Small, Acid-Soluble Spore Proteins of the α/β Type Do Not Protect the DNA in Bacillus subtilis
Spores against Base Alkylation
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Ethyl methanesulfonate (EMS) killed wild-type Bacillus subtilis spores as rapidly as spores lacking small, acid-soluble proteins (SASP) of the α/β type (α-β spores), and 20% of the survivors had obvious mutations. A recA mutation increased the EMS sensitivity of wild-type and α-β spores similarly but reduced their mutagenesis; EMS treatment of dormant spores also resulted in the induction of RecA synthesis during spore germination. EMS generated similar levels of alkylated bases in wild-type and α-β spore DNAs, in purified DNA, or in DNA saturated with α/β-type SASP. Ethylene oxide (EtO) also generated similar levels of base alkylation in wild-type and α-β spore DNAs. These data indicate that EMS and EtO kill spores at least in part by DNA damage but that α/β-type SASP, which protect DNA against many types of damage, do not protect spore DNA from base alkylation.

Spores of Bacillus species are much more resistant than their corresponding growing cells to a variety of treatments, including heat, UV radiation, and oxidizing agents (7, 11). A major factor contributing to spore resistance to these treatments is the saturation of spore DNA with a group of proteins termed small, acid-soluble proteins (SASP) of the α/β type (25, 26). These DNA binding proteins alter spore DNA UV photochemistry, thus contributing to spore resistance to UV radiation, and greatly slow DNA depurination as well as hydroxyl radical-induced DNA backbone cleavage, thus contributing to spore resistance to heat and oxidizing agents (25, 26). The effects of α/β-type SASP on DNA properties in spores generally are quite similar to the effects of purified α/β-type SASP on DNA properties in vitro (5, 12, 21). Studies of alkylation of DNA by dimethyl sulfate have indicated that α/β-type SASP do not significantly protect against this type of DNA damage in vitro (24). However, the effects of these proteins on DNA alkylation in spores have not been studied. Since DNA-alkylating agents, in particular ethylene oxide (EtO), are used for the sterilization of some types of materials (16, 17), we decided to examine the role of α/β-type SASP in the protection of DNA in spores against alkylation.

The alkylation agent we chose to use in most work was ethyl methanesulfonate (EMS), because of both its ease and its relative safety of use and the large amount of knowledge on its mechanism of action (1). The wild-type Bacillus subtilis strain (PS832) used for most experiments was a derivative of strain 168; the isogenic strain lacking the genes coding for the majority of spore α/β-type SASP was PS356 (12) (referred to as α-β). Vegetative cells were prepared by growth at 37°C in 2×YT medium (21) to an optical density of 600 nm (OD600) of ~1.0; spores of various strains were prepared at 37°C in 2×SG medium and purified as described previously (15). Incubation of vegetative cells at 30°C in 0.4 M KPO4 (pH 7.0) with 0.45 M L-alanine to stimulate spore germination. In this medium, ~99% of survivors of EMS treatment occurred at least in part through DNA damage, as there was a high percentage of mutants among the survivors (Table 1), as observed previously (9, 14). A recA mutation decreased the EMS resistance of wild-type and α-β spores by similar amounts (Fig. 1), and the percentage of mutants among the survivors of EMS treatment of recA spores, whether α-β or otherwise wild type, decreased more than sevenfold (Table 1). A recA mutation also decreases the EMS resistance and mutagenesis of Escherichia coli, and these findings have been interpreted to indicate that some of the EMS resistance and much of the mutagenesis are due to the repair of alkylation damage by error-prone DNA repair, such as is induced during the SOS response (2, 6, 10, 19).

To obtain further evidence relative to this interpretation, we used spores of strain PS2271 (prepared as described above), which carry a recA-lacZ fusion but are otherwise wild type (23). Spores treated or not treated with EMS (2 h, 37°C) as described in Table 1 were germinated at 37°C to an initial OD600 of ~0.5 to 0.6 in 25 ml of Spizizen minimal medium (28) with tryptophan (25 μg/ml) and Casamino Acids (0.1%) plus 4 mM L-alanine to stimulate spore germination. In this medium, ~90% of spores had initiated germination after 30 min. [3H]Leucine (10 μCi/50 μCi/μmol) was also added to the medium to allow the measurement of protein accumulation during germination and outgrowth. At various times after the addition of spores to the medium, aliquots (500 μl) were removed for quantitation of the incorporation of [3H]leucine into protein (23). Samples (2 ml) were also harvested by centrifu-
TABLE 1. Survival and mutagenesis of spores after treatment with alkylating agents

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Survival (%)</th>
<th>% of survivors with the following mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>aux</td>
</tr>
<tr>
<td>PS832 (wild type)</td>
<td>—</td>
<td>100</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td>EMS</td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td>PS356 (α−β−)</td>
<td>—</td>
<td>65</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td>EMS</td>
<td>5</td>
<td>9.7</td>
</tr>
<tr>
<td>PS2318b (recA)</td>
<td>—</td>
<td>90</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td>EMS</td>
<td>1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PS2319 (α−β− recA)</td>
<td>—</td>
<td>95</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td>EMS</td>
<td>1.2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PS832b</td>
<td>EtO</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Spores were incubated in 0.4 M KH2PO4 (pH 7.0) with or without 0.45 M EMS. Spores of strains PS832 and PS356 were incubated for 15 h at 30°C, and spores of strains PS2318b and PS2319 were incubated for 2 h at 37°C. In all cases, spores were diluted at least 200-fold into 2.4 ml of 0.1 M Na2S2O3 and then diluted further for analyses of survival on LB medium plates (5, 23). Survivors (at least 200) were picked onto minimal medium or sporulation medium plates to test for the presence of auxotrophic (aux) or sporulation (spo) mutations as described previously (5).

$^{**}$ Spores of strain PS832 were treated with or without EtO for 5 min at 55°C and 60% relative humidity in a Joslyn EO Gas Biological Indicator Evaluator Resistantometer Vessel (Joslyn Sterilization Corporation, Farmington, N.Y.).

$^{a}$ The values for the percentage of survivors with the indicated mutations in a replicate of these experiments were essentially identical (±20%) to those shown here, and values for spores not treated with EMS were also essentially identical to values reported previously (5, 21).

$^{d}$, no EMS treatment.

FIG. 1. EMS resistance of wild-type and α−β− spores with or without a recA mutation. Spores of various strains were incubated at an OD600 of ~1 and 37°C in 0.4 M Na2PO4 (pH 7.0)–0.45 M EMS. At various times, aliquots were diluted ~100-fold into 2.4 ml of 0.1 M Na2S2O3 and then diluted further in 50 mM KPO4 (pH 7)–0.1 M NaCl prior to analysis of viable counts on LB medium plates (4). Symbols: ○, PS832 (wild type); △, PS356 (α−β−); ●, PS2318 (recA); ▲, PS2319 (α−β− recA). Essentially identical results were obtained in a replicate experiment.

FIG. 2. Level of expression of recA-lacZ during germination of spores with or without prior EMS treatment. Spores of strain PS2271 (recA-lacZ::amyE) were germinated as described in the text with or without prior treatment with EMS. This treatment resulted in ~50% killing. At various times during spore germination, samples were taken for analysis of the level of recA-lacZ expression relative to protein accumulated during spore germination and outgrowth as described in the text. Symbols: ○ and ●, OD600; △ and ▲, recA-lacZ specific activity; ○ and △, no EMS treatment; ● and ▲, EMS treatment.
and linear PUB110 (4.5 kb).

20 min, 90 min, 6 h, and 12 h, respectively; 5, incubated without EMS for 12 h. Lanes: 1, 2, 3, and 4, incubated with EMS for 2, 3, and 4) or without (lane 5) 0.45 M EMS. Spore DNA was isolated, treated with piperidine, denatured with glyoxal, and electrophoresed on agarose gels; the DNA was transferred to Hybond N membranes, and PUB110 sequences were detected as described in the text. Lanes: 1, 2, 3, and 4, incubated with EMS for 20 min, 90 min, 6 h, and 12 h, respectively; 5, incubated without EMS for 12 h. The positions of size markers of 3.5 and 1 kb are shown on the left. The two bands in the doublet seen at the top of the gel are circular PUB110 (upper band) and linear PUB110 (4.5 kb).

FIG. 3. Analysis of piperidine-sensitive sites in plasmid PUB110 from EMS-treated wild-type and $\alpha\beta$ spores. Spores of strain PS533 (wild type) (A) and PS578 ($\alpha\beta$) (B) were incubated at 30°C in 0.4 M KPO$_4$ (pH 7) with (lanes 1, 2, 3, and 4) or without (lane 5) 0.45 M EMS. Spore DNA was isolated, treated with piperidine, denatured with glyoxal, and electrophoresed on agarose gels; the DNA was transferred to Hybond N membranes, and PUB110 sequences were detected as described in the text. Lanes: 1, 2, 3, and 4, incubated with EMS for 20 min, 90 min, 6 h, and 12 h, respectively; 5, incubated without EMS for 12 h. The positions of size markers of 3.5 and 1 kb are shown on the left. The two bands in the doublet seen at the top of the gel are circular PUB110 (upper band) and linear PUB110 (4.5 kb).

described above, and ~8 mg (dry weight) was incubated at 30°C in 0.5 ml of 0.4 M KPO$_4$ (pH 7.0)–0.45 M EMS for various times. To stop the reaction, 0.5 ml of 5% Na$_2$S$_2$O$_4$ was added, and the spore pellet was harvested by centrifugation and washed twice with 1 ml of water. The final spore pellet was suspended in 0.5 ml of 50 mM Tris-HCl (pH 8)–1% sodium dodecyl sulfate–8 M urea–50 mM dithiothreitol–10 mM EDTA, incubated for 90 min at 37°C to remove spore coats, and washed extensively by centrifugation (5). The final spore pellet was suspended in 3.5 ml of Quiagen buffer B1 plus RNase as described by Qiagen Inc. (15a) for preparations of bacterial DNA, and DNA was purified on Qiagen columns according to the manufacturer’s instructions. Aliquots of the final DNA were dissolved in 10 mM Tris-HCl (pH 8)–1 mM EDTA or directly in 1 M piperidine. Samples in piperidine were incubated for 30 min at 90°C, and piperidine was removed by repeated hypotitration. Aliquots of the DNA (1 to 2 µg) were electrophoresed on agarose gels after denaturation with glyoxal (5), DNA was transferred to Hybon N membranes (Amersham), and PUB110 sequences were detected by hybridization (21).

Analysis of DNA from spores treated with 0.45 M EMS at 30°C for up to 12 h did not reveal any obvious damage to either bulk chromosomal DNA or PUB110 when the spore DNA was analyzed without piperidine cleavage (data not shown). However, EMS treatment of spores for as little as 20 min resulted in a very large degree of killing of the $\alpha\beta$ spores, even without EMS treatment (data not shown). This result was not totally unexpected, as previous work has shown that $\alpha\beta$ spores are quite sensitive to killing by desiccation and dry heat (4, 22). However, the main DNA alkylation product with EtO is N$^7$-hydroxyethylguanine (20), a modification that sensitizes the DNA backbone to piperidine cleavage.

As found previously (4), desiccation alone caused some fragmentation of the DNA in $\alpha\beta$ spores (Fig. 4A, lanes 1 and 4; DNA from untreated $\alpha\beta$ spores looked like that from desiccated wild-type spores) and resulted in significant (~80%) killing. The mock EtO treatment also caused significant fragmentation of wild-type and $\alpha\beta$ spore DNAs and generated a large number of piperidine-sensitive sites in DNA (Fig. 4, lanes 2 and 5). This mock treatment killed <25% of wild-type spores but >99% of $\alpha\beta$ spores. Consequently, under these conditions, spore killing may not be due to the generation of piperidine-sensitive lesions in DNA. A 2-min EtO treatment which killed ~95% of wild-type spores increased spore DNA fragmentation slightly (Fig. 4A, lanes 3 and 6) and also increased the number of piperidine-sensitive sites (Fig. 4B, lanes 3 and 6). However, the increase in the number of piperidine-sensitive sites generated by EtO treatment appeared similar in both wild-type and $\alpha\beta$ spores (Fig. 4B, compare lanes 2 and 3 and lanes 5 and 6). Thus, $\alpha\beta$-type SASP also did not block EtO alkylation of spore DNA.

The data in this communication allow a number of conclu-
Indeed, we found that EMS treatment of dormant spores induces the SOS response during spore germination, which can lead to error-prone repair (6). Presumably, during the germination of recA spores, EMS lesions are repaired only by a more error-free pathway, possibly via alkyltransferase(s) (2, 19).

Fourth, the data in this communication clearly show that α/β-type SASP do not protect spore DNA from agents that attack either the O6 or the N7 of guanine. Clearly, the structure of the complex between α/β-type SASP and DNA does not hinder access to these two positions, which are both in the major groove of DNA, while at the same time blocking access to the DNA backbone and the glycosyl bond (5, 24) and altering DNA structure and DNA photochemistry (26). It is hoped that detailed analysis of the α/β-type SASP-DNA complex will clarify the mechanisms for these latter changes and the lack of effect of α/β-type SASP on the reactivity of DNA with alkylating agents.

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REFERENCES


