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Heat Killing of *Bacillus subtilis* Spores in Water Is Not Due to Oxidative Damage

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The heat resistance of wild-type spores of *Bacillus subtilis* or spores (termed $\alpha^-\beta^-$) lacking DNA protective α/β-type small, acid-soluble spore proteins was not altered by anaerobiosis or high concentrations of the free radical scavenging agents ethanethiol and ethanedithiol. Heat-killed wild-type and $\alpha^-\beta^-$ spores exhibited no increase in either protein carbonyl content or oxidized bases in DNA. These data strongly suggest that oxidative damage to spore macromolecules does not contribute significantly to spore killing by heat.

Spores of various *Bacillus* species are extremely resistant to heat (16). Although a complete understanding of the mechanism of spore heat resistance has not yet been achieved, it has been established that spore heat resistance is due to a number of different factors, including (i) the growth temperature of the corresponding sporulating cells; (ii) the relative mineralization and dehydration of the spore core; and (iii) the protection of spore DNA against depurination by the binding of small, acid-soluble proteins (SASP) of the α/β type (13, 16, 26, 35, 37). However, we know less about the mechanism(s) by which heat treatment causes spore death. Heat killing of wild-type spores is generally not accompanied by DNA damage, as spore DNA is protected against a variety of types of damage by the saturation of the DNA with α/β-type SASP (37). In contrast, DNA damage, in particular depurination, appears to be a major cause of the heat killing of spores lacking the majority of their α/β-type SASP (termed $\alpha^-\beta^-$ spores) (13, 35, 37). However, heat killing of wild-type spores is associated with protein denaturation and enzyme inactivation (3, 41). While some enzymes are stable even within heat-killed spores, others appear to be inactivated in parallel with spore heat killing (41). Although these data suggest that denaturation of one or more spore proteins may be important in spore heat killing, it is not clear if this protein denaturation (or inactivation) is the cause of spore killing by heat or only an effect. An alternative hypothesis of spore heat killing is that heat causes formation of free radicals within the spore, and that such free radicals result in lethal oxidative damage (26). Support for this hypothesis has come from studies with growing or stationary phase cells of *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae*, which have shown that the loss of enzymes which scavenge intracellular oxidizing agents (hydrogen peroxide and superoxide) results in sensitization of these cells to heat (4, 9). In addition, even wild-type yeast strains are much more heat resistant when heated anaerobically and thus in the absence of a major exogenous source of oxidizing power (9). These findings prompted us to investigate whether heat killing of spores of *B. subtilis* might be due at least in part to oxidative damage. We carried out these analyses with wild-type *B. subtilis* spores as well as $\alpha^-\beta^-$ spores, which are significantly more heat sensitive than wild-type spores and whose DNA is poorly protected against oxidative damage (37).

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If heat killing of spores is due in part to oxidative damage possibly caused by free radicals generated within spores upon heat treatment, an obvious source of free radicals (either directly or indirectly) is oxygen. Consequently, we initially compared spore heat resistance under both aerobic and anaerobic conditions. Spores of the isogenic *B. subtilis* PS832 (wild type) and PS356 ($\alpha^-\beta^-$) (27) strains were prepared at 37°C in 2× SG medium and cleaned and stored as previously described (28). Spores of PS533 and PS578, which are isogenic with strains PS832 and PS356 but contain plasmid pUB110, conferring kanamycin resistance, were prepared and purified similarly. Spores (1 ml) at an optical density at 600 nm of 1.0 were incubated overnight at room temperature in vials either open to the air with no additions (aerobic) or sealed under vacuum and containing 2 mM dithiothreitol (DTT) and 1 μg of resorufin/ml (anaerobic). The resorufin is an indicator of the oxidation-reduction potential of the solution and was generated from resazurin by autoclaving (6). The anaerobic samples were initially pink but turned colorless overnight, indicating a low oxidation-reduction potential (i.e., anaerobiosis [6]), although neither the DTT nor the resorufin should penetrate the spore core (15). Analysis of spore heat killing as described previously (27) showed that there was no difference in the heat resistance of the aerobic and anaerobic samples (Table 1). The effect of ethanethiol and ethanedithiol on spore heat resistance was also analyzed. These compounds were chosen not only because their thiol groups make them potential free radical scavengers, but also because their small size and relative hydrophobicity should allow them to penetrate the spore core (17). Although previous work has shown that thiol compounds protect spores against exogenous oxidizing agents (38), ethanethiol and ethanedithiol had no significant effect on spore heat resistance (Table 2).

The results described above suggest that oxidative damage is not a major factor in heat killing of spores in water. However, to test this point further, we analyzed heat-killed spores directly for oxidative damage to both proteins and DNA. The protein damage for which we analyzed was carbonyl formation, which has been shown to result from metal-catalyzed oxidation of proteins (39, 40). For analysis of protein carbonyls, spores (~30 mg [dry weight]) were suspended in 5 ml of water and then either heated or not heated. The spores were centrifuged, suspended in 2.5 ml of 50 mM Tris-HCl (pH 8.0)–1% sodium dodecyl sulfate–8 M urea–50 mM DTT–10 mM EDTA, and incubated for 90 min at 37°C to remove spore coats and render the spores sensitive to lysozyme (28). The decoated spores were washed five times by successive resuspension and centrif-

TABLE 1. Effect of anaerobiosis on heat killing of wild-type and $\alpha^-\beta^-$ *B. subtilis* spores[a]

Treatment conditions	% of spores surviving[b]	
	Wild type (PS832)	$\alpha^-\beta^-$ (PS356)
90°C, aerobic	2.9 ± 0.4	
90°C, anaerobic	3.0 ± 0.4	
80°C, aerobic		1.0 ± 0.3
80°C, anaerobic		1.2 ± 0.3

[a] Spores were prepared and cleaned as described in the text. Spores were suspended in vials under either aerobic or anaerobic conditions as described in the text. Both types of vials were incubated overnight at 10°C, during which time anaerobiosis was achieved in the anaerobic vials. The aerobic vials were then sealed without evacuation, and vials were either heated for 30 min or not heated, and were cooled on ice for ~30 min. The vials were opened, and viable spores were determined as described in the text.

[b] Values are the averages of duplicate determinations in three separate experiments ± the standard deviations.

ugation in 2.5 ml of 10 mM KPO_4 (pH 7)–10 mM EDTA–150 mM NaCl and suspended in 2.5 ml of the same buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 16 μg of lysozyme/ml. After incubation at 37°C for 60 min to achieve spore lysis, the sample was chilled on ice, and 275 μl of 10% streptomycin sulfate was added to precipitate nucleic acids. After 30 min on ice, the samples were centrifuged, and the supernatant fraction was dialyzed overnight in the cold room against 1 liter of 2 mM KPO_4 (pH 7)–0.1 mM PMSF with one change. The dialyzed material was centrifuged and any precipitate was discarded, the supernatant fluid was lyophilized, and the dry material was dissolved in ~0.5 ml of water. This procedure yielded 2 to 3 mg of soluble protein from unheated spores and 1 to 2 mg of protein from heated spores. A lower recovery of soluble protein from heated spores has been observed previously (41). Protein carbonyl content was determined by a modification of a published procedure (24) and is expressed here relative to total soluble protein determined by the procedure of Lowry et al. (25). Specifically, soluble protein was obtained from heated or unheated spores and carbonyls in soluble protein determined by reaction with 2,4-dinitrophenylhydrazine in 0.4 M HCl for 60 min at 24°C, removal of excess 2,4-dinitrophenylhydrazine by precipitation of proteins with trichloroacetic acid, rinsing of the pellet fraction with ethanol/ethyl acetate (1/1), dissolving of the pellet in 6 M guanidine hydrochloride at pH 2.5, and reading of the optical density at 370 nm against a guanidine hydrochloride blank (24). Heating was done at 90 to 95°C for 30 to 60 min for wild-type spores, giving <0.001 to 3% survival. No differences in levels of protein carbonyls based on the time of treatment were seen. The $\alpha^-\beta^-$ spores were heated for 60 min at 90°C, resulting in <0.01% survival. Values for protein carbonyl content in soluble protein from unheated wild-type and $\alpha^-\beta^-$ spores were similar to values obtained in protein from stationary phase bacteria (18). Specifically, the protein carbonyl levels (expressed in nanomoles per milligram of protein ± the standard deviation) in protein from wild-type and $\alpha^-\beta^-$ spores were 1.3 ± 0.3 (9) and 1.3 ± 0.4 (7) (numbers in parentheses are the numbers of determinations [in duplicate] made; these represent at least four independent protein isolations from both treated and untreated spores). However, there was no significant increase in the level of protein carbonyls in wild-type or $\alpha^-\beta^-$ spores killed at levels of >99% by heat. For wild-type and $\alpha^-\beta^-$ spores these levels were 1.4 ± 0.3 (7) and 1.4 ± 0.4 (8), respectively (numbers in parentheses indicate number of determinations made). In contrast, others have found significant increases in levels of protein carbonyls in organisms which have been subjected to oxidative damage (39, 40).

TABLE 2. Effect of ethanethiol and ethanedithiol on heat killing of wild-type and $\alpha^-\beta^-$ spores of *B. subtilis*[a]

Treatment conditions[b]	% of spores surviving[c]	
	Wild type (PS832)	$\alpha^-\beta^-$ (PS356)
Experiment 1		
90°C, no additions	1.0 ± 0.4	
90°C, plus ethanethiol[d]	0.5 ± 0.2	
80°C, no additions		1.2 ± 0.4
80°C, plus ethanethiol[d]		1.5 ± 0.5
Experiment 2		
90°C, no additions	0.3 ± 0.1	
90°C, plus ethanedithiol[d]	0.3 ± 0.1	
80°C, no additions		2.9 ± 1
80°C, plus ethanedithiol[d]		2.0 ± 1

[a] Spores were prepared and cleaned as described in the text and suspended in water in capped tubes with or without 25 mM ethanethiol or ethanedithiol. After incubation overnight at 10°C, tubes were either heated for 30 min or not heated and cooled in ice for 30 min, and samples were plated to determine viable counts.

[b] Values are percentages ± standard deviations and are the averages of quadruplicate determinations in at least two separate experiments.

[c] Different preparations of spores were used for experiments 1 and 2, and for the experiment in Table 1.

[d] Obtained from the Aldrich Chemical Company.

The DNA damage for which we initially assayed was oxidized or ring-opened guanine residues, which can arise following treatment of DNA with oxidizing agents (10, 20). For these analyses, heated or unheated spores (5 mg [dry weight]) carrying plasmid pUB110 were decoated and washed as described above. Spores were lysed and DNA was purified by using RNase and Qiagen columns as described previously (36), and the final DNA was dissolved in 100 μl of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA. For assay of the percentage of oxidized bases, DNA samples (~200 ng) were digested in 25 μl of 70 mM HEPES-KOH (pH 7.6), 0.1 M KCl, and 1 mM EDTA with 60 ng of purified *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) (5, 15), which was a gift from Jacques Laval (Institute Gustave-Roussy, Villejuif, France). Control experiments showed that this amount of Fpg gave complete nicking at oxidized guanine residues in DNA (data not shown). After incubation at 37°C for 15 min, aliquots (~60 ng) were analyzed by agarose gel electrophoresis, DNA was transferred to Hybond N membranes (Amersham), and plasmid pUB110 sequences were detected by hybridization (34). The percentage of the 4.5-kb pUB110 in nicked and supercoiled forms was then determined by densitometric quantitation of autoradiograms of the hybridizing bands, with calculations performed as previously described (12, 34). Control experiments showed that the amounts of DNA and exposures used resulted in a linear response between autoradiographic exposure and DNA concentration (data not shown). Analysis of DNA by this procedure (Table 3) has led to a number of conclusions. First, untreated DNA had some nicks even without Fpg treatment; the number of nicks was higher in $\alpha^-\beta^-$ spores, and this value increased significantly with heat treatment of $\alpha^-\beta^-$ spores but not wild-type spores. These observations have been made previously, and the increased DNA nicking in $\alpha^-\beta^-$ spores has been ascribed to cleavage in vivo or during DNA isolation at abasic sites, which are generated readily in $\alpha^-\beta^-$ spores on storage or by heat (13, 35). Second, Fpg treatment of DNA from either wild type or $\alpha^-\beta^-$ spores does cause some increased nicking; however, the amount of the Fpg dependent

TABLE 3. Fpg-sensitive damage in DNA from heat-killed *B. subtilis* spores[a]

Spore type[b]	Degree of heat killing[c]	No. of nicks/molecule of pUB110[d]	
		With no treatment	With Fpg added
Wild type	None	0.2	0.5
Wild type	86% (1 h)	0.2	0.6
Wild type	99.98% (4 h)	0.3	0.6
$\alpha^-\beta^-$	None	0.9	1.4
$\alpha^-\beta^-$	98% (30 min)	1.7	2.2

[a] Heated or unheated spores were decoated, DNA was isolated, and the number of nicks per pUB110 molecule with or without Fpg treatment was quantitated as described in the text.

[b] The strains used for this experiment were PS533 (wild type) and PS578 ($\alpha^-\beta^-$), which are isogenic with PS832 and PS356, respectively, but carry pUB110.

[c] Spores were heated at 80°C (PS578) or 85°C (PS533) for various times (shown in parentheses), and the degree of spore killing was determined as described in the text.

[d] All values are averages of quadruplicate determinations except those for the non-heat-treated samples, which are averages of duplicate determinations from four different DNA samples. All values are ±25%.

nicking did not increase with spore heat killing. We do not know the reason for the Fpg-dependent nicking in the DNAs tested; this may be due to cleavage at oxidized guanine residues or abasic sites formed during DNA isolation (Fpg cleaves DNA at abasic sites as well as at modified guanine residues [5, 15]) or at both types of lesions. Third, whatever the source of the Fpg-sensitive sites in spore DNA, it is clear that the number of these sensitive sites does not increase with spore killing by heat. Consequently, these data indicate that 2 to 4 logs of spore killing by heat causes generation of less than one oxidized guanine residue per 25 kb. A similar conclusion was drawn concerning the generation of oxidized thymine residues by spore heat killing by using sensitivity to cleavage by *E. coli* endonuclease III (Nth) (1, 11, 22) to assess the presence of oxidized pyrimidine residues (data not shown).

The data reported in this communication do not support the hypothesis that killing of spores of *B. subtilis* in water by heat occurs through oxidative damage. There are also data in the literature which contradict this hypothesis. Spores of *Clostridium botulinum* and several *Bacillus* species in water were more heat resistant in an aerobic atmosphere than in an anaerobic atmosphere (23). However, the anaerobic atmosphere used contained hydrogen gas, which appeared to stimulate spore heat killing, possibly by generating hydrogen ions or radicals upon reacting with spore metal ions (23). There have also been several thorough studies of the effect of anaerobiosis on heat killing of dry spores (30, 31). These studies showed a very slight increase in the heat resistance of spores treated anaerobically. However, there is good evidence that the mechanisms of heat killing of spores in water and in the dry state are different in at least one respect (37). We also note that loss of enzymes (i.e., catalase and superoxide dismutase, which detoxify oxidizing agents) sensitizes growing *B. subtilis* cells to oxidizing agents and heat (in the case of loss of superoxide dismutase) but has no effect on spore heat resistance (8). These enzymes may be important in the sporulating cell, either in providing resistance to oxidizing agents (catalase) (2) or in the maturation of the spore coat (superoxide dismutase) (21), but it is quite possible that these enzymes are not active within the dormant spore (8).

The lack of any obvious oxidative damage to protein in heat-killed spores is further evidence that spore heat killing is not due to oxidative damage, although we cannot rule out a small amount of oxidative damage to some crucial protein as the mechanism for lethal heat damage. One possible protein target might be some component of the spore germination apparatus. However, spores which are heat killed can often still initiate spore germination (41).

A second possible target for oxidative damage generated by heat is DNA, which is subject to a variety of types of oxidative damage (10, 20). However, the DNA in *B. subtilis* spores is so well protected by α/β-type SASP that heat treatment of wild-type spores generates neither mutations in the survivors nor obvious DNA damage (37). Even killing treatment with oxidizing agents such as peroxides does not generate mutations in wild-type *B. subtilis* spores (33, 34–37). The obvious inference from this work is that heat treatment cannot kill wild-type spores by generation of oxidative damage in spore DNA, and this is consistent with our lack of detection of any increased base oxidation in DNA from heat-killed wild-type spores. In the absence of α/β-type SASP (i.e., in $\alpha^-\beta^-$ spores), heat treatment does generate high levels of both mutations and DNA damage (13, 35). Although much of the latter DNA damage appears to be due to depurination, which is normally blocked by α/β-type SASP (13, 35, 37), it is possible that some of the DNA damage caused by heat treatment of $\alpha^-\beta^-$ spores is due to oxidative reactions. However, we saw no evidence of DNA oxidation in heat-treated $\alpha^-\beta^-$ spores; the lack of effect of anaerobiosis, ethanethiol, and ethanedithiol on the heat resistance of $\alpha^-\beta^-$ spores further argues against the idea that heat treatment of these spores causes oxidative damage to DNA.

If it is not through oxidative damage, what is the mechanism (and the target) for heat killing of spores? As noted above, there is evidence associating protein denaturation and enzyme inactivation with spore heat killing (3, 41). It has also been observed that recovery of soluble protein from heat-killed spores is decreased (reference 41 and this work). These data suggest that protein denaturation takes place in heat-killed spores. Possibly some crucial spore protein is the initial target for heat killing, with this protein being denatured by heat. Although the identity of this protein is unknown, there are other data consistent with a protein as the target for spore heat killing. Heat shock of *Bacillus megaterium* cells at approximately the second hour of sporulation greatly increases the heat resistance of the resulting spores, and it has been suggested that this effect is mediated through the heat shock response (32). Many genes are turned on in response to heat shock in bacteria, which increases the cell's ability to deal with an increase in the amount of denatured proteins by either refolding or degrading them (29, 43). Heat treatment can also cause spore injury from which spores can recover during germination and outgrowth (7). Perhaps this injury is to one or more spore proteins, although spore membranes may also be the target for spore injury (14). It is also possible that the heat inactivation of some key spore protein is not through protein denaturation but through some other reaction such as deamidation of a labile asparagine residue (42). Indeed, deamidation of asparagine residues in protein does accompany spore heat killing (19). Although the identity of the protein(s) deamidated in this latter reaction is not clear, this process might also destroy some key spore protein, thus leading to spore death. Certainly, the preponderance of evidence presently available supports the hypothesis that inactivation of a key protein or proteins is the major mechanism whereby heat kills bacterial spores. The challenges now are to prove this hypothesis conclusively and to identify the protein(s) that is the target in this process.

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