ABSTRACT  Dormant spores of a Bacillus subtilis mutant that lacks two major small, acid-soluble spore proteins are very sensitive to UV irradiation, which in spores generates about half the amount of thymine-containing dimers formed by comparable irradiation of vegetative cells. Irradiation of mutant spores also produces spore photoproducts, but again only about one-half the amount formed in comparably irradiated wild-type spores. These findings suggest (i) that the high UV sensitivity of the mutant spores is due to the production of pyrimidine dimers, which are not found in UV-irradiated wild-type spores, and (ii) that the high level of small, acid-soluble proteins found in wild-type spores is directly involved in spore UV resistance by facilitating a conformational change in spore DNA, preventing pyrimidine dimer formation.

Dormant spores of various Bacillus species are much more resistant to UV irradiation than are their vegetative cell counterparts (1). The major photoproduct in the DNA of UV-irradiated vegetative cells of various Bacillus species is a cyclobutane-type thymine-thymine dimer, with smaller amounts of cytosine-thymine and cytosine-cytosine dimers (2, 3). In contrast, UV irradiation of dormant spores of various Bacillus species produces no detectable thymine-containing dimers, but rather a series of other photoproducts, the most predominant of which has been termed spore photoprodut and is a 5-thyminyl-5,6-dihydrothymine adduct (2, 4). Although high levels of spore photoprodut can be formed in spores, spores have at least two mechanisms that efficiently eliminate this photoprodut, one of which operates only on spore photoprodut (5). Presumably the efficient repair of spore photoprodut is the reason for the high UV resistance of dormant spores as compared to vegetative cells.

Studies on the photochemistry of DNA under various conditions have led to the conclusion that the production of pyrimidine dimers is characteristic of DNA in the B conformation, while spore photoprodut formation is characteristic of DNA in the A conformation (3, 4). This work strongly suggests that DNA in dormant spores is in the A conformation, and there is electron microscopic evidence that spore DNA has a different structure than vegetative cell DNA (6). However, the nature of the factor(s) that might be involved in the change of DNA conformation from the B to A form during sporulation is not known. We have suggested (7, 8) the possibility that the large amount of small, acid-soluble proteins (SASPs) found in dormant spores of various Bacillus species might be involved in spore UV resistance, since the SASPs appear during sporulation at about the time of acquisition of UV resistance and are associated with spore DNA. We have constructed a mutant strain of B. subtilis that lacks genes for two of the major B. subtilis SASPs (9), grows, and sporulates normally; but its spores lack two of the three major SASPs, and levels of no other SAP are altered (9, 10). Strikingly, the mutant spores are more sensitive to UV light than are vegetative cells of either the mutant or wild-type strain, thus strongly implicating SASPs in the UV resistance of bacterial spores (9). In this communication we report the nature of the photoproduts formed in DNA of these mutant spores upon UV irradiation.

MATERIALS AND METHODS

Bacterial Strains and Growth of Cells and Spores. The strains used for this work were Bacillus subtilis 168 (originally obtained from D. J. Tipper), and a derivative of this strain in which genes for two major SASPs [SASP-α and -β (10)] are made nonfunctional by construction of deletion mutations in the cloned genes in vitro and then by inserting these deleted genes in the chromosome of B. subtilis 168 with concomitant loss of the wild-type genes (9, 10). This latter double mutant (termed α-β-) lacks SASP-α and -β (9, 10).

Bacteria were grown and sporulated at 37°C in 2× SG medium (11) supplemented with deoxyadenosine (0.45 mM) and [methyl-3H]thymidine (10 μM; 23 μCi/ml; 1 Ci = 37 GBq) obtained from Amersham. For vegetative cells, cultures (50 ml) were harvested in late exponential phase (OD, 1.5) by centrifugation (10 min, 10,000 × g), washed with an equal volume of 25 mM potassium phosphate, pH 7.5/0.15 M NaCl (PBS) and resuspended in 20 ml of PBS prior to irradiation. For spores, 120-ml cultures were grown for 72 hr with vigorous aeration, and the final cultures was washed repeatedly (at least 20 times) over the course of a week with cold water. The final cleaned spores were suspended in 20 ml of H2O for storage. The cleaned spore preparations were free (<1%) of vegetative cells, of germinated spores, and of visible cell debris.

UV Irradiation and Analysis of Photoproduts. Spores (5 × 107 spores per ml) or cells (2 × 107 cells per ml) were irradiated at room temperature in 2 ml of PBS in a 3-cm diameter Petri dish at a distance of 5 cm from a short-wavelength UV lamp with maximum output at 254 nm (UVS-11, Ultraviolet Products, San Gabriel, CA, with the filter removed). The Petri dish was placed on a rotating Autoplate (Lab-Line Biomedical Products, Berkeley, CA) to ensure mixing of the solution. There was no detectable (<1%) spore germination during the irradiation procedure. The output of the UV lamp was measured and corrected for scatter due to the turbidity of the spore or cell suspension as described (8). UV sensitivity of the [3H]thymidine-labeled wild-type or α-β- spores was also measured as described (9).

Irradiated or unirradiated [3H]thymidine-labeled cells or spores were centrifuged in a microcentrifuge, and the pellets were lyophilized. The cells or spores were then broken by dry

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Abbreviation: SASP, small, acid-soluble protein.
rupture in a dental amalgamator with glass beads as the abrasive (7). Two minutes of rupture sufficed to break vegetative cells, while 8 min were needed for spores. The broken cells or spores were suspended in 2 ml of 10 mM Tris-HCl, pH 7.4/100 mM NaCl/1 mM EDTA, and DNA was purified by two phenol extractions followed by ethanol precipitation. DNA was hydrolyzed with 98% (vol/vol) formic acid, and samples of the hydrolysate (20–50,000 cpm) were chromatographed by descending paper chromatography on Whatman 1 paper in butanol:acetic acid:water, 80:12:30 (vol/vol) to separate uracil-thymine dimers (Rf = 0.2, a breakdown product of cytosine-thymine dimers), thymine-thymine dimers (Rf = 0.3), spore photoproduct (Rf = 0.4), and thymine (Rf = 0.6) (2). After the solvent front had traveled 45–48 cm, the paper was dried and cut into 1-cm strips, which were eluted with water, and the radioactivity was counted in a Triton/toluene scintillation fluid (2).

In some experiments larger samples (80–200,000 cpm) of hydrolysates were chromatographed, and regions of the dried paper were again eluted with water. Appropriate fractions were pooled, and samples of the pooled eluate were reirradiated as described above with 5 kJ/m² to convert any thymine-thymine dimer back to thymine. After the reirradiated and untreated eluates were dried, they were redissolved in small volumes of water (20–50 μl), samples were rechromatographed, and chromatograms were analyzed as described above.

RESULTS AND DISCUSSION

As observed with unlabeled wild-type and αβ− spores (9), the [3H]thymidine-labeled mutant spores were much more UV sensitive than were labeled wild-type spores. Thus, irradiation of labeled wild-type spores with a dose of 40 J/m² resulted in no observable loss in viability. However, the viability of the αβ− spores fell more than 100-fold with this same dose (data not shown).

The major photoproduct observed in chromatograms of DNA hydrolysates from [3H]thymidine-labeled UV-irradiated wild-type spores was the spore photoproduct (Fig. 1), as has been found by other workers (2, 6). Very little radioactivity (<0.15% per fraction) was found in the region of the chromatogram where thymine-thymine dimers migrate (Fig. 1), and this small amount of radioactivity was also found in chromatograms of hydrolysates of DNA from unirradiated spores (data not shown). In contrast, a significant peak of radioactivity in the thymine-thymine dimer region and some in the uracil-thymine region were observed on chromatograms of the DNA hydrolysate from αβ− spores irradiated with the same dose as wild-type spores (Fig. 1). Spore photoproduct was also found in αβ− spores, but the level was decreased by a factor of ≈2 over that seen with wild-type spores. Again, unirradiated αβ− spores gave only small amounts (<0.2% per fraction) of radioactivity migrating in the thymine-thymine dimer position (data not shown). Qualitatively similar results were obtained when spores were irradiated with twice the dose used in Fig. 1, but ≈50% more photoproducts (both thymine-containing dimers and spore photoproduct) were produced (data not shown). In contrast to the results with irradiated spores, chromatograms of UV-irradiated vegetative cells of either the wild-type (Fig. 1 Inset) or αβ− mutant strain (data not shown) had only uracil-thymine and thymine-thymine dimers.

While the chromatographic analysis of the photoproducts formed in αβ− and wild-type spores strongly suggested that significant levels of pyrimidine dimers were produced in the αβ− spores, we felt it essential to prove this point conclusively. To do this we took advantage of the fact that pyrimidine dimers are both formed and cleaved by irradiation at 254 nm (3). Thus, when the pyrimidine residues are not held adjacent to one another by the DNA backbone, reirradiation at 254 nm of, for example, thymine-thymine dimers in solution (i.e., after DNA hydrolysis) will split dimers quantitatively into thymine. Consequently, we eluted the thymine-thymine dimer regions from preparative chromatograms of DNA hydrolysates from unirradiated and irradiated wild-type and αβ− spores and rechromatographed this material before and after reirradiation at 254 nm. Control experiments showed that thymine-thymine dimers from vegetative cells were almost completely (>95%) converted back to thymine by our reirradiation procedure (data not shown). Strikingly, the radioactivity from irradiated αβ− spores that comigrated with thymine-thymine dimers was almost completely (=83%) converted to thymine upon reirradiation (Fig. 2A), while only a minute amount of the radioactivity from the analogous wild-type spore fraction was converted to thymine (Fig. 2B, note different scale). In contrast to the results with irradiated spores, analysis of the small amount of radioactivity comigrating with thymine-thymine dimers in DNA hydrolysates from unirradiated spores revealed no detectable thymine generated upon reirradiation (data not shown). Analysis of the results described above, as well as a similar experiment in which spores were irradiated with a 2-fold higher dose, indicated that UV irradiation of αβ− spores produces half the amount of spore photoproduct formed in wild-type spores, but more than 40-fold more thymine-thymine dimers. This latter number may actually be an underestimate, because the minute amount of thymine-thymine dimers formed upon irradiation of wild-type spores may be due to contamination of the wild-type spores with a small amount of germinated spores (2).

Several conclusions can be drawn from the results presented in this communication. First, it seems likely that in αβ− spores a significant amount of the chromosomal DNA is in the B conformation. This is suggested by the known photochemistry of DNA and the significant production of thymine-containing dimers upon UV irradiation of αβ−
conformational change in chromosomal DNA that results in both the change in the nature of UV-induced photoproducts as well as UV resistance. The only lesions in the α−β− spores are in the SASP genes, and these result in no change in the UV sensitivity of the vegetative cells—only the spores.

Furthermore we have been able to restore UV resistance to α−β− spores by transforming them with a multicopy plasmid carrying the genes coding for either SASP-α or SASP-β (J. M. Mason and P. S., unpublished results). We have also provided strong evidence (8) that SASPs are associated with spore DNA in vivo, consistent with the involvement of SASPs in spore UV resistance (7, 12). The work in this communication strongly suggests that SASPs play an extremely important structural role in spore DNA conformation and, therefore, spore UV resistance. Thus SASPs are not merely amino acid storage proteins, but structural proteins as well, and a structural role for SASP is consistent with the extremely high degree of amino acid sequence conservation among SASPs in different Bacillus species as well as in at least one other Gram-positive spore former (10, 13). However, while SASPs seem certain to be necessary for spore UV resistance, it is clear that SASPs of themselves are not sufficient for this resistance (14) and that SASPs probably act in concert with some other spore property in bringing about UV resistance.

Two major unanswered questions remain about this system. The first concerns the large amount of spore photoproduct still generated by UV irradiation of α−β− spores that suggests much spore DNA remains in the A conformation. One possible explanation for this is that the α−β− spores retain a high level of SASP—in particular SASP-γ(9). Construction of a triple mutant lacking SASP-α, -β and -γ and analysis of the UV sensitivity and photochemistry of its spores might aid in resolving this question. The other major question is how, on a structural level, do SASP-α and/or -β (or other SASPs) cause the change in DNA conformation from B to A. With the genes for these proteins now cloned, it should be possible to prepare these proteins in sufficient quantities for direct examination of this process.

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