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Dipicolinic Acid Greatly Enhances Production of Spore Photoproduct in Bacterial Spores upon UV Irradiation

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Formation of the spore photoproduct (SP) (5-thyminyl-5,6-dihydrothymine) in DNA of dormant spores of *Bacillus subtilis* upon UV irradiation is due to binding of α/β-type small, acid-soluble proteins (SASP). However, the yield of SP as a function of UV fluence is ~15-fold higher in spores than in an α/β-type-SASP–DNA complex in vitro. The yield of SP as a function of UV fluence in forespore DNA from mutants which make α/β-type SASP but not dipicolinic acid (DPA) was 10 to 20 times lower than that in dormant spores. Furthermore, the yield of SP as a function of UV fluence in an α/β-type-SASP–DNA complex in vitro was increased sixfold by DPA. These data provide further support for the idea that the high DPA level in dormant spores increases the yield of SP as a function of UV fluence and thereby sensitizes spores to UV.

Dormant spores of *Bacillus* species are 10 to 50 times more resistant to UV radiation than are the corresponding vegetative cells; this difference in UV resistance is due to a difference in DNA photochemistry (14). Although the major lesion formed in vegetative-cell DNA upon UV irradiation is a cyclobutane-type thymine-thymine dimer (TT), in spores TT is not formed. The most abundant UV photoproduct from DNA in dormant spores of various *Bacillus* species is a 5-thyminyl-5,6-dihydrothymine adduct, which has been termed spore photoproduct (SP). Although the yield of SP in spore DNA as a function of UV fluence is similar to that for TT formation in vegetative cells, SP is repaired extremely efficiently in the early minutes of spore germination, in large part by an SP-specific repair process which converts SP back into two thymine residues (14). The major reason for SP rather than TT formation upon UV irradiation of spores is the binding of α/β-type small, acid-soluble proteins (SASP) to the spore's DNA (14–16). There are multiple α/β-type SASP in spores of all *Bacillus* species that have been analyzed, and the sequences of these proteins have been highly conserved both within and across species (16). α/β-type SASP are relatively nonspecific DNA-binding proteins which convert DNA from a B-like conformation to an A-like conformation, and there is sufficient α/β-type SASP in spores to saturate the DNA (16). However, these proteins are rapidly degraded in the first minutes of spore germination, when spore DNA's UV photochemistry reverts back to that of a vegetative cell (14).

Recent work has shown that UV irradiation of α/β-type-SASP–DNA complexes in vitro produces SP and not TT (11). However, the yield of SP as a function of UV fluence in these in vitro experiments is ~15-fold lower than that found in dormant spores. The reason for this difference was suggested to be the high levels (up to 10% [dry weight]) of dipicolinic acid (DPA) present in the spore core, which is also the site of spore DNA. DPA, which has significant absorption in UV, is accumulated by developing forespores only after α/β-type SASP are synthesized and is excreted in the first minutes of spore germination. A significant role for DPA in spore DNA photochemistry was further suggested by the low yield of SP as a function of UV fluence in germinated spores of a *Bacillus subtilis gpr* mutant in which degradation of α/β-type SASP during spore germination is greatly slowed, although DPA excretion is rapid (12; also see below).

While the forespore-specific accumulation of α/β-type SASP takes place during sporulation in parallel with the acquisition of UV resistance, which in turn parallels the appearance of SP (presumably only in forespore DNA) upon UV irradiation of sporulating cells, DPA is accumulated well after synthesis of α/β-type SASP (14). Consequently, if DPA is responsible for the high SP yield in spores as a function of UV fluence, then this value should be much lower in forespores which have accumulated α/β-type SASP but not DPA. In order to test this prediction, we measured SP production as a function of UV fluence in sporulating cells of wild-type *B. subtilis*, as well as in two mutants with sporulation mutations (*spoVA* and *spoVF*) which allow α/β-type-SASP synthesis but not DPA accumulation. All sporulating cells were harvested at 6 to 7 h of sporulation (t_{6-7}), well after the normal time for α/β-type-SASP and DPA synthesis (9). As expected, cells of all three strains had similar levels of α/β-type SASP when measured as previously described (10; also data not shown). Although wild-type t_{6-7} sporulating cells had accumulated >90% of the dormant-spore level of DPA (measured as described previously [9]), the *spoVA* and *spoVF* mutant strains had <5 and 8% of the dormant-spore level of DPA, respectively (data not shown). The *spoVA* mutation does not block DPA synthesis but does block DPA uptake or retention in the forespore, whereas the *spoVF* mutation blocks the last enzymatic step in DPA synthesis but probably not the small amount of nonenzymatic reaction at this step (2). As expected, wild-type sporulating cells gave significant SP production as a function of UV fluence (Fig. 1). The amount of SP generated with fluences of 5 to 20 kJ/m^2 from wild-type sporulating cells was approximately one-half that generated in wild-type spores (Fig. 1). This is consistent with the sporulating cell containing two nucleoids (15), one in the mother cell which gives TT upon UV irradiation and one in the forespore which gives SP. In contrast to results with the wild-type strain, yields of SP in *spoVA* and *spoVF* sporulating cells as a function of UV fluence were much lower, although higher than those in

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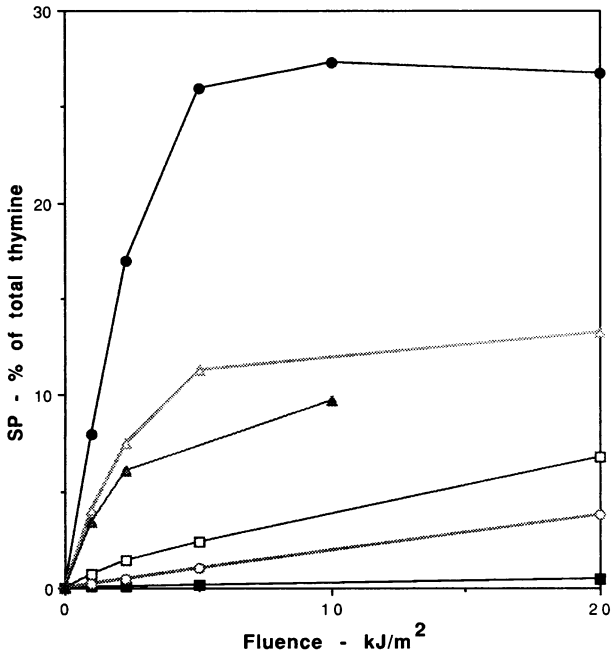

FIG. 1. Formation of SP in DNA of spores and cells as a function of UV fluence. The DNA of cells and spores was labeled with [*methyl*-^{3}H]thymidine and irradiated with UV mostly at 254 nm as described previously (13). Vegetative cells were prepared by growth in 2× YT medium at 37°C, and sporulation was in 2× SG medium (10, 11). Vegetative cells were harvested in late log phase (optical density at 600 nm = 1), and sporulating cells were harvested 6 to 7 h into sporulation (t_{6-7}). Cells were harvested and rinsed prior to irradiation, and spores were isolated and purified as described previously (10, 13). Irradiated cells or spores (1×10^5 to 2×10^5 cpm) were pelleted in a microcentrifuge, the samples were broken by dry rupture, and DNA was extracted and purified (13); DNA was hydrolyzed with 400 μl of 98% (vol/vol) formic acid for 2 h at 175°C. In most cases, portions of the hydrolysate (20,000 to 100,000 cpm) were analyzed directly by paper chromatography to resolve thymine, SP, and TT, which were then quantitated as described previously (11, 13). For extracts from sporulating cells, samples were reirradiated after hydrolysis with 10 kJ/m^2 to monomerize pyrimidine dimers and facilitate analysis of SP on chromatograms. Symbols: ●, dormant spores of strain PS832 (wild type); △, t_{6-7} sporulating cells of strain PS832 (wild type); ▲, dormant spores of strain PS361 ($\alpha^-\beta^-$); □, t_{6-7} sporulating cells of PS1767 (*spoVF*:: *cat7*, obtained from H. Paulus); ○, t_{6-7} sporulating cells of strain PS69 (*spoVA*); ■, vegetative cells of strain PS832 (wild type). While only the data for SP are presented, data on TT yield as a function of UV fluence were collected for strains PS832 (vegetative cells) and PS361.

vegetative cells (Fig. 1). Note that the data in Fig. 1 are presented without correction for the fact that SP formation in the sporulating cell takes place in only one of the cell's two nucleoids, i.e., the forespore nucleoid. When the data were corrected for this fact and then used to calculate SP yield as a function of UV fluence, the values for wild-type spores and sporulating cells were almost identical (Table 1). The value for SP yield as a function of UV fluence in spores lacking most α/β-type SASP ($\alpha^-\beta^-$ spores) was also similar to that in wild-type spores (Table 1) when the data for $\alpha^-\beta^-$ spores (Fig. 1) were corrected for the fact that SP is not formed in much of the DNA because of the low level of α/β-type SASP (13). In contrast, the value for sporulating cells of the *spoVA* mutant was ~20-fold lower than that for

TABLE 1. Yield of DNA photoproducts in vivo and in vitro as a function of UV fluence[a]

Sample tested[b]	Photoproduct formation (% of total thymine/kJ/m^2)	
	TT	SP
Wild-type vegetative cells	5	0.04
Wild-type dormant spores	—[c]	7.7
$\alpha^-\beta^-$ dormant spores	10[d]	7.4[e]
Wild-type sporulating cells (t_{6-7})	ND[f]	7.2[g]
spoVA sporulating cells (t_{6-7})	ND	0.4[g]
spoVF sporulating cells (t_{6-7})	ND	0.8[g]
gpr spores (germinated 40 min)	7[d]	0.6[e]
DNA	>4	0.3[h]
DNA-SspC complex	—[c]	0.5[i]
DNA-SspC-DPA complex	—[c]	3.0

[a] The data from analyses carried out as described in the legends to Fig. 1 and 2 were plotted, and the linear portions of the curves were used to calculate the yield of TT or SP as a function of UV fluence, except for the values for 40-min-germinated *gpr* spores, which were calculated from data in reference 12.

[b] All DNA and DNA complex samples were dry.

[c] —, too little TT formed to calculate a value.

[d] Value calculated only on the basis of the thymine in the DNA whose irradiation does form TT (12, 13). In $\alpha^-\beta^-$ dormant spores and germinated *gpr* spores, maximum TT formation was 60 and 40%, respectively, of that in vegetative cells or germinated wild-type spores.

[e] Value calculated only, on the basis of the thymine in the DNA whose irradiation does not form TT (12, 13). In $\alpha^-\beta^-$ dormant spores and germinated *gpr* spores, maximum TT formation was 60 and 40%, respectively, of that in vegetative cells or germinated wild-type spores.

[f] ND, not determined because TT was largely destroyed by reirradiation during the analysis.

[g] Values calculated only on the basis of thymine in the DNA in the forespore compartment, which was taken as 50% of the total thymine (15).

[h] The corresponding value for DNA in solution is ≤0.07 (11).

[i] The corresponding value for a DNA-SspC complex in solution is 0.4 (11).

wild-type spores, with the value for *spoVF* forespores, which had significant DPA, ~10-fold lower. However, for both *spo* mutants, the value was 10- to 20-fold higher than that found for vegetative cells (Table 1) or for cells early in sporulation prior to synthesis of α/β-type SASP (data not shown). The value for SP formation as a function of UV fluence in germinated *gpr* spores, which retain α/β-type SASP but have lost DPA, was similar to that for the sporulating cells of *spoVA* and *spoVF* mutants (Table 1). One experiment which would have been most informative to carry out at this stage was to produce *spoVF* forespores or spores with increased DPA levels by sporulation with exogenous DPA. This has been reported to give an increase in heat-resistant spores in *spoVF* strains, although generally to levels 1 to 2 log units less than in *spo*$^+$ strains; however, we obtained no significant increase in total *spoVF* forespore DPA by this procedure, as has also been found by others (2).

Although α/β-type SASP appear essential for the switch in forespore DNA's UV photoproduct from TT to SP, the good correlation between a high yield of SP as a function of UV fluence and the presence of DPA is certainly consistent with a casual relationship between these two phenomena. In order to demonstrate this directly, we examined the effects of DPA on SP yield as a function of UV fluence in α/β-type-SASP–DNA complexes in vitro. These experiments were initially carried out in solution, and we found that 1 mM DPA consistently gave a 1.5- to 2-fold stimulation in SP yield from an α/β-type-SASP–DNA complex (data not shown). However, we did not obtain further stimulation with more DPA, possibly because of significant absorption by the DPA; the

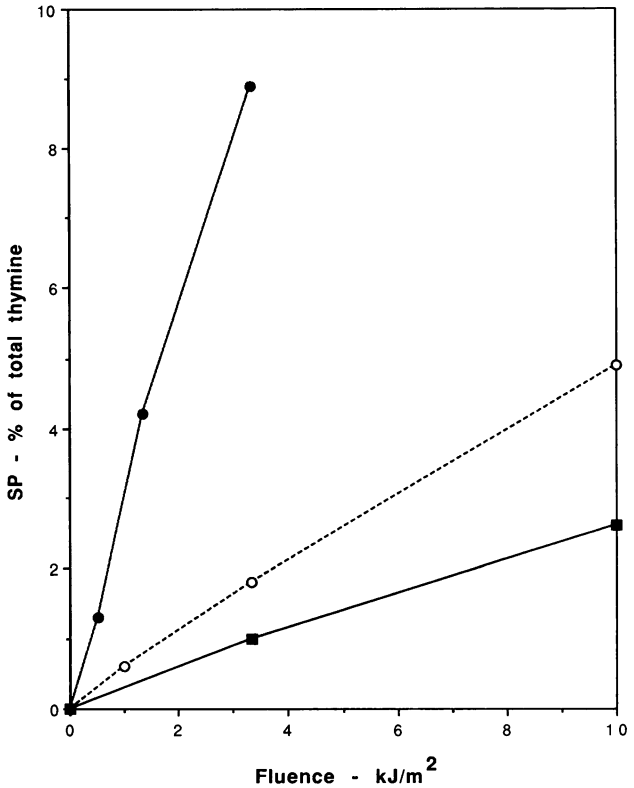

FIG. 2. Formation of SP in DNA with or without SspC and DPA. [*methyl*-^{3}H]thymidine-labeled *Eco*RI-cut plasmid pUC19 (6×10^3 cpm/μg) was prepared as described previously (11). Complexes, as well as DNA by itself, were preincubated for 15 min at 37°C in a total volume of 280 μl of 10 mM Tris-acetate (pH 7.0)–0.1 mM EDTA containing 28 μg of DNA. DPA and $CaCl_2$ were both added to 1 mM; SspC, a purified α/β-type SASP from *B. subtilis*, was added in an amount (200 μg) sufficient to saturate the DNA (11, 16). Aliquots (70 μl) of these incubations were then dried in the dark on the surface of parafilm at 30°C overnight at ~40% relative humidity. The dried spots were then irradiated and analyzed as described elsewhere (11). Symbols: ●, DNA plus SspC, $CaCl_2$, and DPA; ○, DNA plus SspC; ■, DNA alone.

DNA solution we used had an optical density at 254 nm of ~2.0, the α/β-type SASP had an optical density of ~0.5, and 1 mM DPA had an optical density of ~2.5. Since the concentrations of DNA, DPA, and α/β-type SASP in vivo are all probably more than 2 orders of magnitude higher than in the solutions we used (14, 16), it seemed likely that further analyses in solution would be difficult. Consequently, we used dry DNA films for further analyses (Fig. 2; Table 1). This work showed that, as found previously (11), binding of α/β-type SASP to DNA increased SP yield as a function of UV fluence 1.5- to 2-fold and completely suppressed TT formation (Fig. 2; Table 1). Preparation of the SASP–DNA films from a solution 1 mM in both DPA and Ca^{2+} gave a much larger increase in SP yield, to a value ~40% of that seen in dormant spores (Fig. 2; Table 1). Control experiments showed that 1 mM Ca^{2+} alone had no effect, while DPA alone had a lesser effect. Most of the DPA in spores is thought to be complexed with divalent metal ions, predominantly Ca^{2+} (3).

Given the findings noted above as well as those made previously with *B. subtilis*, it seems most likely that in the presence of α/β-type SASP DPA somehow sensitizes spore DNA to UV-induced SP formation. Given this statement, as well as knowledge that SP is a potentially lethal photoproduct (14) (although much less so than TT), one can make several predictions. The first is that increased levels of spore DPA should lower spore UV resistance. Although this prediction has not been tested in *B. subtilis*, with *Bacillus cereus* spores an inverse correlation between spore DPA levels and UV resistance has been found (5). Furthermore, *B. cereus* spores with 12% of their dry weight as DPA have an SP yield as a function of UV fluence which is approximately three times higher than the value for spores with 6% DPA (5). A second prediction is that while forespores which have accumulated α/β-type SASP should be UV resistant, their UV resistance should decrease as DPA is accumulated. While experiments to test the latter prediction are complicated by asynchrony in the cell population of a sporulating culture, results of experiments carried out a number of years ago with *B. cereus* are consistent with this prediction (1, 4). Thus, it seems likely that DPA in spores actually sensitizes DNA to UV-induced SP formation. Note, however, that TT formation as a function of UV fluence is essentially the same in vegetative cells (which lack DPA) and spores that lack the majority of α/β-type SASP (yet retain DPA) ($\alpha^-\beta^-$ spores; Table 1). While the precise role of DPA in spores is not clear, DPA was initially suggested to play a key role in spore heat resistance (3). However, more recent work suggests that DPA plays no major role in spore heat resistance but rather is essential for spore dormancy, as DPA^- spores (including the ones studied here) germinate spontaneously (3).

A final question is what the mechanism whereby DPA sensitizes spore DNA to SP formation is. There have been reports suggesting interaction between DPA and DNA in vitro (7, 8). However, the DNA analyzed in these studies was free DNA, not the form in which DNA exists in spores, i.e., a complex with α/β-type SASP. Furthermore, there is one report suggesting that while DPA is in the spore core (the site of spore DNA), the majority of the DPA is not intimately associated with the DNA (6). Although the extremely high level of DPA in spores might cause sensitization of SP formation by some nonspecific energy transfer mechanism (possibly involving only a small percentage of the DPA), it is also possible that more-specific interactions of DPA with the α/β-type-SASP–DNA complex may take place. Analysis of such interactions seems worthy of further study.

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