus* appeared to have more highly hydrated core regions than untreated spores yet still retained high wet heat resistance. The reasons for the wet heat

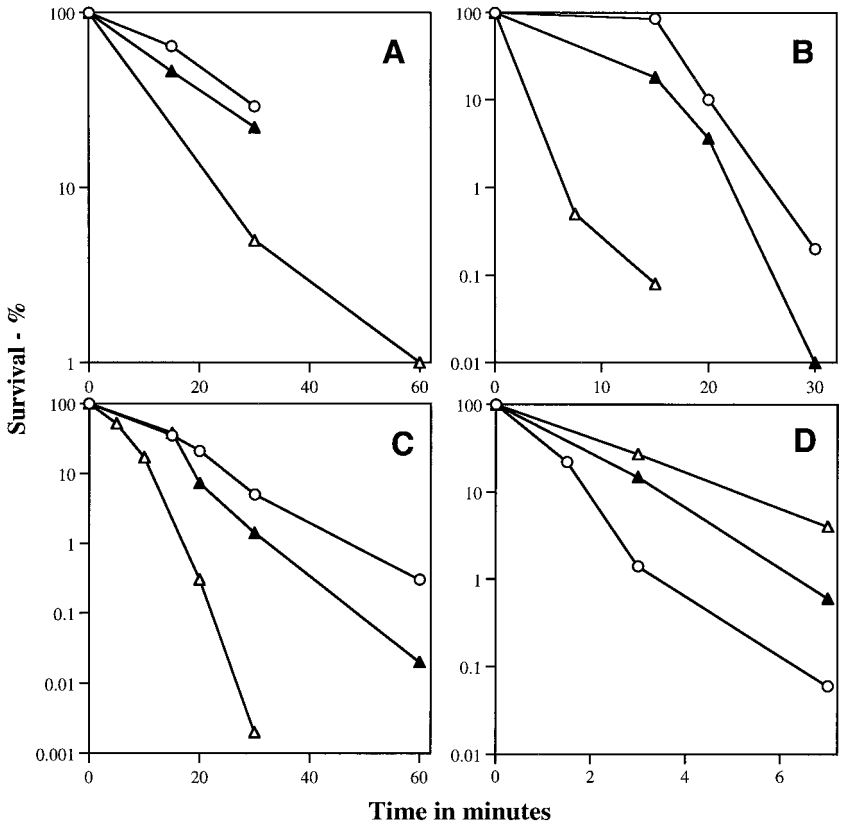

FIG. 5. Resistance of spores with or without DPA to heat (A), hydrogen peroxide (B), formaldehyde (C), or UV radiation (D). Spores were either heated at 85°C (○ and ▲) or 70°C (△) (A), incubated with 0.7 M hydrogen peroxide at room temperature (B), incubated with 0.3 M formaldehyde at room temperature (C), or UV irradiated at 150 J/m^2 · min (D), and the survival was measured as described in Materials and Methods. Symbols: ○, Δ*ger3* spores; △, Δ*ger3 spoVF* spores; ▲, Δ*ger3 spoVF* spores prepared with DPA. All experiments were repeated at least twice with essentially identical results.

resistance of these DPA-less and relatively demineralized spores are not clear, but these data indicate that DPA is not necessarily essential for spore wet heat resistance. However, it is possible that DPA accumulation during sporulation is required for the attainment of some state that is essential for full spore wet heat resistance. In support of this possibility, several studies, including the current one, have found that in *B. subtilis* the loss of the ability to synthesize DPA results in the production of wet heat-sensitive spores which exhibit increased core dehydration (1, 4, 6, 8). However, it is not clear if this effect is due only to a change in spore core hydration or also to the reduction in core mineralization which accompanies the loss of DPA from spores (12). Since spore core mineralization also plays a role in wet heat resistance (11, 20), it is certainly possible that changes in both core hydration and mineral levels contribute to the loss of wet heat resistance of DPA-less spores.

TABLE 2. Spore resistance to dry heat and dessication[a]

Treatment	% Survival		
	FB72 (Δ*ger3*)	FB108 (Δ*ger3 spoVF*) sporulated without DPA	FB108 (Δ*ger3 spoVF*) sporulated with DPA
Dessication[b]	96	92	98
Dry heat (120°C) for 30 min	26	23	24

[a] Spores were produced and analyzed for dessication and dry-heat resistance as described in Materials and Methods.

[b] Spores were freeze-dried once, and the viability was measured after rehydration.

Strains of *Bacillus cereus* and *Bacillus megaterium* with uncharacterized mutations that abolish DPA accumulation in spores also produce heat-sensitive spores, and in at least one case these spores appeared to have increased core hydration (42, 43). While the existence of these latter mutants would seem to support a role for DPA in spore heat resistance, there are several reports (11, 12) that the heat-sensitive DPA-less spores of *B. cereus* can be further mutated to give a strain that produces DPA-less but heat-resistant spores. Unfortunately, the genes responsible for these phenotypes are not known, and the heat-resistant phenotype of the DPA-less spores was extremely unstable (12). There is also an old report that heat-resistant DPA-less spores of *B. subtilis* had been isolated (42); unfortunately, there are almost no details available about this strain and the mutations which gave rise to this phenotype. Since addition of only very small amounts of DPA to *spoVF* cultures can result in production of at least some heat-resistant spores (1), possibly the mutants producing DPA-less, heat-resistant spores are actually oligosporogenous, and the heat-resistant spores arise from the acquisition of sufficient DPA by a fraction of spores, either through synthesis in the surrounding mother cell or from the culture medium (8). If only a fraction of the spore population in a culture acquired only a small amount of DPA, then analysis might well not detect significant DPA in the population as a whole.

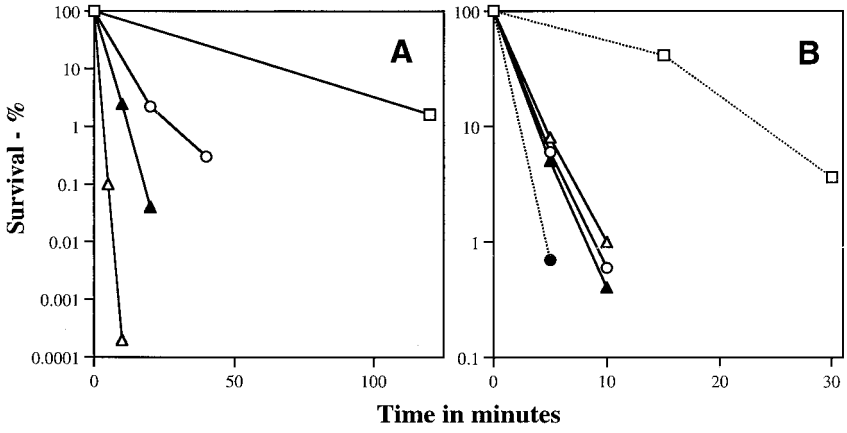

FIG. 6. Betadine and glutaraldehyde resistance of spores with or without DPA. Spores were incubated either with 85% Betadine at 37°C (A) or with 1.8% glutaraldehyde (□ and ●) or 0.5% glutaraldehyde (○, △, and ▲) at room temperature (B), and the survival was measured as described in Materials and Methods. Symbols: □, PS533 (wild-type) spores; ○ and ●, Δ*ger3* spores; △, Δ*ger3 spoVF* spores; ▲, Δ*ger3 spoVF* spores prepared with DPA. All experiments were repeated at least twice with essentially identical results.

The precise role of DPA in spores is still not clear; however, in *B. subtilis* specifically blocking DPA synthesis results in DPA-less spores with significantly less wet heat resistance than wild-type spores. The lack of DPA in these *B. subtilis* spores is accompanied by increased core hydration, and there are abundant data that this increase in core hydration should reduce spore wet heat resistance (11, 27) and, as shown here, it does. However, the DPA-less spores of *B. subtilis* are still significantly more wet heat resistant (and less hydrated) than are growing cells or germinated spores of this organism (26, 27, 40).

In addition to a role for DPA in spore wet heat resistance, two other roles have been proposed. One is to stabilize the dormant spore such that it does not germinate spontaneously (7, 16, 40). This appears to be the case for the *B. subtilis* spores studied in this work, since the Δ*ger3 spoVF* spores germinate spontaneously much more readily than the Δ*ger3* spores. Unfortunately, it is not clear at present either what is involved in "spontaneous" spore germination or how spore DPA could suppress this event. Surprisingly, it has also been reported that some DPA-less spores of *B. cereus*, *B. megaterium*, and *B. subtilis* germinate extremely poorly (12, 42). However, the mutation or mutations giving rise to the DPA-less spores of these strains are not known, and it is certainly possible that, in addition to a mutation blocking DPA synthesis or uptake, these strains have an additional mutation(s) suppressing spore germination, thus allowing the DPA-less spores of these strains to be isolated.

A second specific role for DPA is in allosterically stimulating the processing of GPR from P_{46} to P_{41} such that this processing only takes place very late in spore core maturation, when the core dehydrates; this dehydration also stimulates conversion of P_{46} to P_{41} (14, 32). The coupling of DPA accumulation and core dehydration with generation of active GPR ensures that minimal if any SASP degradation takes place in sporulation, maximizing the levels of these proteins, in particular the α/β-type SASPs which are essential for full spore DNA resistance and long-term spore survival (15, 40). This role of DPA and core dehydration in regulation of P_{46} processing is certainly consistent with the results presented here, since very little P_{46} is processed to P_{41} in spores of the Δ*ger3 spoVF* strain, and this processing is largely restored if these spores are prepared with DPA.

One result which seems to be at odds with the significantly reduced P_{41} generated in Δ*ger3 spoVF* spores is the degradation of the SASP-γ that is accumulated midway in sporulation. Previous work has shown that SASP are normally not degraded during sporulation (38), although this will take place, primarily with SASP-γ, if P_{41} is activated too early or in too high amounts or under conditions of too little core dehydration (12, 27, 32). Although very little P_{41} appears to be present in Δ*ger3 spoVF* spores, there could easily be ~5% of wild-type spore levels, and this would be more than enough to catalyze significant SASP-γ breakdown until sufficient core dehydration precludes further enzyme action. Alternatively, the degradation of SASP-γ in Δ*ger3 spoVF* spores might be catalyzed by proteases other than GPR, which slowly act on SASP-γ in the more hydrated core of these spores. One other possibility that deserves mention is that SASP-γ degradation may actually continue in the mature dormant spore. It is thought that enzyme action in the spore core is precluded by the low level of water in this region of the spore (39). However, the increased hydration of the Δ*ger3 spoVF* spore core may allow some low level of enzyme action. The fact that SASP-γ levels fall only somewhat slowly upon extended incubation of sporulating cells is suggestive of this possibility, but further detailed work on this and other enzyme-substrate pairs (39) in the core of DPA-less spores is needed.

As noted above, the increased hydration and the decreased mineralization of the core of Δ*ger3 spoVF* spores is consistent with their decreased resistance to wet heat (11). The decreased resistance of Δ*ger3 spoVF* spores to formaldehyde was also expected, since this agent kills spores by causing DNA damage in the spore core (17), and the rate of accumulation of this damage would be expected to be more rapid in a more hydrated spore core. Similarly, there are previous data indicating that within a species increasing core hydration is correlated with decreasing spore resistance to hydrogen peroxide (28), and this is consistent with the decreased hydrogen peroxide resistance of Δ*ger3 spoVF* spores. However, the precise target for hydrogen peroxide in spores is not known. It is also possible that the decreased mineralization of DPA-less spores plays some role in their decreased resistance to formaldehyde and hydrogen peroxide, but there are no data available on this point.

The normal resistance of Δ*ger3 spoVF* spores to dry heat and

dessication was also not unexpected, since these resistance properties are independent of core water content in *B. subtilis* spores and depend largely on the presence of α/β-type SASP (9, 35), and levels of these DNA protective proteins are normal in Δ*ger3 spoVF* spores. The presence of normal levels of α/β-type SASP in Δ*ger3 spoVF* spores also explains the UV resistance of these spores, since α/β-type SASPs are the major determinant of spore UV resistance (38, 40). The specific level of core dehydration plays very little if any role in spore resistance to UV radiation at 254 nm as shown previously (28), while DPA actually decreases spore UV resistance by acting as a photosensitizer (33); this latter point explains the increased UV resistance of Δ*ger3 spoVF* spores compared to that of Δ*ger3* spores.

All of the agents discussed above had identical efficiencies in killing wild-type and Δ*ger3* spores. In contrast, glutaraldehyde and the iodine-based disinfectant, Betadine, were much more effective in killing Δ*ger3* spores than wild-type spores. Both of these agents have been shown to kill spores in part by damaging the spore germination apparatus (3, 30, 41). The increased sensitivity of the Δ*ger3* spores to these agents may thus be due to the fact that CaDPA-triggered spore germination requires at least one protein which is in the spore's exterior layers (24) and thus is extremely sensitive to exogenous chemical agents (3, 24, 41). In contrast, wild-type spores appear to have at least one other pathway for triggering spore germination that does not require this sensitive protein (24). In support of this reasoning, the presence of DPA and various levels of core dehydration had no effect on spore resistance to glutaraldehyde, which is thought to block a very early step in spore germination. However, spore Betadine resistance was increased by DPA and increased core dehydration, suggesting that Betadine may also kill spores by inactivating some more interior protein(s).

While the analysis of the properties of the Δ*ger3 spoVF* spores has given us some insight into the role of DPA and core hydration in various aspects of spore resistance and biochemistry, the isolation of moderately stable spores of the Δ*ger3 spoVF* strain of *B. subtilis* also may prove useful in opening up other avenues of research. For example, DPA-less heat-sensitive spores of *B. cereus* have been used as a parent to isolate DPA-less heat-resistant spores (12). However, because of the relative paucity of genetics and techniques for genetic manipulation in *B. cereus*, the nature of the second mutation or mutations restoring heat resistance to these spores is not known. However, given the ease of genetic manipulation with *B. subtilis*, if the Δ*ger3 spoVF* strain can generate DPA-less but now heat-resistant spores, the analysis of the mutation giving this new phenotype should be straightforward and may give us much new insight into the mechanism of spore resistance to wet heat. This work is currently in progress.

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