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# Small, Acid-Soluble Proteins Bound to DNA Protect *Bacillus subtilis* Spores from Killing by Dry Heat

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**Dry *Bacillus subtilis* spores lacking their two major DNA-binding proteins (small, acid-soluble proteins [SASP] α and β) were much more sensitive to dry heat than were wild-type spores. Survivors of dry heat treatment of both wild-type and mutant spores exhibited a high frequency of mutations, and the DNA from the heated spores had increased numbers of single-strand breaks. These data indicate that SASP α and β provide significant protection to spore DNA against the damaging effects of dry heat. This DNA damage may be in part depurination, and a purified α/β-type SASP gave significant protection against dry heat-induced DNA depurination in vitro.**

Dormant spores of various *Bacillus* species are much more resistant than their growing cell counterparts to a variety of treatments, including chemical agents, desiccation, heat, and radiation (11, 12). While radiation undoubtedly kills spores by DNA damage, the precise mechanism(s) by which the other treatments kill spores is not known, although killing is normally not via DNA damage (11, 12). Spore DNA is extremely well protected against many different types of damage through the saturation of the spore chromosome with a novel group of DNA-binding proteins termed α/β-type small, acid-soluble spore proteins (SASP) (11, 12). Spores lacking the majority of their α/β-type SASP (termed $\alpha^-\beta^-$ spores in *Bacillus subtilis*) are much more sensitive than are wild-type spores to all of the treatments noted above, with killing of $\alpha^-\beta^-$ spores due in large part to DNA damage (12). The DNA damage generated in hydrated $\alpha^-\beta^-$ spores by heat includes abasic sites presumably arising from depurination as well as single-strand breaks which may arise at least in part from strand cleavage at abasic sites (4, 9). Although killing of wild-type spores is almost always not by DNA damage, one exception (other than radiation) to this statement appears to be killing by dry heat. Heat killing of wild-type *B. subtilis* spores in water results in no obvious increase in mutations in the survivors and no obvious DNA damage (12). In contrast, several workers have noted that heat treatment of dry wild-type *B. subtilis* spores results in extremely high frequencies (5 to 23%) of obvious mutations in the survivors (2, 7, 14). The latter finding strongly suggests that dry heat kills wild-type *B. subtilis* spores in large part by DNA damage; this DNA damage has been suggested to be depurination (5, 7, 14). Given the known role of α/β-type SASP in protecting spore DNA from a variety of types of damage, including depurination (3), it seemed worthwhile to investigate the role of these DNA-binding proteins in protecting spore DNA from dry heat.

The wild-type *B. subtilis* strain (PS533) used for these experiments was a derivative of strain 168 carrying plasmid pUB110, which provides kanamycin resistance. Vegetative cells of strain PS533 were prepared by growth at 37°C to the late log phase (optical density at 600 nm [$OD_{600}$] of 1.0 in a Milton Roy Genesys 5 spectrophotometer) in 2× YT medium (8) plus kanamycin (10 μg/ml). Multiple aliquots (1 ml) of the culture were centrifuged in a microcentrifuge, washed once with sterile saline phosphate (25 mM potassium phosphate buffer [pH 7.0], 0.1 M NaCl), and suspended in 500 μl of 100 mM sucrose in saline phosphate. Aliquots of three appropriate dilutions were spread on L-broth plates (4) plus kanamycin (10 μg/ml), and the plates were incubated overnight to determine the viable cell count. Samples of the resuspended cells (500 μl) were also added to 5-ml ampoules, frozen, and dried overnight under vacuum (≤ 0.05 mm Hg [ca. ≤ 7 Pa]), and the ampoules were sealed under vacuum. The other *B. subtilis* strains used were also derivatives of strain 168, and spores of all strains were prepared and cleaned as previously described (3, 8) and stored at 10°C in water at an $OD_{600}$ of 2.5. These suspensions were analyzed for viable count as described above, and aliquots (500 μl) were frozen and dried in ampoules, which were sealed under vacuum as described above. Ampoules containing cells or spores were heated in an oil bath for various times as described before (14). The ampoules were cooled to room temperature and opened, cells or spores were suspended in 0.5 ml of sterile water, the $OD_{600}$ was measured to determine cell or spore recovery, and appropriate aliquots were analyzed for viable count. As has been observed previously (2, 7, 14), dry wild-type spores of *B. subtilis* were extremely resistant to heat, with approximately 1 log of killing per 30 min at 120°C (Table 1); hydrated wild-type spores exhibit a similar rate of killing at 90°C (3). The $\alpha^-\beta^-$ spores were much more sensitive to dry heat than were wild-type spores, exhibiting a 10-fold reduction in viability per ~3 min at 90°C (Table 1), which is similar to the level of heat resistance of $\alpha^-\beta^-$ spores in water (3). The dry heat resistance of $\alpha^-\beta^-$ spores was also slightly below that of vegetative cells (Table 1), although the latter may have been given significant protection by the sucrose with which the cells were dried (1); the sucrose was necessary to obtain reasonable survival of vegetative cells after the desiccation step alone, as previously noted (1, 4). In a separate experiment (data not shown), drying $\alpha^-\beta^-$ spores with sucrose had little effect on their dry heat resistance. The similar dry heat resistance of $\alpha^-\beta^-$ spores and vegetative cells strongly suggests that the elevated dry heat resistance of wild-type spores is due predominantly to their α/β-type SASP. Consistent with this suggestion,

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TABLE 1. Survival of spores and cells of various strains during dry heat treatment at different temperatures[a]

| Spores or cells | Treatment temp (°C) | % Survival at given time | | | | |
|---|---|---|---|---|---|---|
| | | 0 min[b] | 5 min | 15 min | 30 min | 60 min |
| Wild-type spores | 120 | 100 | 55 | 35 | 14 | 1.3 |
| | 105 | 100 | ND[c] | 80 | ND | 54 |
| | 90 | 100 | ND | ND | ND | 90 |
| Wild-type vegetative cells | 90 | 100 | 9 | 0.2 | ND | ND |
| $\alpha^-\beta^-$ spores | 105 | 100 | <0.004 | ND | ND | ND |
| | 90 | 100 | 2.1 | 0.03 | ND | ND |
| $\alpha^-\beta^-$ $SspC^{ala}$ spores | 90 | 100 | 1.5 | 0.04 | ND | ND |
| $\alpha^-\beta^-$ $SspC^{tyr}$ spores | 105 | 100 | ND | 2.9 | ND | ND |
| | 90 | 100 | ND | 38 | 12 | ND |

[a] Spores and cells of various strains were prepared and dried in ampoules which were sealed under vacuum, and survival after dry heat treatment was determined as described in the text. The strains analyzed were wild-type strain PS533 (pUB110) ($Km^r$), which is derived from a laboratory strain of 168; $\alpha^-\beta^-$ strain PS578 (Δ*sspA* Δ*sspB* [pUB110]) ($Km^r$), which has deletions in the genes coding for the two major α/β-type SASP (11, 12); $\alpha^-\beta^-$ $Ssp^{tyr}$-producing strain PS1464 (Δ*sspA* Δ*sspB* [p$SspC^{tyr}$]) ($Km^r$), which is analogous to PS578 but expresses high levels of $SspC^{tyr}$ in spores (13); and $\alpha^-\beta^-$ $SspC^{ala}$-producing strain PS1465 (Δ*sspA* Δ*sspB* [p$SspC^{ala}$]) ($Km^r$), which is analogous to PS578 but expresses high levels of $SspC^{ala}$ in spores (13). The results presented are from single representative experiments. However, similar results were obtained in at least one replicate of each experiment.

[b] These values were set at 100%. The freeze-drying step alone gave no killing of spores of strains PS533 and PS1464, but the vegetative cells dried in sucrose lost ~50% viability and strains PS578 and PS1465 spores lost 30 to 50% viability, as noted previously (4).

[c] ND, not done.

$\alpha^-\beta^-$ spores carrying p$SspC^{tyr}$, which directs synthesis of high levels of $SspC^{tyr}$, a variant of a normally minor *B. subtilis* α/β-type SASP (termed $SspC^{wt}$), regained significant dry heat resistance; in contrast, a second mutant form of $SspC^{wt}$, termed $SspC^{ala}$, provided no dry heat resistance to $\alpha^-\beta^-$ spores (Table 1). Previous work has shown that high levels of either $SspC^{wt}$ or $SspC^{tyr}$ restore a significant amount of the heat, UV, hydrogen peroxide, and desiccation resistance to $\alpha^-\beta^-$ spores and that $SspC^{tyr}$ and $SspC^{wt}$ bind equally well to DNA in vitro (10, 12, 13). In contrast, $SspC^{ala}$ does not bind to DNA and is ineffective in restoring heat, UV, hydrogen peroxide, and desiccation resistance to $\alpha^-\beta^-$ spores (10, 12, 13).

The obvious question about the killing of spores by dry heat is the mechanism of this process. As noted above, previous workers have found that survivors of wild-type *B. subtilis* spores treated with dry heat contain large numbers of mutations (2, 7, 14). We analyzed this with our spores by transferring survivors from the rich medium plates used to assess survival to either sporulation or minimal medium agar plates, as described previously (3). Incubation of the latter for 24 to 48 h indicated that a significant percentage (15.5%) of the survivors of dry heat killing of wild-type spores had either auxotrophic or asporogenous mutations (Table 2). In contrast, previous work has shown that survivors of heat killing of wild-type spores in water do not have a significant level (<0.5%) of mutations among the survivors (3). Analysis of the mutagenesis of $\alpha^-\beta^-$ spores by dry heat was more complicated, as freeze-drying alone causes both a small amount of killing and significant mutagenesis of $\alpha^-\beta^-$ spores (4) (Table 2). However, the single freeze-drying used to dry the spores prior to heat treatment gave only a small amount of spore killing and a low level of mutations among the survivors (Table 2). At the higher levels of killing given by dry heat treatment, the levels of mutations observed in $\alpha^-\beta^-$ spore survivors rose approximately fivefold and were similar to that with wild-type spores (Table 2). We also analyzed wild-type vegetative cells dried from sucrose for mutations induced by dry heat. However, at both ~90 and 99% killing there were <1% auxotrophic plus asporogenous mutations among the survivors (data not shown). While dry heat has been reported to generate mutations in vegetative bacteria, the frequency of mutations is much lower than that generated in spores (14). In addition, the latter results were obtained with cells dried from culture medium; possibly drying from sucrose further reduces mutagenesis of cells by dry heat.

TABLE 2. Survival and mutagenesis of wild-type and $\alpha^-\beta^-$ spores after dry heat treatment[a]

| Strain treated | Treatment | Survival (%) | Mutations (%) | | |
|---|---|---|---|---|---|
| | | | aux | spo | aux spo |
| 533 (wt) | None | 100 | <0.3 | <0.3 | <0.3 |
| | 60 min, 120°C | 1.3 | 5.5 | 7.5 | 2.5 |
| 578 ($\alpha^-\beta^-$) | None | 70 | 2.5 | 0.5 | 0 |
| | 5 min, 90°C | 2.1 | 6.5 | 6.5 | 1.5 |
| | 10 min, 90°C | 0.2 | 8.5 | 8.0 | 1 |
| | 15 min, 90°C | 0.03 | 6.0 | 6.0 | 2.0 |

[a] Spores of strain PS533 or PS578 (see Table 1) were dried, sealed under vacuum, and heated for various times. Survivors were quantitated, and the percentage of survivors having mutations causing auxotrophy (aux) or asporogeny (spo) was determined as described in the text. For all treatments, 200 to 400 survivors were tested for mutations. wt, wild type.

The generation of a high percentage of mutations in the survivors of dry heat treatment of both wild-type and $\alpha^-\beta^-$ spores suggested that the dry heat was causing DNA damage. Indeed, when DNA was extracted and purified as described previously (3, 8) from wild-type and $\alpha^-\beta^-$ spores before and after killing by dry heat and the DNA was run on agarose gel electrophoresis, the overall size of the DNA appeared smaller after dry heat treatment (Fig. 1A, cf. lanes 1 and 2 and lanes 3 and 4). In addition, there was clearly significant nicking of the pUB110 plasmid present in the wild-type spores upon dry heat treatment (Fig. 1A, lanes 1 and 2, arrows a and c). This nicking was more evident when the DNA was transferred to nitrocellulose paper after agarose gel electrophoresis and the paper was probed with a pUB110 probe (Fig. 1B). In the untreated wild-type spores, plasmid pUB110 was essentially all supercoiled, while the dry heat treatment converted the majority of the plasmid to the nicked circular form as well as some linear DNA (Fig. 1B, lanes 1 and 2). In the untreated $\alpha^-\beta^-$ spores, there was already some nicking of supercoiled plasmid presumably due to the freeze-drying treatment (4), but the amount of the nicked plasmid increased after dry heat treatment along with generation of some linear DNA (Fig. 1B, lanes 3 and 4). These data suggest that the dry heat treatment is causing single-strand breaks in spore DNA. This was shown further by treating the extracted DNA with *Eco*RI, which linearizes plasmid pUB110, denaturing the DNA and running it on agarose gel electrophoresis, transferring the DNA to nitrocellulose, and again probing with a pUB110 probe (Fig. 1C). There was clearly a large increase in smaller fragments of pUB110 after dry heat treatment of wild-type spores and a significant increase in these smaller fragments in dry heat-treated $\alpha^-\beta^-$ spores (Fig. 1C). However, it was noticeable that there were more single-strand breaks (at least in pUB110) in DNA from wild-type spores killed to 5% survival than in $\alpha^-\beta^-$

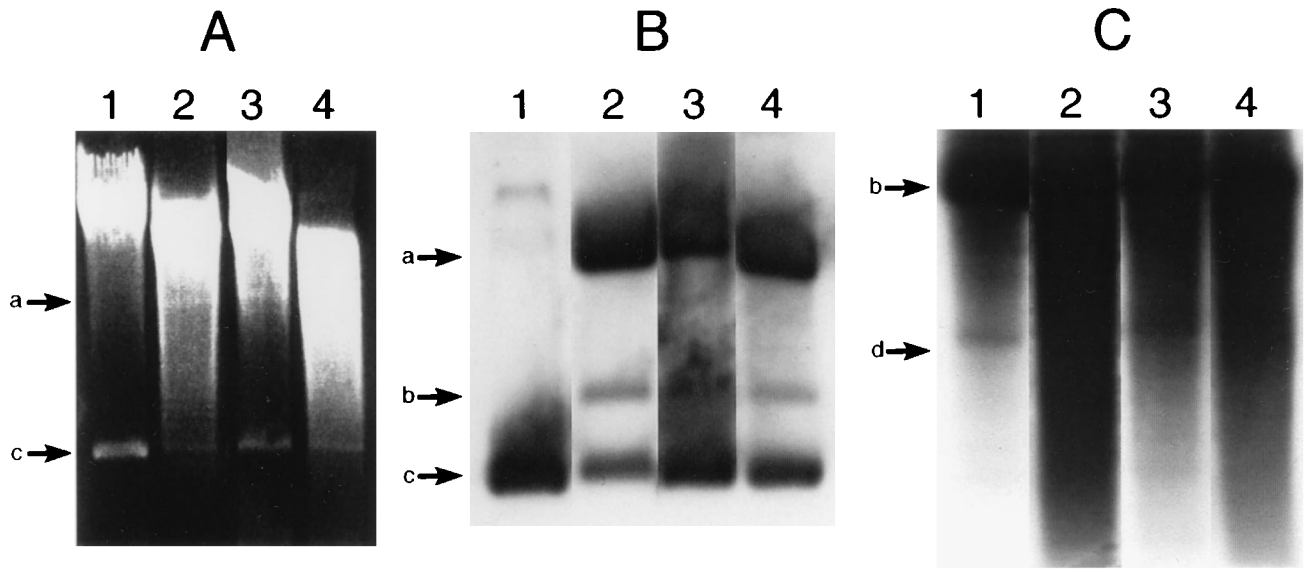


FIG. 1. Agarose gel electrophoretic analysis of DNA from untreated and dry-heated wild-type and $\alpha^-\beta^-$ spores. Fifty $OD_{600}$ units of spores of strain PS533 (wild type) were dried in vacuum and were either not heated or heated for 120 min at 120°C, the latter treatment resulting in 5% survival. Fifty $OD_{600}$ units of strain PS578 ($\alpha^-\beta^-$) were dried in vacuum and were either not heated or heated for 10 min at 90°C, the latter treatment giving 0.7% survival. (A) Aliquots containing 5 μg of DNA were run on a 1% agarose gel and stained with ethidium bromide. (B) Aliquots containing 5 μg DNA were run on a 1% agarose gel, the DNA was denatured and transferred to nitrocellulose paper, the paper was hybridized with a pUB110 probe, and hybridizing bands were detected as described previously (3, 8). (C) Aliquots containing 5 μg of DNA were denatured with glyoxal and run on a 1% agarose gel under conditions preventing DNA renaturation (3). The DNA was then transferred to nitrocellulose paper, and bands hybridizing to a pUB110 probe were detected as described before (3, 8). The sources of the DNA samples run in the various lanes were as follows: 1, wild-type spores, unheated; 2, wild-type spores, heated; 3, $\alpha^-\beta^-$ spores, unheated; 4, $\alpha^-\beta^-$ spores, heated. The horizontal arrows labeled a, b, c, and d denote the migration positions of nicked circular pUB110, linear pUB110 (4.5 kb), supercoiled pUB110, and a 2.3-kb size marker, respectively.

spores killed to 0.7% survival (Fig. 1B and C, cf. lanes 2 and 4). One possible explanation for this finding is that single-strand breaks are not the initial DNA damage caused by dry heat; indeed, single-strand breaks might not be expected to be mutagenic. Rather, the generation of DNA single-strand breaks by dry heat may be a secondary effect of the heat treatment on an initial DNA lesion, possibly DNA depurination (see below).

The data noted above strongly suggest that DNA damage is a major mechanism of spore killing by dry heat. If this is the case, what is the initial DNA damage caused by the dry heat? As noted above, it has been suggested that this damage is depurination (5, 7). Although heat killing of wild-type spores in water is not due to DNA depurination, a major mechanism of heat killing of $\alpha^-\beta^-$ spores in water appears to be generation of abasic sites in DNA by depurination, which can lead either directly to mutations or possibly to DNA strand cleavage (3, 9). Type α/β-SASP slow DNA depurination in aqueous solution at least 20-fold, explaining in large part the higher heat resistance of wild-type versus $\alpha^-\beta^-$ spores (3). While we have not measured DNA depurination in vivo caused by dry heat, we have measured the effect of an α/β-type SASP, $SspC^{wt}$, on DNA depurination in vitro. These measurements utilized poly([$^3$H]dG) · poly(dC), the DNA to which α/β-type SASP bind most strongly, with or without saturating levels (~5:1 weight ratio) of $SspC^{wt}$ (10). The DNA or DNA-$SspC^{wt}$ complexes were dried from solutions at pH 6.5, which is the approximate pH in the region of spore DNA in vivo (11). At the two temperatures tested, the rate of release of guanine from poly(dG) · poly(dC) caused by dry heat was approximately twofold slower than observed previously for total purine release from calf thymus DNA (Table 3) (5); this may be due to differences in the DNAs or conditions used in these different experiments. Strikingly, saturation of poly(dG) · poly(dC) with $SspC^{wt}$ reduced the depurination rate from 2.5- to 4-fold (Table 3). These data are thus consistent with DNA depurination being a major mechanism by which dry heat causes DNA damage in spores. Presumably in wild-type spores the DNA depurination rate is reduced significantly by α/β-type SASP such that spore killing requires high temperatures at which α/β-type SASP perhaps provide less protection against depurination. Possibly at these high temperatures depurination is rapidly followed by DNA strand cleavage, and thus DNA from dry heat-killed wild-type spores is heavily nicked. However, in $\alpha^-\beta^-$ spores DNA is less well protected against depurination, and thus there is significant DNA depurination at much lower temperatures than in wild-type spores. Possibly the abasic sites generated at these lower temperatures in $\alpha^-\beta^-$ spores are not cleaved rapidly; thus, at similar levels of killing by dry heat there is less DNA

TABLE 3. Rate of DNA depurination during dry heating with or without $SspC^{wt}$[a]

| Heating temp (°C) | Rate of guanine release (%) in 24 h[b] | |
|---|---|---|
| | No SspC | Plus SspC |
| 120 | 4.8 (11) | 1.9 |
| 105 | 1.2 (2.7) | 0.3 |

[a] Poly([$^3$H]dG) · poly(dC) (5 μg) in 100 μl of 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.5) without or with $SspC^{wt}$ (25 μg) was lyophilized in an ampoule, and the ampoule was sealed under vacuum. Synthesis of poly([$^3$H]dG) · poly(dC), purification of $SspC^{wt}$, and measurement of the depurination rate upon heating of the dried samples in an oil bath were as described previously (3). The latter measurements were carried out by using quantitation of both ethanol soluble radioactivity and cochromatography of guanine-and-ethanol-soluble radioactivity on a Sephadex G-10 column, as described before (3).

[b] Values in parentheses were calculated from data of Greer and Zamenhof (5) for calf thymus DNA.

single-strand cleavage in $\alpha^-\beta^-$ spores than in wild-type spores, as we have observed (Fig. 1B and C).

The protection afforded to *B. subtilis* spores by α/β-type SASP against killing by dry heat is a further role for these novel proteins in spore resistance and long-term survival (11, 12). However, previous work has shown that α/β-type SASP provide essentially complete protection to spore DNA against lethal damage caused by wet heat, desiccation, and oxidizing agents; therefore, these agents kill spores predominantly by mechanisms other than DNA damage (12). However, this is clearly not the case with dry heat, as this process kills wild-type spores in large part by DNA damage. Consequently, α/β-type SASP only slow DNA damage due to dry heat, for example, depurination, relative to the rates of other mechanisms that may kill spores. In contrast, with hydrated spores α/β-type SASP are much better able to protect DNA from heat damage relative to rates of other killing mechanisms. In this regard it is noteworthy that the DNA in spores dried under vacuum appears to have a structure distinct from that in hydrated spores, as evidenced by major differences in the UV photochemistry of the DNA in spores in these two states (6). Indeed, it has been suggested that some of the DNA in spores in vacuo may be partially denatured (6). Perhaps detailed analysis of the structure of DNA in a vacuum-dried α/β-type SASP-DNA complex and comparison of this structure with that for the hydrated complex may give us some insight into the different effects these proteins have on spore DNA in the two states.

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