Journal of Industrial Microbiology & Biotechnology (1997) 18, 384–388
© 1997 Society for Industrial Microbiology 1367-5435/97/$12.00

Killing bacterial spores by organic hydroperoxides

B Setlow, CA Setlow and P Setlow

Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06032, USA

Killing of wild-type spores of *Bacillus subtilis* by *t*-butyl hydroperoxide, cumene hydroperoxide and peracetic acid was not through DNA damage, as shown by the absence of mutations in the survivors and the identical sensitivity of spores of strains with or without a *recA* mutation. In contrast, *B. subtilis* spores (termed $\alpha^-\beta^-$) lacking the DNA protective α/β-type small, acid-soluble spore proteins (SASP) were more sensitive to *t*-butyl hydroperoxide and cumene hydroperoxide, and their killing was in large part through DNA damage, as shown by the high frequency of mutations in the survivors and the greater sensitivity of $\alpha^-\beta^-$ *recA* spores. Analysis of *t*-butyl hydroperoxide-treated spores showed that generation of DNA damage in $\alpha^-\beta^-$ spores was more rapid than in wild-type spores; α/β-type SASP also protected against DNA strand breakage *in vitro* caused by *t*-butyl hydroperoxide. α/β-Type SASP appeared to play no role in protection of spores from killing by peracetic acid; wild-type and $\alpha^-\beta^-$ spores exhibited identical peracetic acid sensitivity and their killing by this agent appeared to be not through DNA damage.

Keywords: spores; resistance to organic hydroperoxides; *Bacillus*; DNA damage; mutagenesis; DNA protection

Introduction

Organic hydroperoxides such as cumene hydroperoxide (cuOOH), peracetic acid (perOOH) and *t*-butyl hydroperoxide (tbutOOH) kill a wide variety of bacteria [1,2,7]. The mechanism of cell killing by these agents is not clear, but likely involves the production of damaging free radicals. These radicals then kill cells at least in part through DNA damage, as a number of organic hydroperoxides are mutagenic in bacteria [7,11,23].

In addition to killing vegetative cells, organic hydroperoxides also kill spores of Gram-positive bacteria such as those of various *Bacillus* species [9,15,22]. Indeed, perOOH is used in some sterilization/disinfection applications [3]. However, killing of spores by organic hydroperoxides requires harsher treatments than does killing of the corresponding vegetative cells. While there are data consistent with spore killing by organic hydroperoxides being due to free radicals generated from these compounds, the target(s) of these radicals in spores is not clear [9,22]. In contrast, hydrogen peroxide kills spores of wild-type *B. subtilis* possibly by damaging a protein(s), but not through DNA damage [14,16,18]. In wild-type spores the DNA is saturated with a group of small, acid-soluble proteins (SASP) of the α/β-type that protect spore DNA from many types of damage, including hydroxyl radicals generated from hydrogen peroxide [16,17,19,20]. Spores lacking major α/β-type SASP ($\alpha^-\beta^-$ spores) are more sensitive to hydrogen peroxide, and are killed in large part through DNA damage [16]. In this work the ability of various organic hydroperoxides to kill wild-type and $\alpha^-\beta^-$ spores of *B. subtilis* as well as spores of the *recA* derivatives of these strains has been examined; several different methods have also been used to examine the survivors of these treatments for DNA damage.

Correspondence: P Setlow, Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06020, USA
Received 17 December 1996; accepted 13 March 1997

Materials and methods

Spores

The *B. subtilis* strains used [18] are: PS533, wild-type, carrying plasmid pUB110 (conferring kanamycin resistance); PS578, $\alpha^-\beta^-$, with deletions in the two genes (*sspA* and *B*) coding for the two major α/β-type SASP and also carrying pUB110; PS2318, *recA*260, isogenic with PS533 (except for lack of pUB110) and carrying both chloramphenicol (Cm^r) and erythromycin resistance (Ery^r); and PS2319, $\alpha^-\beta^-$*recA*260 (Cm^rEry^r), isogenic with PS578 (except for lack of pUB110). Spores of these strains were prepared at 37°C in 2× SG medium [13,18]; sporulation was in the dark for *recA* strains. Spores were purified [13] over a period of ~2 weeks, and were stored in water in the dark at 10°C. All spore preparations used were free (>95%) of vegetative cells and germinated spores. Cells were grown at 37°C in LB medium (per liter, 10 g NaCl, 1 ml 1 M NaOH, 10 g tryptone, 5 g yeast extract) and harvested at an $OD_{600\ nm}$ of ~0.5 (mid-log phase growth).

Measurement of killing and mutagenesis by organic hydroperoxides

Treatment of cells or spores (5×10^7–10^8 ml^{-1}) by organic hydroperoxides was in 50 mM potassium phosphate (pH 7.0), except that spores treated with perOOH were in 0.2 M potassium phosphate (pH 7.0). PerOOH and tbutOOH were added from water solutions; cuOOH was added from dimethylsulfoxide. There was no killing of cells or spores incubated under killing conditions without hydroperoxides, nor did the dimethylsulfoxide added cause killing. At various times samples of cell or spore incubations were diluted either in water (spores) or LB medium (cells) and plated to determine viable count [9,16,18,22]. At least 200 colonies from spores before or after hydroperoxide treatment were picked onto both minimal medium and sporulation agar plates in order to determine the frequency of mutations [4,16].

Analysis of DNA from treated spores or treated DNA in vitro

Spores (3–5 mg dry wt) were harvested by centrifugation before or after incubation with perOOH or tbutOOH, and their survival was determined. The harvested spores were washed three times with water, spore coats removed, the spores opened with lysozyme, and total spore nucleic acid was isolated [16]. Samples of the total nucleic acid were run on 1% agarose gel electrophoresis, the gel stained with ethidium bromide and photographed, and the nucleic acid transferred to a Hybond-N membrane (Amersham, Arlington Heights, IL, USA). Plasmid pUB110 sequences were detected on the membrane by hybridization with a pUB110 probe [4,16].

Treatment of purified plasmid pUC19 with tbutOOH *in vitro* was as described [21]. Supercoiled pUC19 (0.1 μg μl^{-1}) was incubated in 20 μl of 10 mM potassium phosphate (pH 7.0) with 2 mM reduced glutathione, 10 mM tbutOOH, and 1 mM $NiCl_2$ with or without 500 μg ml^{-1} of SspC. The latter is an α/β-type SASP which saturates pUC19 under these conditions [4,16,19]. SspC was purified as described [12]. After incubation at 50°C for 8 h, samples were made 25 mM in EDTA and 0.4% in N-lauroylsarcosine (sarkosyl), and aliquots were run on 1% agarose gel electrophoresis and the gel stained wtih ethidium bromide.

Results

Killing and mutagenesis of spores by organic hydroperoxides

Wild-type spores of *B. subtilis* were killed by 27 mM cuOOH (~90% killing in 100 min at 60°C) and 0.73 M tbutOOH (70% killing in 90 min at 47°C) (Figure 1a,b). However, growing cells were more sensitive to these agents; 5.5 mM cuOOH gave 90% killing in 5 min at 21°C, while 0.25 M tbutOOH gave 90% killing in 20 min at 21°C (data not shown). The cuOOH- and tbutOOH-resistance of otherwise wild-type spores which carried a *recA* mutation resulting in loss of much DNA repair capacity was nearly the same as that of wild-type spores (Figure 1a,b), indicating that *recA*-dependent DNA repair is not involved in spore resistance to these agents. However, spores lacking the majority of the DNA protective α/β-type SASP ($\alpha^-\beta^-$ spores) were more sensitive to cuOOH and tbutOOH than were wild-type spores (Figure 1a,b; note lower temperatures for treatment of $\alpha^-\beta^-$ spores). In contrast to the results with wild-type spores, the cuOOH and tbutOOH sensitivity of $\alpha^-\beta^-$ spores was increased by a *recA* mutation (Figure 1a,b).

As shown above for cuOOH and tbutOOH, spores were also more resistant to killing by 5.5 mM perOOH (90% killing in 3 min at 28°C (Figure 1c)) than were growing cells (90% killing in 10 min at 20°C in 11 μM perOOH; $>$ 99% killing in 3 min at 28°C in 5.5 mM perOOH (data not shown)). However, there was no significant difference in the perOOH sensitivity of wild-type and $\alpha^-\beta^-$ spores (Figure 1c), and a *recA* mutation had no effect on spore perOOH sensitivity—even in $\alpha^-\beta^-$ spores (data not shown). These data were consistent with cuOOH and tbutOOH killing $\alpha^-\beta^-$ spores but not wild-type spores through DNA damage, and with DNA damage not being important in perOOH killing of both wild-type and $\alpha^-\beta^-$ spores.

To obtain further information on the mechanism(s) of spore killing by organic hydroperoxides, the survivors of spores treated with these agents were analyzed for auxotrophic and asporogenous mutations (Table 1). Survivors of wild-type spores treated with any of the three organic hydroperoxides exhibited no increase in mutants. Survivors of treatment of $\alpha^-\beta^-$ spores with perOOH also exhibited no increase in mutants as compared to untreated spores. However, $\alpha^-\beta^-$ spores treated with either cuOOH or tbutOOH contained at least 13–16% mutants among the survivors.

Analysis of hydroperoxide damage to DNA in vivo and in vitro

Since the data indicated that some organic hydroperoxide treatments killed $\alpha^-\beta^-$ spores by DNA damage, the DNA from treated spores was analyzed for damage. DNA strand breaks were analyzed, since single strand breaks result in

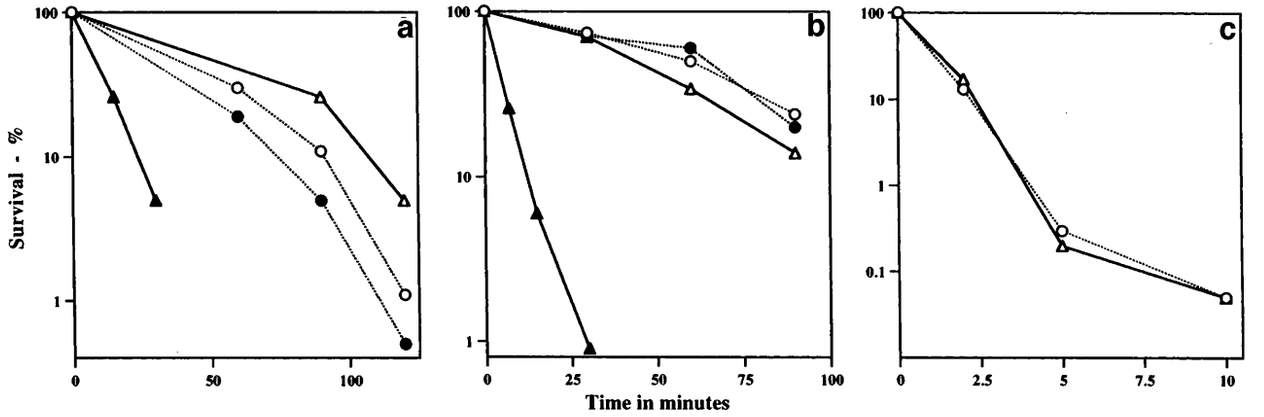

Figure 1 Killing of spores with (a) cuOOH, (b) tbutOOH, and (c) perOOH. Spores were incubated with organic hydroperoxides and spore killing was measured as described in Methods. The conditions used for spore killing were: (a) strains (○) PS533 (wt) and (●) PS2318 (*recA*) – 27 mM cuOOH at 60°C; strains (△) PS578 ($\alpha^-\beta^-$) and (▲) PS2319 ($\alpha^-\beta^-$ *recA*) – 27 mM cuOOH at 50°C; (b) strains (○) PS533 (wt) and (●) PS2318 (*recA*) – 0.73 M tbutOOH at 47°C; strains (△) PS578 ($\alpha^-\beta^-$) and (▲) PS2319 ($\alpha^-\beta^-$ *recA*) – 0.73 M tbutOOH at 37°C; and (c) strains (○) PS533 (wt) and (●) PS578 ($\alpha^-\beta^-$) – 5 mM perOOH at 28°C.

Table 1 Survival and mutagenesis of wild-type and $\alpha^-\beta^-$ spores after organic hydroperoxide treatment[a]

Spores treated	Treatment	Survival (%)	Mutants (%)		
			aux	spo	aux spo
PS533 (wt)	None	100	0	0	0
	120 min, 60°C, cuOOH	4	0.5	0.5	0
	90 min, 50°C, tbutOOH	9	0	0	0
	4 min, 28°C, perOOH	2	0	0	0
PS578 ($\alpha^-\beta^-$)	None	100	0	0.5	0
	120 min, 50°C, cuOOH	2	8	7	1
	90 min, 37°C, tbutOOH	6	7	5	1
	4 min, 28°C, perOOH	3	0	0.5	0

[a]Spores were treated with cuOOH (27 mM), tbutOOH, (0.73 M) or perOOH (5 mM) and survival determined, and the percentage of survivors that were auxotrophic (aux) or asporogenous (spo) or both auxotrophic and asporogenous (aux spo) mutants determined as described in Methods. At least 200 survivors of each treatment were tested for mutations.

conversion of supercoiled plasmid to a nicked form, while double strand breaks (or closely spaced single strand breaks) reduce the size of chromosomal DNA and linearize plasmid DNA; these changes can be assessed by agarose gel electrophoresis. Examination of DNA from untreated wild-type or $\alpha^-\beta^-$ spores on ethidium bromide-stained agarose gels showed that: (i) the chromosomal DNA ran as a smear larger than the 23-kb size marker; and (ii) the majority of plasmid pUB110 was in a supercoiled form, with small amounts of nicked circular plasmid (Figure 2, lanes 1, 3, 5, 7; Figure 3, lanes 1, 3); the amount of nicked circular plasmid was higher in untreated $\alpha^-\beta^-$ spores as found previously [4,16]. However, a significant amount of the chromosomal DNA from wild-type and $\alpha^-\beta^-$ spores

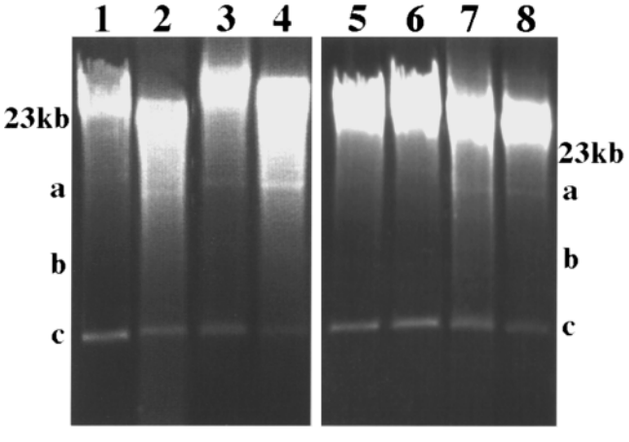

Figure 2 Analysis of total DNA from tbutOOH- and perOOH-treated spores. Nucleic acid was extracted from spores of strains PS533 (wild-type) (lanes 1, 2, 5 and 6) or PS578 ($\alpha^-\beta^-$) (lanes 3, 4, 7 and 8) either before (lanes 1, 3, 5, and 7) or after treatment with tbutOOH (lanes 2 and 4) or perOOH (lanes 6 and 8). The killing of wild-type and $\alpha^-\beta^-$ spores with perOOH was 91% and 84%, respectively; the tbutOOH treatment killed wild-type and $\alpha^-\beta^-$ spores to 99.7% and 91%, respectively. Samples of total nucleic acid (5 μg) were run on 1% agarose gel electrophoresis, stained with ethidium bromide and photographed. The letters adjacent to the gels denote the migration positions of: (a) nicked monomeric plasmid (pUB110); (b) linearized plasmid (4.5 kb); and (c) supercoiled monomeric plasmid. The 23 kb denotes the migration position of a 23-kb size marker.

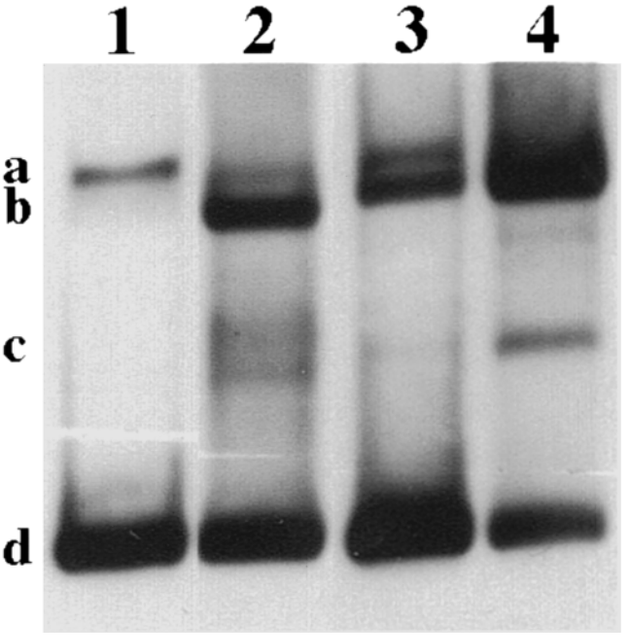

Figure 3 Analysis of plasmid pUB110 from tbutOOH-treated spores. The nucleic acid samples (5 μg) described in the legend to Figure 2 (lanes 1–4) from spores with or without tbutOOH treatment were run on agarose gel electrophoresis, the nucleic acid transferred to a Hybond-N membrane, and pUB110 sequences detected by hybridization as described in Methods. The samples run in the lanes are from: 1, untreated PS533 (wild-type) spores; 2, treated PS533 spores; 3, untreated PS578 ($\alpha^-\beta^-$) spores; and 4, treated PS578 spores. The letters adjacent to the figure denote the migration positions of: (a) supercoiled dimeric plasmid pUB110; (b) nicked monomeric circular plasmid; (c) linear plasmid (4.5 kb); and (d) supercoiled monomeric plasmid.

treated with tbutOOH was smaller than the chromosomal DNA from untreated spores, with much DNA from treated spores running as fragments smaller than 23 kb. TbutOOH treatment also converted much of the supercoiled plasmid into nicked circles, with more plasmid-nicking in $\alpha^-\beta^-$ spores; some linear plasmid DNA was generated as well (Figures 2 and 3; lanes 1–4). TbutOOH treatment of wild-type spores causing less spore killing resulted in less spore DNA damage (data not shown). In contrast to these results obtained with tbutOOH, perOOH treatment of both wild-type and $\alpha^-\beta^-$ spores resulted in no decrease in size of bulk chromosomal DNA and generated no nicked circular plasmid pUB110 as shown both by agarose gel electrophoresis and ethidium bromide staining (Figure 2, lanes 5–8), as well as detection of pUB110 sequences by hybridization (data not shown).

Since generation of DNA damage by tbutOOH required harsher treatment of wild-type spores than of $\alpha^-\beta^-$ spores, this suggests that α/β-type SASP protect the DNA backbone from attack by compounds generated by tbutOOH. To test this point the ability of SspC, a purified α/β-type SASP, to protect DNA from cleavage by tbutOOH in the presence of reduced glutathione and Ni^{2+} [21] was examined. The plasmid pUC19 used for these experiments was predominantly in the supercoiled form (Figure 4, lanes 1 and 2, compare bands labeled a and b). Incubation with tbutOOH resulted in conversion of the majority of the plasmid to a nicked circular form (Figure 4, lane 3). However,

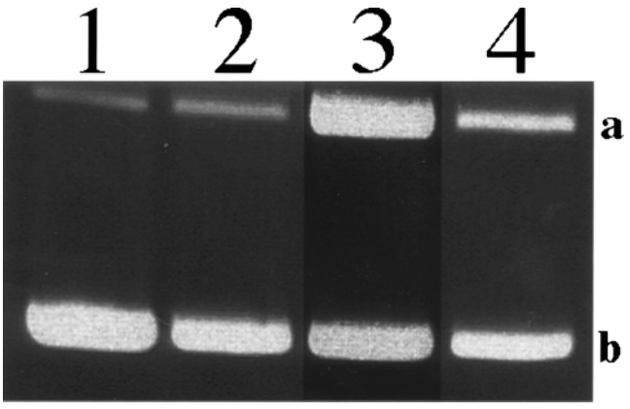

Figure 4 Protection of SspC against tbutOOH-induced DNA strand cleavage *in vitro*. Supercoiled plasmid pUC19 was incubated with reduced glutathione, Ni^{2+} and tbutOOH with or without SspC as described in Methods, and samples taken before and after incubation for 8 h at 50°C. Aliquots (1 μg, except 1.5 μg in lane 3) of DNA were run on 1% agarose gel electrophoresis, the gel was stained with ethidium bromide and photographed. The samples run in the different lanes are: 1, preincubation without SspC; 2, preincubation with SspC; 3, incubation without SspC; and 4, incubation with SspC. The letters (a) and (b) adjacent to the figure give the migration positions of nicked monomers, and supercoiled monomers of pUC19, respectively. The overall intensity in lane 3 has been reduced to facilitate visualization of the differences in bands (a) and (b) in this lane.

saturation of the plasmid DNA with SspC protected the majority of the DNA against tbutOOH-induced strand cleavage (Figure 4, lane 4). Previous work has shown that SspC protects DNA *in vitro* against strand cleavage caused by hydrogen peroxide as well as a variety of other types of DNA damage [4,16,17,19,20].

Discussion

Killing of wild-type spores of *B. subtilis* by wet heat or dessication is not due to DNA damage, as the saturation of spore DNA with α/β-type SASP prevents DNA damage associated with these treatments [4,5,19]. However, in the absence of α/β-type SASP (ie in $\alpha^-\beta^-$ spores), wet heat and dessication cause spore killing by DNA damage. Similar results have been obtained in studies of spore killing by hydrogen peroxide [16]; binding of α/β-type SASP to DNA also protects the DNA backbone against cleavage by hydroxyl radicals *in vitro* [16,19]. In the present study we have shown that killing of wild-type spores by cuOOH, perOOH, and tbutOOH is: (i) not accompanied by significant mutagenesis; and (ii) not increased by a *recA* mutation. These findings are consistent with those made previously for killing of wild-type spores by wet heat, dessication and hydrogen peroxide [4,5,16]. The conclusion from these results is that cuOOH, perOOH and tbutOOH do not kill wild-type spores by DNA damage. While this conclusion is consistent with the data, we cannot rule out the possibility that the organic hydroperoxides tested do cause lethal DNA damage in wild-type spores but that the DNA damage: (i) is not mutagenic; (ii) is not repaired in a *recA*-dependent manner; and (iii) in the case of perOOH, causes no strand breaks in DNA. While this is possible, DNA damage to growing bacteria and $\alpha^-\beta^-$ spores induced by tbutOOH and cuOOH is mutagenic and is repaired in a *recA*-dependent manner [7,22, and this work]. Furthermore, tbutOOH treatment induces the SOS response in growing bacteria [7], and induction of the SOS response is dependent on *recA*. Consequently, it is likely that DNA damage is not a significant cause of killing of wild-type spores by organic hydroperoxides, but that cuOOH and tbutOOH kill $\alpha^-\beta^-$ spores in large part by DNA damage. In addition, the DNA damage generated in $\alpha^-\beta^-$ spores by these agents is likely repairable by the SOS system, which is presumably induced during subsequent spore germination. Previous work has shown that several treatments of $\alpha^-\beta^-$ (but not wild-type) spores result in induction of SOS repair genes during spore germination [18].

The mechanism protecting spore DNA from cuOOH and tbutOOH (but not perOOH, see below) appears to be the saturation of the DNA with α/β-type SASP, as killing of $\alpha^-\beta^-$ spores by cuOOH and tbutOOH was accompanied by a high frequency of mutagenesis in the survivors and was greatly increased by a *recA* mutation. In addition, α/β-type SASP protected against tbutOOH-induced DNA cleavage *in vitro* and *in vivo*. However, some strand cleavage of DNA was induced by tbutOOH in wild-type spores, as seen previously with hydrogen peroxide [16]. Presumably, in wild-type spores the tbutOOH treatment needed to obtain DNA damage is so harsh, that the spores are already dead due to killing mechanisms that do not involve DNA damage. In $\alpha^-\beta^-$ spores the tbutOOH treatment needed for DNA damage is mild enough that the spores die at least in part from the DNA damage.

An obvious question then concerns the nature of the DNA damage induced by cuOOH and tbutOOH in $\alpha^-\beta^-$ spores. The only DNA damage analyzed for was strand breaks, in particular single strand breaks. While tbutOOH causes DNA single strand breaks both *in vivo* and *in vitro* [8,21], this may be neither a mutagenic nor a lethal event, and may not be the only DNA damage generated. The major type of mutagenic DNA damage generated by tbutOOH has been suggested to be formation of 7,8-dihydro-8-oxoguanine [10]. However, the levels of this oxidized base were not measured and is not clear if all organic hydroperoxides cause this DNA modification. Previous work has indicated that cuOOH and tbutOOH act via generation of free radical species [9,22], although the free radicals which effect spore killing have not been identified. Indeed, a large number of different free radicals may be generated from these agents [1,3,6]. As noted above the nature of the initial DNA damage caused by these free radicals in $\alpha^-\beta^-$ spores is also not known, although hydroxyl radicals can result in the cleavage of the DNA backbone both *in vivo* and *in vitro*. It is also possible that DNA strand cleavage is the result of a secondary reaction at some initial DNA lesion; as noted above it is likely that there is also DNA damage generated in $\alpha^-\beta^-$ spores by cuOOH and tbutOOH other than strand cleavage.

One surprising result from this work is that perOOH killing of spores differed significantly from that by cuOOH and tbutOOH. The identical sensitivity of $\alpha^-\beta^-$ and wild-type spores (with or without *recA* mutations) to perOOH, and the lack of any notable mutagenesis or DNA damage associated with spore killing by this agent suggests that perOOH does not kill spores, even $\alpha^-\beta^-$ spores, by DNA

damage. Previous studies have suggested that peracetic acid kills spores by a free radical-based mechanism [9]. The free radicals generated are not clear, although it has been suggested that they are produced inside the spore, since per-OOH may penetrate the spore core. Possibly the free radical(s) generated from this agent is extremely reactive with a spore target other than DNA.

Previous work has shown that hydrogen peroxide can result in enzyme inactivation in wild-type spores [14], and this may also be the case for the organic peroxides studied in the current work. While inactivation of a key spore enzyme could result in killing of wild-type spores, it is not clear why different organic hydroperoxides should exhibit such different mechanisms of killing of $\alpha^-\beta^-$ spores. The elucidation of the mechanisms whereby these organic hydroperoxides kill spores is an interesting subject for future research.

Acknowledgements

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

References

1 Akaike T, K Sato, S Ijiri, Y Miyamoto, M Kohno, M Ando and H Maeda. 1992. Bactericidal activity of alkyl peroxyl radicals generated by heme-iron-catalyzed decomposition of organic peroxides. Arch Biochem Biophys 294: 55–63.
2 Baldry MGC. 1983. The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. J Appl Bacteriol 54: 417–423.
3 Block SS. 1991. Peroxygen compounds. In: Disinfection, Sterilization and Preservation (Block SS, ed), pp 167–181, Lea and Febiger, Philadelphia, PA.
4 Fairhead H, B Setlow and P Setlow. 1993. Prevention of DNA damage in spores and *in vitro* by small, acid-soluble proteins from *Bacillus* species. J Bacteriol 175: 1367–1374.
5 Fairhead H, B Setlow, WM Waites and P Setlow. 1994. Small, acid-soluble proteins bound to DNA protect *Bacillus subtilis* spores from killing by freeze-drying. Appl Environ Microbiol 60: 2647–2649.
6 Hix S, M da Silva Morais and O Augusto. 1995. DNA methylation by tert-butyl hydroperoxide-iron (II). Free Rad Biol Med 19: 293–301.
7 Kato T, M Watanabe and T Ohta. 1994. Induction of the SOS response and mutations by reactive oxygen-generating compounds in various *Escherichia coli* mutants defective in the *mutM*, *mutY* or *soxRS* loci. Mutagenesis 9: 245–251.
8 Latour I, JB Demoulin and P Buc-Calderon. 1995. Oxidative DNA damage by *t*-butyl hydroperoxide causes DNA single strand breaks which is not linked to cell lysis. A mechanistic study in freshly isolated rat hepatocytes. FEBS Lett 373: 299–302.
9 Marquis RE, GC Rutherford, MM Faraci and SY Shin. 1995. Sporicidal action of peracetic acid and protective effects of transition metal ions. J Ind Microbiol 15: 486–492.
10 Michaels ML and JH Miller. 1992. The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). J Bacteriol 174: 6321–6325.
11 Muller J and S Janz. 1992. Assessment of oxidative DNA damage in the *oxyR*-deficient SOS chromotest strain *Escherichia coli* PQ300. Environ Mol Mutagen 20: 297–306.
12 Nicholson WL, B Setlow and P Setlow. 1990. Binding of DNA *in vitro* by a small, acid-soluble spore protein and its effect on DNA topology. J Bacteriol 172: 6900–6906.
13 Nicholson WL and P Setlow. 1990. Sporulation, germination and outgrowth. In: Molecular Biological Methods for Bacillus (Harwood CR and SM Cutting, eds), pp 391–450, John Wiley and Sons, Chichester, UK.
14 Palop A, GC Rutherford and RE Marquis. 1996. Hydroperoxide inactivation of enzymes within spores of *Bacillus megaterium* ATCC 19213. FEMS Microbiol Lett 142: 283–287.
15 Sagripanti JL and A Bonifacino. 1996. Comparative sporicidal effects of liquid chemical agents. Appl Environ Microbiol 62: 545–551.
16 Setlow B and P Setlow. 1993. Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. Appl Environ Microbiol 59: 3418–3423.
17 Setlow B and P Setlow. 1995. Small, acid-soluble proteins bound to DNA protect *Bacillus subtilis* spores from killing by dry heat. Appl Environ Microbiol 61: 2787–2790.
18 Setlow B and P Setlow. 1996. Role of DNA repair in *Bacillus subtilis* spore resistance. J Bacteriol 178: 3486–3495.
19 Setlow B, D Sun and P Setlow. 1992. Studies of the interaction between DNA and α/β-type small, acid-soluble spore proteins: a new class of DNA binding protein. J Bacteriol 174: 2312–2322.
20 Setlow P. 1995. Mechanisms for the prevention of damage to the DNA in spores of *Bacillus* species. Ann Rev Microbiol 49: 29–54.
21 Shi S, Y Mao, N Ahmed and H Jiang. 1995. HPLC investigation on Ni(II)-mediated DNA damage in the presence of *t*-butyl hydroperoxide and glutathione. J Inorg Biochem 57: 91–102.
22 Shin SY and RE Marquis. 1994. Sporicidal activity of tertiary butyl hydroperoxide. Arch Microbiol 161: 184–190.
23 Urios A, G Herrera and M Blanco. 1995. Detection of oxidative mutagens in strains of *Escherichia coli* deficient in the OxyR or MutY functions: dependence on SOS mutagens. Mut Res 332: 9–15.