Binding of Small, Acid-Soluble Spore Proteins to DNA Plays a Significant Role in the Resistance of Bacillus subtilis Spores to Hydrogen Peroxide

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Dormant spores of Bacillus subtilis which lack the majority of the αβ-type small, acid-soluble proteins (SASP) (termed α−β− spores) that coat the DNA in wild-type spores are significantly more sensitive to hydrogen peroxide than are wild-type spores. Hydrogen peroxide treatment of α−β− spores causes DNA strand breaks more readily than does comparable treatment of wild-type spores, and α−β− spores, but not wild-type spores, which survive hydrogen peroxide treatment have acquired a significant number of mutations. The hydrogen peroxide resistance of wild-type spores appears to be acquired in at least two incremental steps during sporulation. The first increment is acquired at about the time of αβ-type SAP synthesis, and the second increment is acquired approximately 2 h later, at about the time of dipicolinic acid accumulation. During sporulation of the α−β− strain, only the second increment of hydrogen peroxide resistance is acquired. In contrast, sporulation mutants which accumulate αβ-type SAP but progress no further in sporulation acquire only the first increment of hydrogen peroxide resistance. These findings strongly suggest that binding of αβ-type SAP to DNA provides one increment of spore hydrogen peroxide resistance. Indeed, binding of αβ-type SAP to DNA in vitro provides strong protection against cleavage of DNA by hydrogen peroxide.

The DNA in dormant spores of Bacillus species, including Bacillus subtilis, is covered with a group of small, acid-soluble proteins (SASP) of the αβ-type (18). The αβ-type SAP are products of a multigene family, and their amino acid sequences have been very highly conserved both within and across species (17). These proteins are synthesized midway in sporulation and only in the developing spore (17). One function of the αβ-type SAP is to be degraded in the first minutes of spore germination, thus providing amino acids for protein synthesis at this time (17). However, recent work has shown that a second major function of αβ-type SAP is to protect spore DNA from various types of damage, including damage caused by heat and UV irradiation (4, 5, 18). Indeed, spores lacking major αβ-type SAP (termed α−β− spores) because of the loss of genes coding for these proteins (termed sps) exhibit greatly decreased UV resistance, as well as heat resistance at a variety of temperatures (5).

In addition to increased resistance to heat and UV radiation compared with their vegetative cell counterparts, dormant spores of Bacillus species are also quite resistant to a variety of chemicals, in particular, hydrogen peroxide (14). While the mechanism by which hydrogen peroxide kills spores is not known, killing of vegetative bacteria involves DNA damage, much of which is DNA backbone cleavage caused by hydroxyl radicals generated from the hydrogen peroxide (8). Since studies in vitro have shown that binding of αβ-type SAP can protect DNA against damage by a variety of agents which attack the DNA backbone (16), it seemed likely that αβ-type SAP would also do this in vivo. Consequently, in this report we present the results of studies of the effect of hydrogen peroxide on wild-type (wt) and α−β− spores of B. subtilis. The data from these studies strongly suggest that binding of αβ-type SAP to spore DNA in vivo is an important component of spore hydrogen peroxide resistance.

MATERIALS AND METHODS

Strains used and growth of cells and spores. All strains used are derivatives of B. subtilis 168 and are as follows: PS533 (wt) trpC2 Km'(pUB110), PS578 (α−β−) ΔspsA ΔspsB trpC2 Km'(pUB110), PS904 spoIII94 trpC2 Km'(pUB110), PS1450 ΔspsA ΔspsB Δspp trpC2 Km'(pSSpCμ), PS1465 ΔspsA ΔspsB trpC2 Km'(pSSpCμ), and PS1914 spoV489 trpC2 Km'(pUB110). All strains were grown at 37°C in 2× SG medium (7) containing kanamycin (10 μg/ml). Vegetative cells were harvested in mid-log phase (optical density at 600 nm = ~0.7), and spores were harvested after 24 to 48 h of growth. Spores were purified by repeated centrifugation and washing with water as previously described (11).

Measurement of hydrogen peroxide resistance. For analysis of spore hydrogen peroxide resistance, spore suspensions at an optical density at 600 nm of ~1.0 were diluted 1:100 in 20 mM KPO4 (pH 6.8) with or without 4 M hydrogen peroxide. At various times, 10-μl aliquots of these incubations were added to 1 ml of saline phosphate (0.15 M NaCl, 25 mM KPO4 [pH 7.0]) containing ~5,000 U of bovine liver catalase (Sigma). This amount of catalase destroys the hydrogen peroxide in <5 s. Aliquots of the final dilution were plated on 2× YT medium plates (4) containing kanamycin (10 μg/ml) and incubated at 37°C for 24 to 48 h prior to enumeration of survivors. Determination of hydrogen peroxide resistance of vegetative or sporulating cells was as described above, but cells (100 to 200 μl) were first harvested by centrifugation, washed with 1 ml of saline phosphate, and suspended in saline phosphate at an optical density at 600 nm of 1.0 prior to dilution into hydrogen peroxide. The time of initiation of sporulation was determined as described previously (13).

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wt or \( \alpha^- \beta^- \) spores which survived hydrogen peroxide treatment were picked onto minimal medium or sporation agar plates as described previously (4), and auxotrophic and asporogenous mutants were identified.

**Extraction and analysis of spore DNA.** For analysis of possible DNA damage upon hydrogen peroxide treatment of intact spores, 10 to 15 mg (dry weight) of spores was suspended in 1 ml of saline phosphate containing 4 M hydrogen peroxide. After various incubation times, the sample was centrifuged for 1 min in a microcentrifuge and resuspended in 1 ml of saline phosphate. Survival was then determined as described above, just prior to the addition of 10,000 U of catalase and further incubation for 30 min. More than 90% of the hydrogen peroxide was destroyed in <5 s. After incubation, the spores were washed once with water by centrifugation; resuspended in 0.5 ml of 50 mM Tris-HCl (pH 8.0) containing 8 M urea, 1% sodium dodecyl sulfate, 10 mM EDTA, and 50 mM dithiothreitol; and incubated for 90 min at 37°C to remove spore coats (4). After these coat-stripped spores were washed by repeated centrifugation, the spores were disrupted by lysozyme and then treated with Sarkosyl (1%) and proteinase K (100 \( \mu g \)/ml), after which total nucleic acid was purified by phenol extraction, isolated by ethanol precipitation, and dissolved in 25 \( \mu l \) of \( H_2O \). Untreated samples of the nucleic acid were resolved by electrophoresis on a 1% agarose gel, and then the gel was stained with ethidium bromide and the nucleic acid was transferred to nitrocellulose paper. Plasmid pUB110 sequences on the paper were detected by hybridization with a digoxigenin-labeled pUB110 probe prepared by the random priming method with EcoRI-linearized pUB110 (4, 11).

**Analysis of hydrogen peroxide degradation of DNA in vitro.** For analysis of hydrogen peroxide cleavage of DNA in vitro, plasmid pUC19 cut with HindIII and Psrl was labeled at the HindIII end with [\( \alpha^-\)P]dATP and reverse transcriptase as described previously (16). Labeled plasmid DNA (6 \( \mu g \); 10\(^6\) cpm) with or without 48 \( \mu g \) of purified SspC\(^{wt}\) (12) (an amount sufficient to saturate the DNA [16]) was preincubated in 50 \( \mu l \) of 10 mM Bis-Tris-HCl (pH 8.0) with 0.1 mM EDTA. Cleavage was initiated by sequential addition of Fe\(^{3+}\)-EDTA, hydrogen peroxide to 0.25 or 2.5 M, and 20 mM Na ascorbate (16, 21). After incubation for 2 min at room temperature, cleavage was stopped by addition of 6 \( \mu l \) of 0.1 M thiourea, protein was dissociated from the DNA by addition of 100 \( \mu l \) of 1.25% sodium dodecyl sulfate and 25 mM EDTA, and DNA was precipitated by addition of 10 \( \mu l \) of 5 M NaCl and 240 \( \mu l \) of ethanol. The precipitated DNA was rinsed with 70% ethanol, dried, dissolved in 25 \( \mu l \) of gel-loading buffer containing formamide, boiled briefly, and run on a 6% acrylamide-sequencing gel; DNA was then detected by autoradiography (16).

**Analytical methods.** Samples (1 ml) of sporulating cells were taken for analysis and assays of glucose dehydrogenase and dipicolinic acid, as previously described (10).

**RESULTS**

**Effects of hydrogen peroxide on spore survival and mutation.** As found previously with many *Bacillus* as well as *Clostridium* species (14), dormant spores of wt *B. subtilis* are much more resistant to hydrogen peroxide than are the corresponding vegetative cells (Fig. 1). However, \( \alpha^- \beta^- \) spores were more hydrogen peroxide sensitive than wt spores, although they were still significantly more resistant to hydrogen peroxide than vegetative cells were (Fig. 1). Removal of spore coats by treatment with urea, sodium dodecyl sulfate, and dithiothreitol (see Materials and Methods) had no effect on the hydrogen peroxide resistance of either wt or \( \alpha^- \beta^- \) spores (data not shown). Spores of strain PS1450, which lacks the two major \( \alpha^-\beta^- \) type SASP found in wt spores but possesses a high level of a normally minor \( \alpha^-\beta^- \) type SASP termed SspC\(^{wt}\), exhibited a level of hydrogen peroxide resistance only slightly lower than that of wt spores (Table 1). Previous work has shown that the synthesis of high levels of SspC\(^{wt}\) in spores of strain PS1450 results in near-wt levels of spore UV and heat resistance (20). However, spores of strain PS1465, which is also \( \alpha^- \beta^- \) but which has high levels of an SspC variant termed SspC\(^{ala}\) which no longer binds DNA in vivo or in vitro, are at least as hydrogen peroxide sensitive as the \( \alpha^- \beta^- \) parent (Table 1), as found previously when the heat and UV resistance of spores of this strain were measured (20). Analysis of the wt spores which survived hydrogen peroxide treatment showed that these survivors had accumulated no readily detectable mutations.

**TABLE 1. Hydrogen peroxide resistance of spores of various *B. subtilis* strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Survival at time of ( H_2O_2 ) treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>PS33 (wt)</td>
<td>ND*</td>
</tr>
<tr>
<td>PS78</td>
<td>34</td>
</tr>
<tr>
<td>PS1450(pSspC(^{wt}))</td>
<td>88</td>
</tr>
<tr>
<td>PS1465(pSspC(^{ala}))</td>
<td>13</td>
</tr>
</tbody>
</table>

* Spores were prepared and survival after hydrogen peroxide treatment was measured as described in Materials and Methods.

* The last three strains are \( \alpha^- \beta^- \).

* ND, not determined.

FIG. 1. Survival of spores and vegetative cells during hydrogen peroxide treatment. Spores or vegetative cells (mid-log phase) were incubated at room temperature in hydrogen peroxide (4 M), aliquots were diluted into a catalase-containing solution, and samples were plated to determine survival as described in Materials and Methods. Symbols: ●, vegetative cells of strain PSSS3 (wt); ○, spores of strain PS578 (\( \alpha^- \beta^- \)); ▲, spores of strain PS533 (wt).
which survived were enumerated as DNA plasmid pUB110. In order to determine the state of plasmid pUB110 DNA from untreated and hydrogen peroxide-treated wt and α− β− spores (Fig. 2). While plasmid pUB110 DNA extracted from untreated spores was essentially all (>95%) in supercoiled form, as found previously (4).

The presence of significant numbers of mutations among α− β− spores surviving hydrogen peroxide treatment suggested that this treatment might be killing spores by DNA damage. Indeed, hydrogen peroxide is known to cause DNA damage in growing cells, damage that can be mutagenic (8). In order to test this point directly, we determined the state of plasmid pUB110 DNA from untreated and hydrogen peroxide-treated wt and α− β− spores (Fig. 2). While plasmid pUB110 DNA extracted from untreated spores was essentially all (>95%) in supercoiled form, as found previously (4).

(Fig. 2, lane A, and data not shown), a hydrogen peroxide treatment of α− β− spores giving ~30% survival resulted in at least one single-strand break in approximately one-half of the plasmid molecules and even a small amount of linear plasmid (Fig. 2, lane C). More extensive hydrogen peroxide treatment of α− β− spores gave an even higher degree of plasmid cleavage (Fig. 2, lane D). In contrast, a hydrogen peroxide treatment of wt spores giving ~1% survival resulted in single-strand breaks in less than one-third of plasmid pUB110 and little if any linear plasmid (Fig. 2, lanes A and B). Examination of an ethidium bromide-stained agarose gel of total nucleic acid from untreated and hydrogen peroxide-treated spores also showed significant chromosomal DNA cleavage in hydrogen peroxide-treated α− β− spores, with much less in wt spores (data not shown), as found previously when DNA from heated wt and α− β− spores was analyzed (4).

The results given above strongly suggest that, as found previously for heat killing of α− β− spores (4), hydrogen peroxide killing of α− β− spores is due in large part to DNA damage. That α/β-type SASP can provide strong protection against cleavage of DNA by compounds generated from hydrogen peroxide was readily shown by an in vitro experiment (Fig. 3). While treatment of 32P-labeled pUC19 DNA with 0.25 M and 2.5 M hydrogen peroxide gave significant or complete degradation of the intact plasmid (Fig. 3, lanes 1, 2, and 3), saturation of the plasmid DNA with the α/β-type SASP SspC′W gave complete protection against 0.25 M hydrogen peroxide and significant protection against 2.5 M hydrogen peroxide (Fig. 3, lanes 4 and 5). Previous work has shown that SspC′W, as well as a number of other wt α/β-type SASP, protects the DNA backbone against attack by a variety of agents (16). However, SspC′Na, which does not bind to DNA, is ineffective in this regard (20).

**Table 2. Induction of mutations by H2O2 treatment of spores**

<table>
<thead>
<tr>
<th>Strain and time (min) of H2O2 treatment</th>
<th>% Survival (no. of survivors)</th>
<th>No. of survivors with mutationa: aux spo aux spo</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS533 (wt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (no H2O2 treatment)</td>
<td>100 (200)</td>
<td>0 0 0</td>
</tr>
<tr>
<td>20</td>
<td>50 (79)</td>
<td>0 0 0</td>
</tr>
<tr>
<td>60</td>
<td>6 (200)</td>
<td>0 1 0</td>
</tr>
<tr>
<td>PS578 (α− β−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (no H2O2 treatment)</td>
<td>100 (300)</td>
<td>0 0 0</td>
</tr>
<tr>
<td>5</td>
<td>32 (168)</td>
<td>11 5 ND</td>
</tr>
<tr>
<td>10</td>
<td>10 (158)</td>
<td>16 11 5</td>
</tr>
<tr>
<td>15</td>
<td>3 (100)</td>
<td>7 6 3</td>
</tr>
</tbody>
</table>

a Spores were treated with hydrogen peroxide and survivors and mutations were enumerated as described in Materials and Methods.

b ND, not determined.

to DNA damage. Indeed, hydrogen peroxide is known to cause DNA damage in growing cells, damage that can be mutagenic (8). In order to test this point directly, we determined the state of plasmid pUB110 DNA from untreated and hydrogen peroxide-treated wt and α− β− spores (Fig. 2). While plasmid pUB110 DNA extracted from untreated spores was essentially all (>95%) in supercoiled form, as found previously (4).

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**Appearance of hydrogen peroxide resistance during sporulation.** The data given above indicated that saturation of spore DNA with α/β-type SASP is one (although certainly
not the only) cause of spore hydrogen peroxide resistance. If so, one would expect to see acquisition of significant hydrogen peroxide resistance during sporation at about the time of synthesis of the αβ-type SASP. Indeed, during sporation of the wt B. subtilis, significant hydrogen peroxide resistance was acquired approximately in parallel with synthesis of glucose dehydrogenase (Fig. 4), a biochemical marker whose appearance parallels that of αβ-type SASP (10). The level of hydrogen peroxide resistance which appeared at this time in sporulation of the wt strain was not as high as that of wt dormant spores, but a much higher level of hydrogen peroxide resistance was acquired ~2 h later in sporulation, at approximately the time of synthesis of dipicolinic acid (Fig. 4). In contrast to the results obtained with the wt B. subtilis strain described above, during sporation of the α−β− strain only one increment of hydrogen peroxide resistance appeared, just prior to synthesis of dipicolinic acid (Fig. 5). The reason for the slight difference in the timing of acquisition of maximum hydrogen peroxide resistance relative to dipicolinic acid accumulation in the wt and α−β− strains is not clear. Similar analyses of two sporulation mutants, spoIIIIC (Fig. 6) and spoIVA (data not shown), which make αβ-type SASP but do not accumulate dipicolinic acid in the forespore (5), showed that the increment of hydrogen peroxide resistance appearing in parallel with glucose dehydrogenase was acquired, but there was no further increase in hydrogen peroxide resistance.

**DISCUSSION**

Previous work concerning the mechanism of heat resistance of bacterial spores has indicated that while spore coats play no role in this process, there are at least four factors contributing incrementally to spore heat resistance (6, 18). On the basis of data obtained in this work, it appears that spore coats are also not involved in resistance of B. subtilis spores to hydrogen peroxide. This was found previously with spores of Bacillus cereus (22), but it may not be a general rule for all spore formers, as the coats of Clostridium bifermantans spores do provide a significant increment of hydrogen peroxide resistance (2). Another possible contributor to the hydrogen peroxide resistance of spores could be catalase, which can protect growing cells against hydrogen peroxide (8). Indeed, B. subtilis spores contain a form of catalase not found in growing cells (9). However, the level of this spore type catalase is significantly below that in growing cells, and the spore type enzyme is actually synthetized late in growth, long before we observe the first significant increment in hydrogen peroxide resistance during sporulation (9). While changes in catalase levels seem unlikely to be involved in acquisition of hydrogen peroxide resistance.

**FIG. 4.** Appearance of hydrogen peroxide resistance during sporulation of the wt strain (PS533). Sporulation was carried out, and samples were taken for analyses of dipicolinic acid, glucose dehydrogenase, and survivors of a 1- or 10-min hydrogen peroxide treatment (4 M), as described in Materials and Methods. The values for relative survival after hydrogen peroxide treatment have been normalized so that the highest percentage of survival is given as 100%. The actual values were ~80% for the 1-min treatment and ~40% for the 10-min treatment. Symbols: ○, glucose dehydrogenase (gdh); △, dipicolinic acid (DPA); ●, survival after the 1-min hydrogen peroxide treatment; and ▲, survival after the 10-min hydrogen peroxide treatment.

**FIG. 5.** Appearance of hydrogen peroxide resistance during sporulation of the α−β− strain (PS578). The procedures used are identical to those described in the legend to Fig. 4 except that a 30-s incubation with hydrogen peroxide was used. The actual maximum percentage of survival after the 30-s hydrogen peroxide treatment was ~40%. Symbols: ○, glucose dehydrogenase (gdh); △, dipicolinic acid (DPA); ●, survival after the 30-s hydrogen peroxide treatment. Similar kinetic results were obtained in this experiment when a 1-min hydrogen peroxide treatment was used (data not shown), but the maximum percentage of survival was ~7%.
SASP protect the DNA backbone from hydroxyl radical cleavage is not clear. However, since the hydroxyl radicals are likely generated in vivo from hydrogen peroxide by the Fenton reaction with Fe²⁺ (8) (see below also), α/β-type SASP binding to DNA might exclude Fe²⁺ from the immediate vicinity of the DNA, thus reducing DNA backbone cleavage indirectly. The protection of spore DNA against damage due to hydrogen peroxide by the binding of α/β-type SASP to the DNA backbone is another addition to the effects these proteins have on the properties of bacterial spores. Previous work has shown that these proteins also protect spore DNA against lethal or mutagenic damage due to UV light or heat (18), and it would not be surprising if these proteins protect spore DNA against other damaging agents as well. Recent work has shown that a new DNA-binding protein synthesized during stationary-phase growth of Escherichia coli also provides significant hydrogen peroxide resistance to this organism, presumably by protecting the DNA from attack by hydroxyl radicals (1).

While α/β-type SASP binding is clearly one component of spore hydrogen peroxide resistance, there must be at least one other component, and possibly several others, as α -spores are much more hydrogen peroxide resistant than vegetative cells. Indeed, the acquisition of hydrogen peroxide resistance by α - spores occurs prior to dipicolinic acid accumulation while high-level hydrogen peroxide resistance of wt spores is acquired after dipicolinic acid accumulation, which suggests that there are multiple factors responsible for the hydrogen peroxide resistance acquired late in sporulation, well after α/β-type SASP synthesis. There are a number of possible causes of this increment of hydrogen peroxide resistance acquired late in sporulation, including dipicolinic acid itself, as well as the reduction in forespore water content which takes place around the time of dipicolinic acid accumulation. Unfortunately, there is no available evidence to allow assignment of a role for these factors in spore hydrogen peroxide resistance. However, there is another event which takes place approximately in parallel with dipicolinic acid accumulation and for which there is good evidence in other systems for a role in hydrogen peroxide resistance. In growing cells of E. coli, much of the killing effect of hydrogen peroxide is mediated by hydroxyl radicals generated by the Fenton reaction (8). Continued operation of the Fenton reaction in cells requires a source of reducing equivalents which are ultimately derived from NADH, and NADH-depleted cells are much more hydrogen peroxide resistant than are normal cells (8). Strikingly, the NADH pool of the developing forespore is lost completely by its conversion to NAD at about the time of dipicolinic acid accumulation (19); much of the spore’s low-molecular-weight thiol pool is converted to disulfide form at this time as well (15). Consequently, the loss of reducing equivalents available to drive the Fenton reaction at about the time of dipicolinic acid accumulation may greatly slow generation of hydroxyl radicals and thus provide another component of spore hydrogen peroxide resistance. If this is true, it will be a further example of how the extreme dormancy of the bacterial spore contributes to its long-term survival.

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