Role of Dipicolinic Acid in Resistance and Stability of Spores of *Bacillus subtilis* with or without DNA-Protective α/β -Type Small Acid-Soluble Proteins

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Dipicolinic acid (DPA) comprises ~10% of the dry weight of spores of *Bacillus* species. Although DPA has long been implicated in spore resistance to wet heat and spore stability, definitive evidence on the role of this abundant molecule in spore properties has generally been lacking. *Bacillus subtilis* strain FB122 (*sleB spoVF*) produced very stable spores that lacked DPA, and sporulation of this strain with DPA yielded spores with nearly normal DPA levels. DPA-replete and DPA-less FB122 spores had similar levels of the DNA protective α/β -type small acid-soluble proteins (SASP), but the DPA-less spores lacked SASP- γ . The DPA-less FB122 spores exhibited similar UV resistance to the DPA-replete spores but had lower resistance to wet heat, dry heat, hydrogen peroxide, and desiccation. Neither wet heat nor hydrogen peroxide killed the DPA-less spores by DNA damage, but desiccation did. The inability to synthesize both DPA and most α/β -type SASP in strain PS3664 (*sspA sspB sleB spoVF*) resulted in spores that lost viability during sporulation, at least in part due to DNA damage. DPA-less PS3664 spores were more sensitive to wet heat than either DPA-less FB122 spores or DPA-replete PS3664 spores, and the latter also retained viability during sporulation. These and previous results indicate that, in addition to α/β -type SASP, DPA also is extremely important in spore resistance and stability and, further, that DPA has some specific role(s) in protecting spore DNA from damage. Specific roles for DPA in protecting spore DNA against damage may well have been a major driving force for the spore's accumulation of the high levels of this small molecule.

Spores of *Bacillus* species are dormant and extremely resistant to many environmental stresses including, heat, desiccation, radiation, and a variety of toxic chemicals (9, 17, 33). As a consequence, spores can survive for extremely long periods, certainly hundreds of years and perhaps much longer (5, 15, 37). Spore resistance is due to a variety of factors, including the outer spore coats and the relative impermeability of the spore's inner membrane (6, 9, 17). There are also novel features of the spore's central region or core, the site of spore DNA, which play major roles in spore resistance. These include the relatively low content of core water and the saturation of spore DNA with a group of small acid-soluble proteins (SASP) of the α/β type (9, 11, 23, 31). The low core water protects spores against wet heat, while the α/β -type SASP protect DNA against damage due to wet and dry heat, desiccation, and genotoxic chemicals (6, 17, 31, 33). The binding of the α/β -type SASP also drastically alters the DNA's UV photochemistry, and this change is a major factor in the elevated resistance of dormant spores to UV light (17, 18).

Another novel feature of the spore core is the presence of high levels (~20% of core dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), that likely exists in the core as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (11). One role of DPA is to lower the core water content, since DPA loss early in germination is paralleled by its replacement

with water, and genetically DPA-less dormant spores have more core water than wild-type dormant spores (10, 21, 32). The role of DPA in spore properties has long been unclear. There are data indicating that increasing spore DPA levels are associated with increased spore wet heat resistance (11, 21). However, mutants have been isolated that produce heat-resistant but DPA-less spores (4, 12). Unfortunately, there has never been a thorough genetic analysis of these strains, and they may well have had multiple mutations.

DPA-less spores of *B. subtilis* can also be generated by mutation of the *spoVF* operon that encodes DPA synthetase (7, 11, 21). However, the DPA-less *spoVF* spores are unstable and lyse during sporulation (21, 36). In contrast, when sporulated with exogenous DPA, *spoVF* spores accumulate near wild-type DPA levels and are stable (21, 36). DPA-less *spoVF* spores can also be stabilized somewhat, albeit not completely, by deleting the genes encoding the three receptors that trigger spore germination in response to nutrient germinants (termed the *ger3* mutations) (21). DPA-less and DPA-replete *ger3 spoVF* spores have relatively similar resistance to UV radiation, but the DPA-less spores are more sensitive to wet heat and hydrogen peroxide (21).

Although the latter differences in resistance between spores with or without DPA are striking, it is not clear whether these differences are due only to the higher core water content of the DPA-less spores or to specific effects of DPA on spore resistance. Indeed, DPA can have striking effects on spore properties, in particular spore DNA properties (8, 26). In particular, UV irradiation of spores lacking DPA is much less efficient in generating the thymine-thymine adduct termed the spore pho-

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TABLE 1. *B. subtilis* strains used

Strain	Genotype	Phenotype ^a	Construction ^b and/or source or reference
AD28	$\Delta cotE::cam$	Cot ⁻	A. Driks (41)
FB122	$\Delta sleB::spc \Delta spoVF::tet$	DPA ⁻ Sp ^r Tc ^r	20
PS683	$\Delta sigG$	Asporogenous	35
PS832		Wild type	Laboratory stock
PS2318	$\Delta recA::cam$	Rep ⁻ Cm ^r	30
PS3664	$\Delta sleB::spc \Delta spoVF::tet \Delta sspA \Delta sspB$	$\alpha^- \beta^-$ DPA ⁻ Sp ^r Tc ^r	8
PS3747	$\Delta cotE::cam \Delta sleB::spc \Delta spoVF::tet \Delta sspA \Delta sspB$	$\alpha^- \beta^-$ Cot ⁻ DPA ⁻ Cm ^r Sp ^r Tc ^r	AD28→PS3664
PS3754	$\Delta recA::cam \Delta sleB::spc \Delta spoVF::tet$	DPA ⁻ Rep ⁻ Cm ^r Sp ^r Tc ^r	PS2318→FB122
PS3755	$\Delta recA::cam \Delta sleB::spc \Delta spoVF::tet \Delta sspA \Delta sspB$	$\alpha^- \beta^-$ DPA ⁻ Rep ⁻ Cm ^r Sp ^r Tc ^r	PS2318→PS3664

^a $\alpha^- \beta^-$, spores lack SASP- α and - β and thus ~80% of the spore's pool of α/β -type SASP; Cm^r, resistance to chloramphenicol (3 mg/ml); Cot⁻, spores have a defective coat; DPA⁻, spores lack DPA; Rep⁻, lacks the capacity for much DNA repair; Sp^r, resistance to spectinomycin (100 μ g/ml); Tc^r, resistance to tetracycline (10 μ g/ml).

^b Strains to the right of the arrow were transformed to appropriate drug resistance with chromosomal DNA from the strain to the left of the arrow.

toproduct in DNA than is UV irradiation of spores containing normal DPA levels (8). This difference is even larger in spores with or without DPA that also lack α/β -type SASP (8). These latter studies were carried out with spores of a strain lacking both *spoVF* and *sleB*. The latter gene encodes one of the two redundant enzymes responsible for the hydrolysis of the spore's peptidoglycan cortex in the first min of germination (32). The second cortex-lytic enzyme, CwlJ, is present in *sleB spoVF* spores. However, CwlJ requires DPA for its activation (20). Consequently, DPA-less *sleB spoVF* spores do not germinate either spontaneously or with nutrients and are much more stable than *ger3 spoVF* spores (20, 21). However, DPA-less *sleB spoVF* spores can be germinated either by exogenous Ca²⁺-DPA or by lysozyme treatment of decoated spores in a hypertonic medium (20, 21).

Given the extreme stability of the *sleB spoVF* spores that lack DPA, we have reexamined the role of DPA in the resistance of these spores. We have also examined the survival and resistance of spores that lack both DPA and α/β -type SASP. We report here the results of these studies.

MATERIALS AND METHODS

Strains used. All strains used are isogenic with PS832, a prototrophic laboratory strain of *B. subtilis* 168 and are listed in Table 1. Strain PS3664 (8) lacks the *sspA* and *sspB* genes which encode SASP- α and - β that together constitute ~80% of the spore's pool of α/β -type SASP; consequently, PS3664 spores are termed $\alpha^- \beta^-$. Strains PS3664 and FB122 also lack the *spoVF* operon that encodes DPA synthetase, and the DPA-less spores produced by these strains are stabilized by the loss of the *sleB* gene that encodes one of the spore's cortex lytic enzymes (8, 20). Strains made in current study were constructed by transformation to appropriate drug resistance with chromosomal DNA (1). Transformants were screened to ensure that they retained all antibiotic markers and, in the case of strains PS3747 and PS3755, that the spores of these strains lacked SASP- α and - β .

Spore formation. Spores of FB122 were routinely prepared by growth at 37°C on 2xSG medium agar plates (19) without antibiotics for 72 h. The plates were then incubated at 23°C for 3 to 4 days, and spores were harvested, cleaned over 10 to 14 days at 4°C, and stored as described previously (19). In some experiments strain FB122 was sporulated on 2xSG medium plates containing 200 μ g of DPA/ml. FB122 spores made without added DPA had <2% of the DPA of wild-type spore DPA levels, whereas spores made with DPA had ~85% of wild-type spore DNA levels, as found previously (20, 21). Spores of strain FB112 used in this study were free (>98%) of growing or sporulating cells, germinated spores, or cell debris as observed in a phase-contrast microscope. In one experiment FB122 spores were prepared at 37°C in liquid 2xSG medium (19) without DPA.

Spores of strain PS3664 were initially prepared on plates with or without DPA as described above. However, while spores of this strain made with DPA were

fully viable after cleaning, the DPA-less spores were not (see Results). Consequently, in further work this strain was grown at 37°C in liquid 2xSG medium (19) with or without DPA. At various times in sporulation 1-ml aliquots were harvested by centrifugation in a microcentrifuge and treated with decoating solution at an optical density at 600 nm (OD₆₀₀) of ~5 (see below). The decoated preparation was washed, suspended in hypertonic medium at an OD₆₀₀ of 1, and germinated with lysozyme, and viable counts were determined as described below. In other experiments, washed decoated preparations of sporulating cells of various strains were subjected to wet heat treatment at an OD₆₀₀ of 2, and viable counts were determined as described below.

Measurement of spore resistance. Spores of strain FB122 or PS3664 with or without DPA were decoated and washed (3) prior to measurement of resistance to heat and oxidizing agents to allow spore germination by lysozyme treatment in a hypertonic medium. This method was chosen for spore germination rather than germination with Ca²⁺-DPA, since CwlJ, the enzyme activated by Ca²⁺-DPA, is relatively sensitive to treatments such as wet heat and oxidizing agents, more sensitive than is spore viability (2, 39, 40). The decoating procedure was as described previously (3) but at 45°C for 60 min. The spores at an OD₆₀₀ of 1 to 20 in hypertonic medium were germinated with lysozyme (22), diluted serially in hypertonic medium, and aliquots were spotted onto LB medium plates (21) containing an appropriate antibiotic, generally spectinomycin at 100 μ g/ml. The plates were incubated for ~36 h at 37°C, and colonies were counted, although generally >90% of colonies appeared in 24 h. Decoating has no significant effect on *B. subtilis* spore resistance to wet or dry heat or UV radiation but does decrease spore resistance to hydrogen peroxide slightly (17, 18, 24).

For wet heat resistance, decoated spores at an OD₆₀₀ of 2 were incubated in water at various temperatures. At various times samples (0.5 ml) were diluted with 0.5 ml of cold water, centrifuged, and suspended in 1 ml of hypertonic medium. For UV resistance, decoated spores at an OD₆₀₀ of 1 in water were irradiated in small petri dishes rotating beneath a UVS-11 lamp (UVP, Inc., San Gabriel, CA) with a maximum output at 254 nm. At various times aliquots (1 ml) were centrifuged and suspended in 1 ml of hypertonic medium. For hydrogen peroxide resistance, spores at an OD₆₀₀ of 1 were incubated at 23°C in 25 mM KPO₄ buffer (pH 7.4) with 1.5% H₂O₂. At various times samples (1 ml) were centrifuged (1 min) and rapidly suspended in 1 ml of hypertonic medium containing catalase (40 μ g/ml) as described previously (21, 27). For dry heat resistance, decoated spores (200 μ l of a suspension with an OD₆₀₀ of 10) were lyophilized in glass tubes and exposed to dry heat in an oil bath. After heating, the tubes were cooled on ice, 1 ml of hypertonic medium added, and the suspension sonicated in a bath sonicator to disperse the spores, followed by lysozyme treatment.

For desiccation resistance, 2 ml of intact spores at an OD₆₀₀ of 10 were pelleted in a 2-ml plastic centrifuge tube and freeze-dried overnight. The dried spores were hydrated in 2 ml of water in a cold room at 4°C overnight, and the OD₆₀₀ of the suspension measured to assess spore recovery. An aliquot of the spores was diluted 1/10 in 50 mM Ca²⁺-DPA at pH 8.3 and incubated for 2 h at 23°C (20), and viability was assessed by plating as described above. The centrifugation, freeze-drying, and hydration steps were then repeated.

Analytical procedures. Extraction of SASP from spores and their analysis by polyacrylamide gel electrophoresis at low pH was as described previously (21). Spore core water content was determined on decoated spores by isopycnic density gradient centrifugation as described previously (11). The level of muta-

tions in spores of different strains with or without various treatments was determined by transferring colonies of either untreated or treated spore preparations obtained from LB medium plates to either 2xSG medium plates or Spizizen minimal medium plates without Casamino Acids as described previously (10, 34). A lack of growth on the minimal medium plates indicates an auxotrophic mutation, while the colonies on 2xSG medium plates were examined for sporulation.

RESULTS

Characterization of DPA-less *sleB spoVF* spores. Previous work on DPA-less spores (i.e., *spoVF* spores) of *Bacillus* species has shown that such spores lyse readily and are thus extremely difficult if not impossible to work with (21, 36). Deletion of the genes encoding the three functional nutrient germinant receptors (termed the *ger3* mutations) stabilizes *spoVF* spores to some degree, such that they can be isolated and characterized (21). However, even these *ger3 spoVF* spores germinate spontaneously much more readily than wild-type spores (21). In contrast to the *ger3* mutations, a *sleB* mutation stabilizes *spoVF* spores completely, since *sleB spoVF* spores exhibit no spontaneous germination (20; data not shown). Indeed, these spores even germinate extremely poorly with nutrients, because the remaining spore cortex-lytic enzyme, CwlJ, requires DPA for its activation (20). However, DPA-less *sleB spoVF* spores do germinate with exogenous Ca^{2+} -DPA, and decoated *sleB spoVF* spores can also be germinated by lysozyme treatment in a hypertonic medium (20).

Previous work found that DPA-less *ger3 spoVF* spores had very low levels of SASP- γ compared to SASP- γ levels in wild-type or DPA-replete *ger3 spoVF* spores (21). SASP- γ was also absent from DPA-less *sleB spoVF* spores (Fig. 1). In contrast, the levels of the DNA binding SASP- α and - β were similar in DPA-less and DPA-replete *sleB spoVF* spores (Fig. 1), as found previously with *ger3 spoVF* spores (21). The DPA-less *sleB spoVF* spores also had ~20% higher levels of core water than did DPA-replete spores (data not shown), similar to results with *ger3 spoVF* spores (21).

Resistance of *sleB spoVF* spores. The higher level of core water in DPA-less *sleB spoVF* spores made it likely that these spores would be more sensitive to wet heat than their DPA-replete counterparts (11, 16), and this was the case (Fig. 2A). The DPA-less spores were also more sensitive to hydrogen peroxide and dry heat than were the DPA-replete spores (Fig. 2C and D). Since the levels of SASP- α and - β were similar in spores with or without DPA, it seemed likely that these spores would have similar UV resistance (17, 18, 31), and this was again the case (Fig. 2B). While DPA-less *sleB spoVF* spores were more sensitive to wet heat and hydrogen peroxide than their DPA-replete brethren, the DPA-less spores were not killed by DNA damage. Analysis of auxotrophic and asporogenous mutations in survivors of DPA-less *sleB spoVF* spores killed ~95% by wet heat or hydrogen peroxide revealed no significant increase in mutation frequency (Table 2). In contrast, 15 to 20% of the survivors of $\alpha^- \beta^-$ spores treated with hydrogen peroxide or wet heat, treatments that kill these spores via DNA damage, have auxotrophic or asporogenous mutations (10, 27). Dry heat is also known to kill even wild-type spores by DNA damage (28).

The greater sensitivity of the DPA-less *sleB spoVF* spores to dry heat suggested that these spores might also be sensitive to desiccation alone. Indeed, while DPA-replete *sleB spoVF*

FIG. 1. Levels of SASP in *sleB spoVF* spores prepared with or without DPA. SASP were extracted from spores of strain FB122 (*sleB spoVF*) prepared at 37°C on plates with or without DPA. Extracts were prepared and subjected to polyacrylamide gel electrophoresis at low pH, and the gel was stained with Coomassie blue as described in Materials and Methods. The samples run in the lanes are from 0.5 mg of dry spores and are presented as spores with DPA (lane 1) and spores without DPA (lane 2). The symbols α , β , and γ denote the migration positions of SASP- α , - β , and - γ , respectively.

spores were resistant to at least 10 cycles of freezing, desiccation, and rehydration, the DPA-less spores lost ~50% of their viability in each cycle (Fig. 3). However, these DPA-less spores were not sensitive to freezing and thawing (data not shown). Examination of the survivors of four freeze-dry cycles showed that the DPA-replete spores had acquired no mutations, whereas the survivors of the DPA-less spores had (Table 2 and data not shown). These results suggested that desiccation had killed DPA-less *sleB spoVF* spores by DNA damage. This was suggested further by the greater sensitivity of DPA-less *recA sleB spoVF* spores to desiccation, while DPA-replete *recA sleB spoVF* spores remained resistant (Fig. 3). Previous work found that a *recA* mutation alone does not sensitize *B. subtilis* spores to desiccation (30).

Loss of viability of DPA-less *sspA sspB sleB spoVF* spores. Another factor that is important in spore resistance and long-term survival is the α/β -type SASP (9, 17, 18, 31, 33). Consequently, it was of interest to analyze spores that lacked both DPA and α/β -type SASP. A strain, PS3664, that produces such spores has been generated and has been used to show that both DPA and α/β -type SASP play major roles in determining the UV photochemistry of spore DNA (8). However, initial attempts to measure the resistance properties of cleaned DPA-less PS3664 spores made on plates failed because these cleaned spores at an OD_{600} of 1 routinely gave $\leq 0.001\%$ of the CFU/ml given by the DPA-replete spores at the same concentration (Table 3). The low viability of the DPA-less PS3664 spores when the spores were plated directly on nutrient plates was not surprising, since these spores are expected to germi-

FIG. 2. Resistance of *sleB spoVF* spores prepared with or without DPA. Spores of strain FB122 (*sleB spoVF*) prepared on plates at 37°C with or without DPA were decoated (A, C, and D) or not (B) and treated with wet heat (70°C) at an OD₆₀₀ of 2 (A), UV radiation at an OD₆₀₀ of 1 (B), hydrogen peroxide at an OD₆₀₀ of 1 (C), or dry heat (200 µl at an OD₆₀₀ of 10) at 105°C (D). Spores were germinated with lysozyme in hypertonic medium (A, C, and D) or by Ca²⁺-DPA treatment (B), and viability was determined as described in Materials and Methods. Symbols: ○, spores with DPA; ●, spores without DPA.

nate very poorly with nutrients, as noted above (20). However, these DPA-less spores were also not viable when either germinated with Ca²⁺-DPA or first decoated and then germinated with lysozyme in a hypertonic medium (Table 3). In contrast, DPA-replete PS3664 spores at an OD₆₀₀ of 1 gave ~10⁷ CFU/ml when decoated and germinated with lysozyme (Table 3). This latter value is similar to that obtained in the present study and previously with spores of several *B. subtilis* strains germinated by this procedure (Table 3) (22, 25). Purified spores of strain PS3747 (*cotE sspA sspB sleB spoVF*) prepared without DPA were also not viable when germinated with lysozyme in a hypertonic medium (Table 3). Spores of *cotE* strains are germinated by lysozyme without prior decoating since, due to the lack of the morphogenic CotE protein, their defective coat is permeable to lysozyme (9). Thus, the lack of recovery of viable spores of DPA-less PS3664 spores was not due to their destruction by the decoating regimen used. De-

TABLE 2. Level of mutations in DPA-less *sleB spoVF* spores exposed to various treatments

Treatment	Level of killing (%)	No. of colonies tested	No. of mutations ^a			% ^b
			aux	spo	aux and spo	
70°C, 30 min	85	416	0	1	0	0.2
H ₂ O ₂ , 20 min	94	416	1	0	0	0.2
Four freeze-dry cycles	95	432	8	20	1	6.7 (<0.2)
None	0	355	0	0	0	0

^a Spores of strain FB122 prepared on plates without DPA were treated in various ways, and survivors were tested for mutations to auxotrophy (aux) or sporogony (spo) as described in Materials and Methods. One colony surviving freeze-drying was both auxotrophic and sporogenous (aux and spo). Spores treated with wet heat at an OD₆₀₀ of 2 and with hydrogen peroxide at an OD₆₀₀ of 1 were decoated prior to treatment and recovered by lysozyme germination in hypertonic medium. Samples subjected to no treatment or to freeze-drying were not decoated and were recovered by Ca²⁺-DPA treatment.

^b This value is the percentage of mutants in the survivors and was calculated as: 100 × (the number of colonies with mutations/the total number of colonies tested). The value in parentheses is the percentage of mutations in DPA-replete FB122 spores given four cycles of freeze-drying.

FIG. 3. Effect of freezing, desiccation, and rehydration on spores prepared with or without DPA. Spores of strains FB122 (*sleB spoVF*) (circles) or PS3754 (*recA sleB spoVF*) (2 ml at an OD_{600} of 10) prepared at 37°C with (open symbols) or without (solid symbols) DPA were subjected to freeze drying cycles as described in Materials and Methods. Spores were germinated by Ca^{2+} -DPA treatment, and viability was determined as described in Materials and Methods. This procedure gave 2×10^7 to 8×10^7 CFU of spores/ml at an OD_{600} of 1 at time zero for the four types of spores.

spite the extremely low viability of the DPA-less PS3664 spores, the spores were bright and looked normal in the phase-contrast microscope (data not shown).

Although the cleaned DPA-less PS3664 spores were not viable, it was possible that these spores were simply extremely labile and died during the ~72 h of sporulation on plates at 37°C, the several days of incubation of the plates at 23°C, and the ~10 days of incubation at 4°C after the spores were harvested and were being cleaned. Spores of strain PS3664 harvested after 36 h of sporulation at 37°C in liquid medium without DPA and then cleaned over a period of ~10 days were also not viable (Table 3 [values in parentheses]). However, we did recover viable spores early in the sporulation of such cultures by decoating, followed by lysozyme treatment in hypertonic medium (Fig. 4). In contrast, no viable forms ($\leq 10^3$ CFU/ml at an OD_{600} of 1; data not shown) were recovered in this manner from cultures of strain PS683, an asporogenous *B. subtilis* strain blocked late in sporulation (data not shown). The maximum viable count recovered from a culture of strain

FIG. 4. Viability of DPA-less PS3664 spores during sporulation. Strain PS3664 (*sspA sspB sleB spoVF*) was sporulated at 37°C in liquid without DPA, at various times samples (2 ml) were harvested, decoated, and germinated with lysozyme at an OD_{600} of 1 in hypertonic medium, a process that took ≤ 3 h, and viability was determined as described in Materials and Methods. Cultures were also examined in the phase-contrast microscope. The culture was inoculated ~10 h prior to time zero, and arrows a and b denote the times at which surviving spores were tested for mutations. The number of phase bright spores in the culture did not decrease ($\leq 20\%$) after 75 h as determined by phase-contrast microscopy.

PS3664 sporulated without DPA (1.4×10^6 CFU/ml at an OD_{600} of 1; Fig. 4) was only ~4-fold lower than for this strain sporulated with DPA recovered similarly (5×10^6 CFU/ml at an OD_{600} of 1; data not shown). However, the DPA-replete PS3664 spores maintained their viability throughout sporulation, harvesting, and cleaning (Table 3), whereas the DPA-less spores lost viability continually as sporulation proceeded (Fig. 4). In contrast to strain PS3664 sporulated without DPA, strain FB122 sporulated without DPA did give fully viable spores after harvesting and cleaning (Table 3).

Analysis of mutations in the PS3664 spores prepared in liquid medium with or without DPA showed that <1% of the DPA-replete spores had auxotrophic or asporogenous mutations (Table 4). However, of the colonies from DPA-less PS3664 spores harvested at several times after the point of

TABLE 3. Viability of spores of various strains prepared with or without DPA^a

Strain (genotype)	Sporulated with DPA	CFU/ml of spores at an OD_{600} of 1 as determined by various methods for the assessment of spore viability ^b		
		Nutrients	CaDPA	Decoat/lysozyme
FB122 (<i>sleB spoVF</i>)	Yes	10^8	10^8	10^7
FB122 (<i>sleB spoVF</i>)	No	10^4	10^7	9×10^6
PS3664 (<i>sspA sspB sleB spoVF</i>)	Yes	9×10^7	10^8	8×10^6
PS3664 (<i>sspA sspB sleB spoVF</i>)	No	$<10^3$	$<10^{-3}$ ($<10^{-3}$)	$<10^{-3}$ ($<10^{-3}$)
PS3747 (<i>cotE sspA sspB sleB spoVF</i>)	No	NT	$<10^{-3}$	$<10^{-3}$

^a Spores of strains FB122 and PS3664 were prepared on plates at 37°C with or without DPA and were cleaned as described in Materials and Methods. The viability of the cleaned spores was measured by plating on LB plates alone (Nutrients), on LB plates after pretreatment with Ca^{2+} -DPA (CaDPA), or on LB plates after decoating and germinating with lysozyme in hypertonic medium (Decoat/lysozyme).

^b NT, not tested. Values in parentheses are for spores prepared in liquid medium at 37°C and then cleaned as described in Materials and Methods.

TABLE 4. Levels of mutations in spores of strain PS3664 prepared with or without DPA^a

Time (h) in sporulation (+/-DPA) ^b	No. of colonies tested	Mutation(s)			% ^c
		aux	spo	aux and spo	
35 (+DPA)	416	1	0	0	<0.2
8 (-DPA)	290	10	19	8	13
25 (-DPA)	237	18	49	11	33

^a Samples (2 ml) were harvested from cultures of strain PS3664 (*sspA sspB sleB spoVF*) sporulating at 37°C in liquid with or without DPA. The spores were deoated, germinated with lysozyme at an OD₆₀₀ of 1, and plated on LB medium plates as described in Materials and Methods. Colonies on LB medium plates were picked onto appropriate plates for identification of auxotrophic (aux) or asporogenous (spo) mutants, including colonies that had acquired both auxotrophic and asporogenous mutations (aux and spo).

^b For the culture sporulated without DPA, the sporulation times are as denoted by the arrows in Fig. 4. For the culture sporulated with DPA, the time is equivalent to hour 35 in Fig. 4.

^c That is, the percentage of mutants in survivors, was calculated as $100 \times (\text{the number of colonies with mutations} / \text{the total number of colonies tested})$.

maximum spore viability, 12 to 34% had obvious mutations (Table 4). This finding suggested that DPA-less PS3664 spores were dying because of DNA damage. Consistent with this suggestion, when sporulated without DPA, the *recA* derivative of strain PS3664 (strain PS3755) lost viability more rapidly than did strain PS3664 (Fig. 5).

Wet heat resistance of DPA-less PS3664 spores. Given the relatively rapid loss in viability of PS3664 spores during sporulation without DPA, it was reasonable to assume that these spores would have much lower resistance than DPA-replete PS3664 spores or DPA-less spores that contained α/β -type SASP. Indeed, when sporulating cultures were harvested

FIG. 5. Effect of a *recA* mutation on the viability of DPA-less α - β - spores during sporulation. Strains PS3664 (*sspA sspB sleB spoVF*) and PS3755 (*recA sspA sspB sleB spoVF*) were sporulated at 37°C in liquid 2xSG medium without DPA. At various times samples were harvested and deoated, spores were germinated at an OD₆₀₀ of 1 with lysozyme in hypertonic medium, and the viability was determined as described in Materials and Methods. Cultures were inoculated ~10 h prior to time zero, and the times of development in the two cultures were aligned by examination with a phase-contrast microscope. Symbols: ○, strain PS3664; ●, strain PS3755.

FIG. 6. Resistance of spores of various strains to wet heat. Strains FB122 (*sleB spoVF*) and PS3664 (*sspA sspB sleB spoVF*) were sporulated with or without DPA, and samples were harvested after ~80% of spores had been released from the sporangium, treated with deoating solution, washed, treated with wet heat at 65°C, and germinated with lysozyme in hypertonic medium. The spore viability was determined as described in Materials and Methods. Symbols: ■, PS3664 sporulated with DPA; □, PS3664 sporulated without DPA; ○, FB122 sporulated without DPA.

shortly after the achievement of maximum spore viability, DPA-less PS3664 spores died rapidly upon incubation at 65°C, a temperature to which the DPA-replete PS3664 spores were relatively resistant (Fig. 6). DPA-less FB122 spores were also much more resistant to this temperature than were the DPA-less PS3664 spores (Fig. 6; see also Fig. 2). Given the significant DNA damage in DPA-less PS3664 spores even just after their release from the sporangium, we carried out no further studies of the resistance of these DPA-less spores.

DISCUSSION

Some of the results presented here with DPA-less spores of strain FB122 (*sleB spoVF*) confirm results obtained with DPA-less spores of a *ger3 spoVF* strain (21). These confirmatory results with DPA-less FB122 spores include (i) the absence of SASP- γ , (ii) the presence of normal levels of SASP- α and - β , and (iii) their increased sensitivity to wet heat and hydrogen peroxide compared to that of their DPA-replete brethren. It was suggested previously (21) that the increased sensitivity to wet heat and hydrogen peroxide of DPA-less *ger3 spoVF* spores is due to the increased water content of these DPA-less spores, since increased core water content increases spore sensitivity to both wet heat and hydrogen peroxide (11, 23). The increased core water content of DPA-less FB122 spores is certainly consistent with this suggestion. The increased core water content is also the likely reason for the absence of SASP- γ from DPA-less FB122 spores. Presumably, the SASP-specific germination protease (GPR) that becomes activated late in forespore development is able to digest SASP- γ in the devel-

oping DPA-less forespore due to these forespore's higher levels of hydration (14, 21, 28). However, the binding of SASP- α and - β to DNA protects these proteins against GPR digestion (29, 31). In contrast, SASP- γ does not bind to spore DNA and is thus not well protected against GPR digestion (31).

Previous work showed that DPA-less *ger3 spoVF* spores were slightly more UV resistant than were DPA-replete spores, but DPA-less *sleB spoVF* spores exhibited similar UV resistance to the comparable DPA-replete spores. The retention of spore UV resistance in the DPA-less spores is not surprising, since these spores retain their α/β -type SASP, the major factor causing spore UV resistance (17, 18, 31, 33). Previous work (21) also found very little difference in the dry heat resistance of DPA-less and DPA-replete *ger3 spoVF* spores, while we now observed significantly lower dry heat resistance of DPA-less *sleB spoVF* spores. We do not know the reason for the differences between previous and current findings. Possible contributing factors may be that (i) our current measurements were more extensive than those made previously and (ii) the DPA-less *sleB spoVF* spores are much more stable than the DPA-less *ger3 spoVF* spores.

Another new finding was that DPA-less *sleB spoVF* spores were sensitive to repeated desiccation, a treatment that was not investigated thoroughly with DPA-less *ger3 spoVF* spores (21). The high level of mutations in survivors of DPA-less *sleB spoVF* spores subjected to multiple freeze-drying cycles suggests that desiccation is killing these spores by DNA damage. This is consistent with the sensitization of these DPA-less spores to desiccation by a *recA* mutation that eliminates much DNA repair in *B. subtilis* (30, 38).

Certainly the most striking finding in the present study was that spores that lacked both DPA and α/β -type SASP rapidly lost viability during sporulation and storage. In contrast, spores that lacked only DPA or only α/β -type SASP are stable for weeks to years during sporulation and subsequent storage, as found previously and in current study (10, 20, 21; data not shown). $\alpha^- \beta^-$ spores stored in water at 12°C do lose viability on extended storage, since 90% viability is lost in ~2 years (10). However, DPA-less $\alpha^- \beta^-$ spores lost ~90% viability in 10 to 15 h at 37°C. The high level of mutations in DPA-less $\alpha^- \beta^-$ spores recovered early in sporulation suggests that these spores were dying largely because of DNA damage, and again this was consistent with the more rapid decline in viability of DPA-less *recA* $\alpha^- \beta^-$ spores. $\alpha^- \beta^-$ spores do accumulate DNA damage during sporulation, harvesting, and cleaning (10, 39, 40). However, the levels of obvious mutations in $\alpha^- \beta^-$ spores just after the spores are cleaned, a process that can take up to 2 weeks, are only ~1% of the population (39, 40), a value more than 10-fold lower than for DPA-less $\alpha^- \beta^-$ spores immediately after their release from the sporangium. The heightened sensitivity of DPA-less $\alpha^- \beta^-$ spores to wet heat is a further indication of the potential lability of DNA in DPA-less spores, since wet heat treatment kills $\alpha^- \beta^-$ spores by DNA damage (10, 28, 30).

Although DPA was identified as a major component of spores of *Bacillus* species over 50 years ago, the role of this compound in spores has been difficult to clarify. This has been in large part because of the instability of spores from strains whose only lesion is that they cannot make DPA. The results in this communication as well as previous work indicate that

stable DPA-less spores can be prepared from *B. subtilis* strains that have defined mutations in genes of known function (20, 21). In current and previous work the analysis of these spores has shown that DPA plays a number of roles in spores including the following. (i) DPA in the spore core displaces water such that DPA-replete spores have less core water than isogenic DPA-less spores (21). This is a major reason for the role of DPA in spore wet heat resistance, since there is an inverse correlation between core water content and spore wet heat resistance (11). (ii) DPA plays a major role in the UV photochemistry of spore DNA, affecting both the photoreactivity of spore DNA and the nature of the photoproducts produced (8, 26). Since at least some of the effects of DPA on spore DNA are most likely via energy transfer from an excited state of DPA to DNA (8), DPA must be in close proximity to spore DNA. (iii) The likely close proximity of DPA and DNA noted above is consistent with spore protection against dry heat damage by DPA, since dry heat kills spores by DNA damage (28). (iv) The loss in viability of DPA-less spores upon desiccation, with killing of these DPA-less spores due in large part to DNA damage, is further evidence that DPA plays a major role in stabilizing spore DNA. (v) Finally, the extreme lability of DPA-less $\alpha^- \beta^-$ spores indicates again that DPA is an important factor in protecting spore DNA from damage, since in $\alpha^- \beta^-$ spores the strong protective effect of α/β -type SASP on DNA is lost.

DPA in spores thus has two main general functions. One is to lower the content of core water by replacing some core water with DPA. This elevates spore wet heat resistance by protecting core proteins from inactivation or denaturation by wet heat. The second function of DPA in spores is to contribute to the protection of spore DNA against many types of damage. In wild-type spores, the protection of DNA against wet heat by DPA may not be especially important, since this is provided by α/β -type SASP (10). However, when α/β -type SASP are absent, DNA protection by DPA becomes important even in hydrated spores. In dry spores, DNA is not completely protected against heat damage by the α/β -type SASP (28). Consequently, DPA protects the DNA in dry spores from heat damage and also against desiccation. The mechanism whereby DPA protects DNA against dry heat and desiccation is not known. However, the DNA damage caused by dry heat and desiccation treatment of spores is different than that caused by wet heat or spontaneous mutations (13).

Since core water content is significantly higher in DPA-less spores, this raises the possibility of there being a low level of aerobic metabolic activity in such spores, and aerobic metabolism can generate compounds such as superoxide and hydrogen peroxide that directly or indirectly can cause DNA damage. In otherwise wild-type spores, the α/β -type SASP would protect DNA against such oxidative damage (27), but in the absence of these protective proteins such DNA damage could lead to spore death. Currently available evidence suggests that metabolism in dormant spores, even DPA-less spores, is minimal, if it takes place at all (25). However, a very low level of metabolism in DPA-less spores would be extremely difficult to detect.

Spores of most *Bacillus* species are soil organisms and thus are likely to spend a large amount of time in the dry state. Consequently, the protection of spore DNA against damage

due to dry heat and desiccation may be extremely important in spore survival in the environment. The ability of DPA to contribute significantly to spore DNA protection may thus have been a major driving force in the evolution of the capacity for DPA synthesis during sporulation and the accumulation of high DPA levels in spores.

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