JOURNAL OF BACTERIOLOGY, Apr. 1994, p. 2111–2113
0021-9193/94/$04.00+0

Vol. 176, No. 7

Heat Inactivation of *Bacillus subtilis* Spores Lacking Small, Acid-Soluble Spore Proteins Is Accompanied by Generation of Abasic Sites in Spore DNA

BARBARA SETLOW AND PETER SETLOW*

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06030

Received 13 December 1993/Accepted 28 January 1994

Previous work has shown that lethal heat treatment of *Bacillus subtilis* spores lacking the major DNA-binding proteins SASP-α and -β ($\alpha^-\beta^-$ spores) causes significant DNA damage, including many single-strand breaks. In this work we have used a reagent specific for aldehydes present in abasic sites in DNA to show that DNA from wild-type spores killed by heat treatment to levels of <0.05% survival had at most two aldehydes (i.e., abasic sites) per 10^4 nucleotides, while DNA from $\alpha^-\beta^-$ spores killed to similar levels had 7 to 20 times as many abasic sites per 10^4 nucleotides. These data were generally consistent with the level of single-strand breaks in DNA from these heated spores and strongly suggest that a major mechanism responsible for the heat killing of $\alpha^-\beta^-$ (but not wild-type) spores is DNA depurination followed by strand breakage at the resultant abasic site. In contrast, hydrogen peroxide killing of $\alpha^-\beta^-$ spores was not accompanied by generation of a high level of DNA aldehydes.

Dormant spores of *Bacillus* species are much more resistant to a variety of killing treatments, including heat and oxidizing agents, than are the corresponding growing cells (3, 13, 17). There appear to be multiple factors responsible for spore resistance to heat and oxidizing agents (e.g., hydrogen peroxide), including low spore permeability to small molecules and reduced spore core hydration (2, 3, 15). One additional factor common to spore resistance to heat and hydrogen peroxide is the saturation of spore DNA with a group of nonspecific DNA-binding proteins termed α/β-type small, acid-soluble spore proteins (α/β-type SASP) (2, 11, 15–17). These proteins are encoded by a multigene family in *Bacillus* species, are synthesized only in the developing forespore in amounts sufficient to saturate spore DNA, and are degraded in the first minutes of spore germination when spore resistance properties are lost (16, 17). Spores of *Bacillus subtilis* which lack the majority of α/β-type SASP (termed $\alpha^-\beta^-$ spores) as a result of mutations in genes coding for major proteins of this type have significantly lower heat and hydrogen peroxide resistance than wild-type spores (11, 15, 17). In addition, the inactivation of $\alpha^-\beta^-$ spores by heat or hydrogen peroxide is associated with induction of a high level of mutations, as well as significant DNA single-strand breakage, in particular in DNA from heat-treated $\alpha^-\beta^-$ spores (2, 15). In contrast, killing of wild-type spores by these reagents is associated with no significant mutagenesis and much less DNA backbone cleavage. While the precise causes of the DNA strand cleavage and mutagenesis induced by heat and hydrogen peroxide in $\alpha^-\beta^-$ spores are not known, heat treatment has been suggested to cause DNA depurination followed by strand cleavage (2). Indeed, binding of α/β-type SASP to DNA in vitro slows DNA depurination at least 20-fold (2). While depurination is certainly an attractive explanation for the DNA damage caused by heat treatment of $\alpha^-\beta^-$ spores, there is no direct evidence for this process in vivo. Clearly, it would be most informative to analyze the level of abasic sites in DNA from heat-inactivated $\alpha^-\beta^-$ spores directly.

Recently, an elegant, specific, and sensitive assay for abasic sites in DNA that detects the aldehyde moieties generated upon base removal has been developed (5, 7). This assay uses a biotin-tagged reagent termed the aldehyde reactive probe (ARP), which reacts specifically and completely with DNA-aldehydes, giving biotin covalently bound to the DNA (5, 7). The bound biotin tag is then quantitated by an enzyme-linked-immunosorbence-like assay and an avidin-biotin-horseradish peroxidase complex. Previous work has shown that the ARP is specific for DNA damage which results in an alkyl or allyl aldehyde group and has a sensitivity of 0.3 to 1 abasic site per 10^4 nucleotides (nt) (5).

Use of the ARP with DNA from heated and unheated wild-type spores detected only very low aldehyde levels, as was also the case with DNA from unheated $\alpha^-\beta^-$ spores (Table 1). In contrast, DNA from heated $\alpha^-\beta^-$ spores had much higher aldehyde levels at the same level of killing than did wild-type spores (Table 1). Presumably the aldehydes in DNA from heated spores represent abasic sites, as base loss (predominantly depurination) is by far the predominant means whereby heat can generate an aldehyde in DNA (1, 9, 10, 19). In contrast to the results when heat was used to inactivate spores, hydrogen peroxide treatment (15) of wild-type and $\alpha^-\beta^-$ spores giving 0.1 and 1% survival, respectively, generated <1.5 aldehydes per 10^4 nt in spore DNA (data not shown). This is perhaps not unexpected, given the products generated by attack of the hydroxyl radical (presumably the active agent generated from hydrogen peroxide) on DNA (4, 6). However, the precise nature of the products generated in spore DNA by hydrogen peroxide treatment was not studied further.

Previous work has shown that heat treatment of $\alpha^-\beta^-$ spores, but to a much lesser extent wild-type spores, generates significant numbers of DNA single-strand breaks which were suggested to be the result of strand cleavage at abasic sites (2, 8). Analysis of our spore DNA samples by agarose gel electrophoresis showed a reasonable qualitative agreement between the level of aldehydes and the degree of DNA fragmentation

* Corresponding author. Mailing address: Department of Biochemistry MC-3305, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030. Phone: (203) 679-2607. Fax: (203) 679-3408.

TABLE 1. Abasic sites in DNA from heat-treated wild-type and $\alpha^-\beta^-$ spores[a]

Time (min) of heat treatment	Wild-type spores		$\alpha^-\beta^-$ spores	
	% Survival	No. of abasic sites/10^4 nt	% Survival	No. of abasic sites/10^4 nt
0	100	<0.3	100	0.4
3	–[b]	–	20	2
5	–	–	10	4.2
10	–	–	1	9
30	4	1.2	<0.05	42
60	<0.05	2.2	–	–

[a] Spores of *B. subtilis* PS533 (strain 168 pUB110 Km^r) (wild-type) and PS578 (strain 168 Δ*sspA* Δ*sspB* pUB110 Km^r) ($\alpha^-\beta^-$) (12) were prepared and cleaned as previously described (11). Spores at an optical density at 600 nm of ~100 were heated at ~85°C for various lengths of time and cooled in ice, and DNA was isolated and purified as previously described by proteinase K and ribonuclease digestion and exhaustive dialysis (5, 7, 12). The viability of spores after heat treatment was determined as described previously (2). The number of aldehydes in DNA isolated from spores was determined by using the ARP reagent (a generous gift of H. Ide) and a calibration curve obtained with calf thymus DNA depurinated under defined conditions, as previously described (5). We have assumed that DNA aldehydes represent abasic sites.

[b] Samples treated under these conditions were not analyzed.

(Fig. 1). Plasmid pUB110 from untreated wild-type spores was almost completely in a supercoiled form, and even after 60 min of heating (<0.05% survival), only one-third to one-half of the plasmid had acquired a single nick (Fig. 1A, band a). While the plasmid isolated from untreated $\alpha^-\beta^-$ spores was slightly

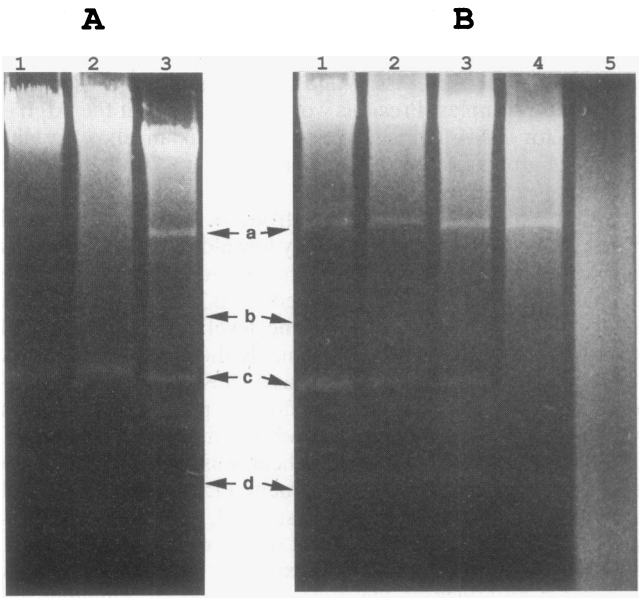

FIG. 1. Agarose gel analysis of DNA from heated and unheated wild-type and $\alpha^-\beta^-$ spores. DNA samples were obtained and purified from spores treated as described in footnote *a* of Table 1, samples were subjected to 1% agarose gel electrophoresis, and the gel was stained with ethidium bromide and photographed. The DNA samples (~3 μg) run on the various lanes were from heated and unheated wild-type (A) and $\alpha^-\beta^-$ (B) spores. (A) Lanes: 1, unheated; 2, heated for 30 min; 3, heated for 60 min. (B) Lanes: 1, unheated; 2, heated for 3 min; 3, heated for 5 min; 4, heated for 10 min; 5, heated for 30 min. The arrows labeled a, b, c, and d denote the migration positions of relaxed pUB110, linear pUB110 (4.5 kb), supercoiled pUB110, and a 1.9-kb marker, respectively.

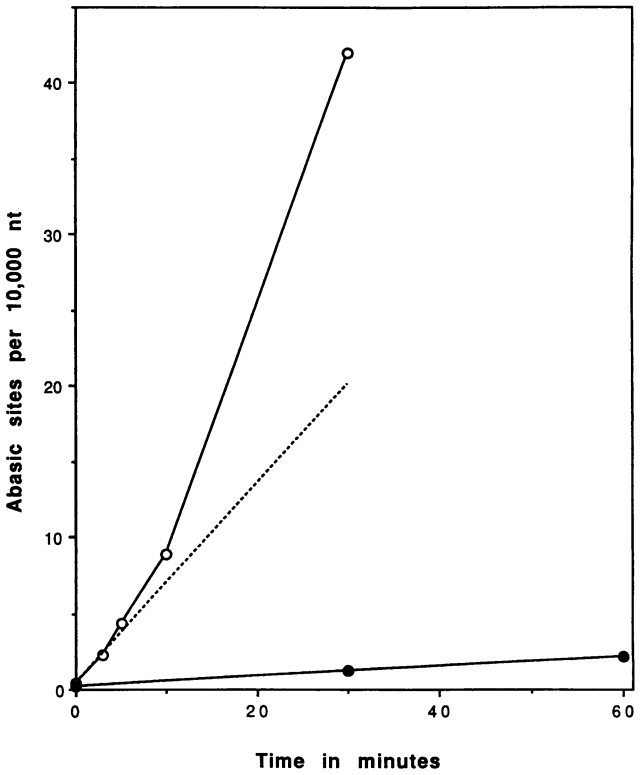

FIG. 2. Accumulation of abasic sites in DNA from $\alpha^-\beta^-$ (○) and wild-type (●) spores as a function of time of heating at 85°C. The data are from Table 1. The first three datum points for $\alpha^-\beta^-$ spores (Table 1) have been used to generate a linear rate of generation of abasic sites as a function of time (dashed line).

nicked, the plasmid from $\alpha^-\beta^-$ spores heated for 10 min (1% survival) was essentially completely nicked (Fig. 1B, band a in lanes 1 and 4). Significant fragmentation of chromosomal as well as plasmid DNA was also obtained with even shorter heating times of $\alpha^-\beta^-$ spores, while 30-min heating resulted in massive DNA fragmentation (Fig. 1B). Analysis of these DNA samples by electrophoresis on denaturing agarose gels following *Eco*RI digestion also showed that there was little breakdown of DNA from heated wild-type spores but much more breakdown in heated $\alpha^-\beta^-$ spores (data not shown), as found previously (2). The general agreement between the level of DNA aldehydes and the degree of single-strand breakage further suggests that there is strand cleavage at the majority of the abasic sites generated by heat treatment of $\alpha^-\beta^-$ spores, with retention of the aldehyde adjacent to a 2,3-unsaturated deoxyribose in the DNA (see below). Previous work (5) has shown that aldehydes adjacent to double bonds are detected by the ARP reagent in DNA.

The rate of generation of abasic sites (i.e., aldehydes) increased with the time of heating of $\alpha^-\beta^-$ spores, but in wild-type spores abasic sites were generated only at a slow constant rate (Fig. 2). Increasing rates of generation of abasic sites at longer heating times have been seen previously in vitro (10). Presumably DNA which has undergone significant base loss with accompanying strand cleavage at the abasic site denatures readily, and denatured DNA undergoes base loss at a rate four- to fivefold faster than native DNA does (8–10). Using the initial more-linear portion of the curve for base loss as a function of heating time of $\alpha^-\beta^-$ spores and the data for

wild-type spores, the calculated rates are 40 and 2 abasic sites generated per 10^4 nt per h in $\alpha^-\beta^-$ and wild-type spores, respectively, at 85°C (Fig. 2). These rates indicate that α/β-type SASP retard the rate of DNA base loss in vivo by approximately a factor of 20, which is similar to the minimum value found previously for retardation of DNA depurination in vitro by α/β-type SASP (2). DNA depurination should represent the majority of the base loss in vivo since depyrimidination is much slower (9). By using published data for the dependence of the DNA depurination rate in vitro on pH and temperature (10), the rate at 85°C and pH 6.3 (that of the *Bacillus megaterium* spore [14]) can be calculated as ~10 abasic sites generated per 10^4 nt per h. This rate is significantly slower than determined in vivo for $\alpha^-\beta^-$ spores. However, the conditions inside the spores during heating are certainly extremely different from the conditions under which DNA depurination rates were measured in vitro. Consequently, the difference between depurination rates in vitro and in $\alpha^-\beta^-$ spores may not be unreasonable.

From the data presented in this communication, it is clear that base loss from DNA is much faster in $\alpha^-\beta^-$ spores than in wild-type spores during heating; presumably, there is also rapid phosphodiester bond cleavage at these abasic sites. While cleavage at the abasic sites may not be quantitative, previous work in which DNA from heated $\alpha^-\beta^-$ spores was analyzed by agarose gel electrophoresis before and after treatment with an apurinic endonuclease indicated that the number of uncleaved abasic sites in this DNA was <50% of the number of single-strand breaks (2). Although it is formally possible that a DNA glycosylase might catalyze DNA cleavage at an abasic site in spores, this seems unlikely, given the general absence of activity of enzymes in spores (18). Consequently, we suggest that rapid phosphodiester bond cleavage in the DNA in heated $\alpha^-\beta^-$ spores takes place nonenzymatically, as is not unreasonable at the elevated temperature used in these experiments (8). The aldehyde generated by the initial depurination event remains with the DNA after strand cleavage by β-elimination (1, 8). While a second β-elimination reaction will remove the aldehyde from the DNA, this second cleavage reaction is generally significantly slower than the first at near-neutral pH (1, 8). Given the high degree of mutagenesis accompanying the heat killing of $\alpha^-\beta^-$ spores and since abasic sites with or without accompanying single-strand breakage can be mutagenic, it is certainly reasonable to suggest that it is the initial generation of abasic sites during heat treatment of $\alpha^-\beta^-$ spores that results in their mutagenesis and killing. In contrast, wild-type spores accumulate many fewer abasic sites at similar levels of heat killing. Presumably wild-type spores are killed by heat through damage other than that to DNA, as we have argued previously (2, 17). It is of course very difficult to prove definitively that the abasic sites generated by heat treatment of $\alpha^-\beta^-$ spores are not simply an effect of spore heat inactivation, rather than the direct cause of heat killing and mutagenesis. However, since heat inactivation of wild-type spores does not result in high levels of abasic sites in spore DNA, it seems more likely that α/β-type SASP play a direct role in retarding DNA depurination and depyrimidination in spores, thus slowing spore heat killing and greatly reducing heat-induced mutagenesis. Given the long periods over which spores of *Bacillus* species can survive and the relative instability of the N glycosylic bond in DNA (1, 2, 10, 19), it is possibly not unexpected that mechanisms such as α/β-type SASP binding have evolved to stabilize this potentially labile bond in a molecule whose integrity is essential to spore survival.

We are extremely grateful to and appreciative of Hiroshi Ide for the generous gift of the ARP reagent and instructions in its use. Without his gift this work would not have been completed.

This work was supported by a grant from the Army Research Office.

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