Killing of spores of *Bacillus subtilis* by peroxyxynitrite appears to be caused by membrane damage

Peter C. Genest, Barbara Setlow, Elizabeth Melly and Peter Setlow

Author for correspondence: Peter Setlow. Tel: +1 860 679 2607. Fax: +1 860 679 340.
e-mail: setlow@sun.uhc.edu

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

During an infection of a higher eukaryote, dormant spores of a *Bacillus* species have been previously shown to be present in cells that can generate the toxic agent peroxyxynitrite (PON). Dormant spores of *Bacillus subtilis* were much more resistant to killing by PON than were growing cells, and spore-coat alteration or removal greatly decreased PON resistance. Spores were not killed by PON through DNA damage and lost no dipicolinic acid (DPA) during PON treatment. However, PON-killed spores lost DPA during subsequent heat treatments that caused much less DPA release from untreated spores. Although dead, the PON-killed spores germinated and initiated metabolism but never went through outgrowth; the great majority of germinated PON-killed spores also took up propidium iodide, indicating that they had suffered significant membrane damage and were dead. Together these data suggest that spore killing by PON is through some type of damage to the spore's inner membrane.

Keywords: spore resistance, spore killing, membrane damage

INTRODUCTION

Among the many human defences against bacteria is the generation of reactive oxygen species by a number of cell types, most notably phagocytes. Whilst a variety of reactive oxygen species are generated by these cells, production of the superoxide anion is most notable as this compound can react with nitric oxide to form the peroxyxynitrite (PON) anion, an extremely reactive nitrogen species (Hozz *et al.*, 1992; Beckman & Koppenol, 1996). PON can react with proteins to cause oxidation of sulfur-containing amino acids and iron–sulfur centres as well as nitration of tyrosine residues, and can also cause a variety of types of damage to DNA and lipids, because PON is a strong oxidant (Beckman & Koppenol, 1996; Keyer & Imlay, 1997; Perrin & Koppenol, 2000). A number of studies have shown that PON can kill and mutagenize growing bacteria (Brunnelli *et al.*, 1995; Hurst & Lymar, 1997; Kuwahara *et al.*, 2000; Routledge, 2000). However, no such studies have been carried out with bacterial endospores, which are generally much more resistant than growing bacteria to toxic chemicals (Russell, 1982, 1990; Bloomfield & Arthur, 1994; McDonnell & Russell, 1999; Setlow, 2000). Another reason for analysis of the effect of PON

on spores is recent work showing that a major route for infection with spores of a human pathogen, *Bacillus anthracis*, is via uptake and subsequent germination of spores of this organism within alveolar macrophages, cells which can generate PON (Guidi-Rontani *et al.*, 1999). Consequently, we have undertaken a study of the mechanism of spore resistance to and killing by PON using the model spore-forming organism *Bacillus subtilis*.

METHODS

Bacterial strains, and isolation of spores and growing cells.

The *B. subtilis* strains used were all derivatives of strain 168 (originally obtained from D. J. Tipper, University of Massachusetts Medical School, Worcester, MA, USA), and all contained drug-resistance markers to facilitate accurate identification of survivors of spore-killing experiments. Strain PS533 carries plasmid pUB110 which also carries a kanamycin-resistance marker, and strain PS578 (termed $\alpha\beta^-$) is isogenic with PS533, but lacks the genes encoding the spore's two major DNA-protective, small, acid-soluble spore proteins (SASPs) α and β (Setlow & Setlow, 1996; Setlow, 2000). Strain PS3328 has been described previously (Loshon *et al.*, 2001) and is isogenic with strain PS533 but lacks pUB110 and contains a deletion of most of the *cotE* gene whose product is involved in spore-coat assembly and maturation (Driks, 1999; Driks & Setlow, 1999). In strain PS3328, the *cotE* gene is replaced with a tetracycline-resistance marker (Loshon *et al.*, 2001). Chromosomal DNA from strain PS3328 was used to transform strains

Abbreviations: DPA, dipicolinic acid; PON, peroxyxynitrite; SASP, small acid-soluble protein.

PS533 and PS578 to tetracycline resistance, giving strains PS3394 (*cotE* pUB110) and PS3395 ($\alpha^- \beta^-$ *cotE* pUB110). Strains PS2318 and PS2319 are isogenic derivatives of PS533 and PS578, respectively, which lack plasmid pUB110, have also lost their *recA* gene encoding a protein responsible for much DNA repair in this organism and carry a chloramphenicol-resistance marker (Yasbin *et al.*, 1993; Setlow & Setlow, 1996). Strain PS3388 is isogenic with strain PS578 but lacks plasmid pUB110 and carries an erythromycin-resistance marker as well as the *luxAB* genes from *Vibrio harveyi* under control of the strong *B. subtilis* forespore-specific *sspB* promoter (Hill *et al.*, 1994). Spores of strain PS3388 accumulate the *luxAB* gene products in dormant spores, but only generate light using these proteins during spore germination when metabolism begins (Hill *et al.*, 1994; Ciarciaolini *et al.*, 2000; Setlow *et al.*, 2001). Spores of all these strains were prepared by growth at 37 °C on 2× SG medium plates without antibiotics, and spores were harvested, purified and stored as described (Nicholson & Setlow, 1990; Paidhungat *et al.*, 2000). All spore preparations used were free (>97%) of growing or sporulating cells or germinated spores. Cells growing at 37 °C in 2× YT medium (Loshon *et al.*, 2001) were harvested at an OD₆₀₀ of 1, washed with 25 mmol potassium phosphate l⁻¹ (pH 7.0) and resuspended in this buffer at an OD₆₀₀ of 4 immediately prior to PON treatment.

PON synthesis and treatment of cells or spores. PON was synthesized by rapid mixing of 10 ml ice-cold 250 mM sodium nitrite and 270 µl 22% hydrogen peroxide followed by rapid addition of 5 ml ice-cold 1 mol HCl l⁻¹ and 5 ml 1.5 mol NaOH l⁻¹. Unreacted hydrogen peroxide was removed and the PON was stored as described (Beckman *et al.*, 1994). PON concentrations were determined immediately prior to use by measurement of the OD₃₀₂ of appropriate dilutions in 1.2 M NaOH (Beckman *et al.*, 1994).

Spores and growing cells were incubated with PON at an OD₆₀₀ of 1 in 200 mmol potassium phosphate l⁻¹ (pH 7.4) for 15 min at 24 °C, with PON added last. For control incubations, PON was diluted into the buffer prior to addition of spores or growing cells, the mix incubated for 15 min at 24 °C to allow PON breakdown, spores or growing cells added, and the mix incubated a further 15 min at 24 °C. Aliquots of PON-treated or control mixtures were diluted into phosphate-buffered saline (0.2 g KCl l⁻¹, 0.24 g KH₂PO₄ l⁻¹, 8 g NaCl l⁻¹, 1.44 g Na₂HPO₄ l⁻¹, pH 7.4), appropriate dilutions plated on LB agar plates (Setlow & Setlow, 1996) and colonies counted after 24–36 h incubation at 30–37 °C; incubation for longer times gave no increase in colonies. All killing experiments were repeated at least twice with two independent spore preparations, always with essentially identical results, as the slopes of killing curves for the same strain never varied by more than 25%. However, in all experiments reported here, killing of spores of different strains was always carried out at the same time. In some experiments, catalase or pyruvate was added to the plates used for analysis of PON killing as described (Flowers *et al.*, 1977; Hood *et al.*, 1990).

The disinfectant Sterilox was prepared and analysed as described previously (Loshon *et al.*, 2001); Sterilox preparations had pH values of ~6.3 and available free chlorine levels of ~240 mg l⁻¹. Spores were treated with Sterilox without prior decoating, the Sterilox inactivated and spores recovered and killing measured as described (Loshon *et al.*, 2001).

Spore decoating, spore germination and analytical procedures. Spores were decoated by incubation for 30 min at 65 °C in 100 mmol NaOH l⁻¹, 100 mM NaCl, 5 g SDS l⁻¹ and

100 mmol DTT l⁻¹, and the spores washed as described (Bagyan *et al.*, 1998). Note that this decoating procedure also removes much, if not all, of the spore's outer membrane (Buchanan & Neyman, 1986). Spores with or without PON treatment were tested for mutagenesis to asporogeny or auxotrophy as described by Fairhead *et al.* (1993). The pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] content of spores with or without PON treatment was assayed after DPA extraction by boiling as described (Rotman & Fields, 1967; Nicholson & Setlow, 1990). Spores with or without prior PON or Sterilox treatment were incubated in water at an OD₆₀₀ of 5–9 for 30 min at various temperatures, cooled on ice for 10 min, centrifuged in a microcentrifuge and DPA in the supernatant fluid assayed directly.

After a heat shock for 30 min at 70 °C in water, spores were germinated at an OD₆₀₀ of 1 in either 2× YT medium (Setlow & Setlow, 1996) plus 4 mmol L-alanine l⁻¹ or 10 mmol Tris/HCl l⁻¹ (pH 8.3) plus 8 mmol L-alanine l⁻¹. DPA release, hexosamine release from spore cortex peptidoglycan and the percentage of spores that had turned phase dark or swelled were determined as described (Popham *et al.*, 1996; Loshon *et al.*, 2001). For analysis of light production during germination of spores carrying the *V. harveyi luxAB* genes, spores were germinated in 2× YT medium plus L-alanine as described above; at various times aliquots of 500 µl were mixed with 500 µl fresh medium and dodecanal added to 0.1 g l⁻¹ as described (Hill *et al.*, 1994; Loshon *et al.*, 2001). Light production was measured in a Turner TD-20/20 Luminometer over three consecutive intervals of 10 s and values extrapolated to the time of dodecanal addition, since light production from the *V. harveyi* LuxA and B gene products in *B. subtilis* decays, as seen previously (Karp, 1989; Ciarciaolini *et al.*, 2000; Loshon *et al.*, 2001). Recovery of decoated spores by treatment with lysozyme in a hypertonic medium was as described by Popham *et al.* (1996). Spores were stained with acridine orange and examined by fluorescence microscopy as described by Setlow *et al.* (2001).

RESULTS

In preliminary experiments we found that incubation of spores with 5 mmol 3-morpholinolysidnonimine-*N*-ethylcarbamide (SIN-1) l⁻¹, a compound which decomposes slowly to superoxide and nitric oxide, generating low levels of PON (Beckman & Koppenol, 1996), gave no killing of intact or decoated *B. subtilis* spores (data not shown). Since 5 mmol SIN-1 l⁻¹ is very rapidly lethal to growing bacteria (Brunelli *et al.*, 1995), it appeared that spores were not particularly sensitive to PON and that we would have to use high concentrations of this agent to get spore killing. Indeed, incubation with up to 16 mmol PON l⁻¹ gave no (<15%) detectable killing of intact dormant *B. subtilis* spores of either wild-type (PS533) or $\alpha^- \beta^-$ (PS578) strains (Fig. 1a). In contrast, germinated wild-type and $\alpha^- \beta^-$ spores were readily killed by PON and growing wild-type and $\alpha^- \beta^-$ cells were even more PON sensitive (Fig. 1a and data not shown). A major factor in spore PON resistance was the spore-coat structure, as PON resistance of either wild-type or $\alpha^- \beta^-$ spores was greatly decreased by removal of much coat protein by a decoating regimen (Fig. 1a, b). Similarly, a *cotE* mutation that disrupts spore-coat assembly as well as much coat maturation (Driks, 1999; Driks & Setlow, 1999) also decreased spore PON resistance greatly (Fig.

Fig. 1. Killing of dormant and germinated spores and growing cells of *B. subtilis* strains by PON. Intact dormant or germinated spores or growing cells (a); decoated dormant spores (b); or intact or decoated *cotE* spores (c) of *B. subtilis* were treated with various concentrations of PON and survival measured as described in Methods. The symbols used are: (a) ○, intact wild-type spores (PS533); △, intact $\alpha^- \beta^-$ spores (PS578); ●, germinated (3 h in Tris/alanine) wild-type spores (PS533); □, growing wild-type cells (PS533); (b) ○, decoated wild-type spores (PS533); △, decoated $\alpha^- \beta^-$ spores (PS578); ●, decoated *recA* spores (PS2318); ▲, decoated $\alpha^- \beta^-$ *recA* spores (PS2319); and (c) ○, intact *cotE* spores (PS3394); △, intact $\alpha^- \beta^-$ *cotE* spores (PS3395); ●, decoated *cotE* spores (PS3394); and ▲, decoated $\alpha^- \beta^-$ *cotE* spores (PS3395). Note that (a) and (b) have the same scales on their vertical axes.

Table 1. Killing and mutagenesis of spores with PON

Spores with or without PON treatment were analysed for mutation to asporogeny (*spo*), auxotrophy (*aux*) or both (*aux spo*) as described in the text.

Mutations		Survival (%)	Total tested	<i>aux</i>	<i>spo</i>	<i>aux spo</i>	%
Spores	Treatment						
Wild-type (PS533)	None	100	475	0	0	0	0
Wild-type (PS533)	8 mmol PON l ⁻¹	22	689	0	0	0	0
$\alpha^- \beta^-$ (PS578)	None	100	727	4	3	0	1
$\alpha^- \beta^-$ (PS578)	8 mmol PON l ⁻¹	6	533	3	2	0	1

1a, c). However, even the decoated *cotE* spores were more PON resistant than were growing cells (Fig. 1a, c). There was only a slight difference in the PON resistance of decoated spores of wild-type or $\alpha^- \beta^-$ strains (Fig. 1b) and even this slight difference was not observed with intact or decoated *cotE* or $\alpha^- \beta^-$ *cotE* spores (Fig. 1c).

SASP- α and β protect spore DNA from many types of damage (Setlow, 2000), so the observation that wild-type and $\alpha^- \beta^-$ spores exhibited generally similar PON resistance suggested that PON killing of spores was not through DNA damage. Indeed, there was no increase in mutations in the survivors of PON treatment of wild-type or $\alpha^- \beta^-$ spores, although the untreated $\alpha^- \beta^-$ spores did have a significant level of mutations undoubtedly acquired during sporulation, spore purification and storage, as seen previously (Table 1) (Fairhead *et al.*, 1993; Loshon *et al.*, 2001). That spore killing by PON was not through DNA damage was further indicated by the lack of sensitization of spores to PON by a mutation

in the *recA* gene which abolishes repair of a number of types of DNA damage during spore germination and outgrowth (Fig. 1b) (Yasbin *et al.*, 1993; Setlow & Setlow 1996). A *recA* mutation actually sensitizes spores to agents that kill spores through damage to spore DNA (Setlow & Setlow, 1996). Decoated spores of the *recA* derivatives were actually more PON resistant than their counterparts with a wild-type *recA* gene (Fig. 1b). The reason for the higher PON resistance of *recA* spores is not clear, but this has been seen previously for a number of chemicals that do not kill spores by DNA damage, and has been suggested to be due to significant differences in the sporulation of wild-type and *recA* strains which in turn causes significant differences in the properties of the resultant spores (Tennen *et al.*, 2000; Loshon *et al.*, 2001). The slightly decreased PON resistance of decoated $\alpha^- \beta^-$ spores relative to that of decoated wild-type spores (Fig. 1b) may also be due to slight differences in the sporulation of wild-type and $\alpha^- \beta^-$ strains causing differences in the properties of the

Table 2. DPA release from PON- or Sterilox-killed spores

Spores of strains PS533 or PS578 were either not treated, or treated with PON or Sterilox with (PON treatment) or without (Sterilox treatment) prior decoating, and spore killing and subsequent DPA release after various treatments analysed as described in Methods.

Treatment	Survival (%)	DPA release (%)		
		No further treatment	30 min, 80 °C	30 min, 85 °C
Decoated*	> 90	< 5	5	8
PON*	1	< 5	17	57
None†	100	< 3	4	6
Sterilox†	5	< 5	15	31

* PS578 spores.

† PS533 spores.

Table 3. Analysis of germinated PON- and control PON-treated spores

Spores of strain PS578 ($\alpha^- \beta^-$) were decoated, and either not treated, control PON treated or PON treated; spore killing was determined as described in Methods, as were DPA and hexosamine release upon spore germination and germinated spore viability.

	PON-treated (% killing)	Control-treated*	Decoated*
DPA release upon spore germination (% of total)†	89 (96)	92	93
Hexosamine release upon spore germination (% of total)‡	3 (95)	5	25
Viability of germinated spores (%)†§	8 (94)	97	96

* There was <10% spore killing by decoating with or without the control treatment.

† Spores were germinated for 2 h in 2× YT medium.

‡ Spores were germinated for 3 h in Tris/alanine.

§ Germinated spore viability measured using the BacLite viability stain (Molecular Probes).

spores produced, as suggested previously (Setlow *et al.*, 2000; Tennen *et al.*, 2000; Loshon *et al.*, 2001). However, these differences are not evident in intact or decoated spores of *cotE* strains (Fig. 1c).

The data given above indicate that spores are not killed by PON through DNA damage and this prompted us to look at other mechanisms of spore killing. Most of this work was carried out with spores of strain PS578 because of the slightly greater PON sensitivity of the decoated spores of this strain, but results with spores of strain PS533 were similar where examined (data not shown). Initially we examined the disruption of the spore's inner-membrane permeability barrier, as this can accompany spore killing by wet heat (Russell, 1982). However, analysis of decoated dormant spores killed by PON indicated that the major small molecule found in the spore core, DPA, was not released by PON treatment (Table 2). Thus PON killing of spores is not through a severe disruption of the dormant spore's inner mem-

brane. However, this latter permeability barrier appeared to be at least slightly damaged by PON treatment, as PON-killed spores released much more DPA upon subsequent heat treatment than did untreated spores (Table 2). Interestingly, spore killing by the disinfectant Sterilox (Selkon *et al.*, 1999; Shetty *et al.*, 1999; Middleton *et al.*, 2000; Loshon *et al.*, 2001) also resulted in DPA release upon subsequent heat treatment (Table 2), although Sterilox killing alone again resulted in no DPA release, as shown previously (Table 2).

A third possible mechanism for spore killing is by inactivation of one or more components of the spore germination apparatus and several chemicals have been shown to kill spores at least in part by this mechanism (Williams & Russell, 1993a, b; Tennen *et al.*, 2000). However, PON-killed spores did release their DPA upon incubation of spores with germinants (Table 3). PON-killed spores also became dark in the phase-contrast microscope upon spore germination, and at a rate only

Fig. 2. Germination of and light production from PON- and control PON-treated spores. (a) Decoated spores of strain P5578 ($\alpha^- \beta^-$) either control PON-treated or treated with 8 mmol PON l⁻¹ were germinated in Tris/HCl plus L-alanine as described in Methods. At various times ≥ 100 spores were examined under the phase-contrast microscope and the percentage of phase-dark spores determined as a measure of spore germination. (b) Decoated spores of strain P53388 ($\alpha^- \beta^-$ luxAB), either PON-treated or control PON-treated, or untreated intact spores of strain P5533 were germinated in 2 $\times$ YT medium plus L-alanine and light production was measured after dodecanal addition at various times as described in Methods. The symbols used are: (a) $\circ$, control PON-treated $\alpha^- \beta^-$ spores (P5578); $\triangle$, $\alpha^- \beta^-$ spores (P5578) treated with 8 mmol PON l⁻¹ giving 94% killing; (b) $\bullet$, untreated wild-type spores (P5533); $\circ$, control PON-treated $\alpha^- \beta^-$ luxAB spores (P53388); $\square$, $\alpha^- \beta^-$ luxAB spores (P53388) treated with 5 mmol PON l⁻¹ giving 64% killing; and $\triangle$, $\alpha^- \beta^-$ luxAB spores (P53388) treated with 8 mmol PON l⁻¹ giving 96% killing.

slightly slower than did control-treated spores (Fig. 2a); the central regions or cores of PON-killed (99%) $\alpha^- \beta^-$ spores germinated for 215 min in Tris/alanine were also stained completely with the nucleic acid stain acridine orange which does not penetrate the core of dormant spores (data not shown). PON-killed spores initiated metabolism upon mixing of spores with germinant, as shown by measuring light production catalysed by the *V. harveyi* luxAB gene products (Fig. 2b), as this process requires production of reduced flavin mononucleotide, which is absent from dormant spores (Karp, 1989; Hill *et al.*, 1994; Setlow, 1994). Whilst there was slightly reduced light production from germinating PON-treated spores compared to that from control PON-treated spores, the reduction in light production was much, much less than the degree of spore killing (Fig. 2b) and light production in this assay is linear with the amount of untreated spores assayed (data not shown). All these data thus suggest that PON-killed spores can undergo the normal early events in the spore germination process (Paidhungat & Setlow, 2001; Setlow *et al.*, 2001). These events include the disruption of the peptidoglycan cortex that restricts the approximately twofold expansion of the spore core, an early germination event which is essential for initiation of spore metabolism and staining of the spore core by acridine orange (Setlow *et al.*, 2001). Surprisingly, analysis of both PON- and control PON-treated spores indicated that significant amounts of cortex fragments, as measured by assays of hexosamine, were not released into the medium during germination of these spores, although decoated spores did release cortex fragments during germination (Table 3). However, the cortex must

be disrupted during germination of PON- and control PON-treated spores since upon mixing with a germinant, both types of spores initiated metabolism, became dark under phase-contrast microscopy, stained fully with acridine orange, and colony formation by the control-treated spores was normal. Previous work has shown that cortex disruption during spore germination is necessary for all these latter events (Setlow *et al.*, 2001). That a block in cortex disruption during germination is not the reason for PON killing of spores was shown further by the inability of lysozyme treatment in hypertonic medium to recover PON-killed spores (data not shown). This treatment has been shown previously to recover spores in which cortex disruption during spore germination is blocked (Popham *et al.*, 1996; Setlow *et al.*, 2001). Possibly some enzyme needed only for release of cortex fragments from spores but not for cortex disruption is inactivated by a PON breakdown product. PON-killed spores were also not recovered by addition of either catalase or pyruvate to the plating medium (data not shown). These additions have been shown to increase the recovery of Gram-positive bacteria subjected to a variety of harsh treatments, although the reasons for these effects are not known (Flowers *et al.*, 1977; Hood *et al.*, 1990).

Although PON-killed spores do initiate early events in spore germination, even in a rich medium the volume of the cores of these spores did not increase significantly (<25% increase in spore diameter; data not shown) and the germinated spores never gave rise to colonies. Indeed, analysis of germinated spore viability using the BacLite stain from Molecular Probes as described by

Murray *et al.* (1998) indicated that the great majority of PON-killed spores appeared dead in this assay as they took up propidium iodide, while >90% of the control-treated spores were alive (Table 3). This latter finding was made previously in examining spores killed by Sterilox (Loshon *et al.*, 2001).

DISCUSSION

There are two major but related questions arising from the results presented in this report: (i) what are the factors involved in the high resistance of spores to PON and (ii) what is the mechanism whereby spores are killed by PON? In examining factors involved in spore PON resistance, the spore coat clearly plays a major role, given the much greater PON sensitivity of decoated or *cotE* spores. The *B. subtilis* spore coat is exterior to the outer spore membrane, contains a large number of proteins and is important in spore resistance to both lysozyme and a number of chemicals (Russell, 1990; Bloomfield & Arthur, 1994; Driks, 1999; Driks & Setlow, 1999; Nicholson *et al.*, 2000; Setlow, 2000). While the spore coat may provide lysozyme resistance simply by blocking the access of this enzyme to the peptidoglycan cortex, the precise mechanism whereby the spore coat protects against chemicals is not clear. The spore coat is thought to be permeable to small molecules such as PON and possibly the spore coat proteins provide a reactive barrier restricting access of the short-lived PON to more sensitive areas of the spore. The outer membrane could also restrict PON access to the spore's inner regions, although this structure may not be a complete membrane in the dormant spore (Driks & Setlow, 1999). Indeed, since decoating of *cotE* spores had only a moderate effect on their PON resistance and decoating largely if not completely removes the spore's outer membrane (Buchanan & Neyman, 1986), it appears most likely that the outer spore membrane plays no role in spore PON resistance.

While removal of much coat protein by either mutation or decoating greatly reduced spore PON resistance, decoated or *cotE* spores are much more PON resistant than are growing cells. Although the coat protein remaining in decoated or *cotE* spores could provide significant protection against PON, it seems more likely that there are factors in addition to the spore coat which are involved in spore PON resistance. Other factors that have been identified as involved in spore resistance to one or more chemicals include the relative impermeability of the spore's inner membrane, the dehydration and mineralization of the spore core, the protection of spore DNA from damage by its saturation with α/β -type SASP, and the repair of damage, in particular to DNA, in the early minutes of spore germination (Setlow, 2000). Neither DNA repair nor the α/β -type SASP appears to play any role in spore PON resistance, as PON treatment of neither wild-type nor $\alpha^- \beta^-$ spores caused mutagenesis and a *recA* mutation did not decrease spore PON resistance. Since growing bacteria are mutagenized by PON and DNA repair is a crucial factor in their PON resistance (Routledge, 2000; Spek *et al.*, 2001), these

findings imply that PON causes no significant DNA damage in spores and thus that PON levels in the spore core, the site of spore DNA, must never become very high. Consequently, the dehydration and mineralization of the spore core, which probably reduce the rates of reactions of toxic chemicals with spore core targets (Setlow, 2000), may not be particularly important in spore PON resistance. This analysis thus suggests that a major factor in spore PON resistance may be the relative impermeability of the spore's inner membrane to PON. This latter compound is largely an anion at neutral pH and previous work has shown that charged molecules penetrate the spore core extremely poorly (Gerhardt *et al.*, 1972; Khairutdinov *et al.*, 2000). In contrast, the protonated form of PON, peroxyntous acid, is a neutral species which is thought to readily cross biological membranes (Khairutdinov *et al.*, 2000), and the spore core is permeable to small uncharged molecules <150 Da in size (Gerhardt *et al.*, 1972). However, the rate of permeation of small uncharged molecules, for example unprotonated methylamine, into the spore core is extremely slow, possibly because of the compressed state of the spore's inner membrane (Setlow & Setlow, 1980; Driks & Setlow, 1999). Consequently the slow permeation of peroxyntous acid across the inner spore membrane may restrict most PON action to targets on the outer surface of or exterior to the spore's inner membrane. It might be expected that PON would be more effective in killing bacteria at lower pH values because the higher levels of the more permeable peroxyntous acid at lower pH values would result in more PON inside cells. However, this has not been observed by others, perhaps due to the greater lability of peroxyntous acid relative to PON (Hurst & Lyman, 1997; Khairutdinov *et al.*, 2000). It is also worth noting that enzymic detoxification of PON by reduction, a significant factor in the PON resistance of growing bacteria (Chen *et al.*, 1998; Bryk *et al.*, 2000), is almost certainly not important in spore PON resistance. Whilst at least one enzyme that is reported to detoxify PON, alkyl hydroperoxide reductase subunit C, is present in the spore core, this enzyme as well as catalase has been shown to play no role in dormant spore resistance to peroxides, most likely because of the inactivity of enzymes in the dormant spore core (Casillas-Martinez & Setlow, 1997; Chen *et al.*, 1998).

A tentative conclusion from the material presented above is that spores are most likely killed by PON action on the outer surface of, or exterior to, the spore's inner membrane. This region of the spore contains the receptors for germinants as well as the enzymes that are involved in the depolymerization of the spore cortex (Paidhungat & Setlow, 2001). However, spore germination is not abolished in PON-killed spores, as these spores release DPA and initiate metabolism upon mixing of spores with germinants. It is true that cortex fragments are not released into the medium during this latter process with PON-killed spores. However, the cortex of PON-killed germinated spores must be significantly depolymerized, since initiation of metabolism

and complete staining of the germinated spore by acridine orange are observed, and both these processes have been shown to require cortex depolymerization (Setlow *et al.*, 2001). However, whilst PON-killed spores do initiate germination, these germinated spores have severe permeability defects as shown by their uptake of propidium iodide, a hallmark of bacteria with a damaged cytoplasmic membrane; in germinated spores this latter membrane is derived from the spore's inner membrane. The precise nature of the inner membrane damage caused by PON is not clear, but PON is a strong oxidant and at concentrations well below those used in the current work can damage both membrane lipids and proteins (Soszynski & Bartosz, 1996; Gadhela *et al.*, 1997; Mallozzi *et al.*, 1997). That PON-killed dormant spores have suffered some type of membrane damage is also suggested by their poor retention of DPA upon subsequent heat treatment. The precise barrier to DPA loss from dormant spores is not known, but a major role for the inner spore membrane in the process seems most likely. Therefore, a likely scenario for PON killing of *B. subtilis* spores is that this agent causes some type of damage to the spore's inner membrane that does not breach the permeability barrier of this membrane, perhaps because the inner membrane is extremely compressed in the dormant spore. However, the permeability barrier of this membrane is weakened by the PON damage, resulting in more rapid loss of DPA on spore heating. Upon spore germination and the inner membrane expansion to accommodate the rapid two-fold expansion of the core's volume in the absence of membrane synthesis (Paidhungat & Setlow, 2001), the effects of the inner-membrane damage caused by PON become much more severe, resulting in spore death even though early events in spore germination are relatively normal. Interestingly, essentially all the findings reported here on PON killing of *B. subtilis* spores are extremely similar to those made both previously and in the current work examining spore killing by the super-oxidized water, Sterilox (Loshon *et al.*, 2001). This suggests that both these agents kill *B. subtilis* spores by similar mechanisms, possibly through damage to the inner spore membrane as we suggest here. The challenge now is to identify and quantitate this membrane damage.

ACKNOWLEDGEMENTS

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

REFERENCES

- Bagyan, I., Noback, M., Bron, S., Paidhungat, M. & Setlow, P. (1998). Characterization of *yhcN*, a new forespore-specific gene of *Bacillus subtilis*. *Gene* **212**, 179–188.
- Beckman, J. S. & Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good the bad and the ugly. *Am J Physiol* **271**, C1424–C1437.
- Beckman, J. S., Chen, J., Ischiropoulos, H. & Crow, J. P. (1994). Oxidative chemistry of peroxynitrite. *Methods Enzymol* **233**, 229–240.
- Bloomfield, S. F. & Arthur, M. (1994). Mechanisms of inactivation and resistance of spores to chemical biocides. *J Appl Bacteriol* **76**, 91S–104S.
- Brunelli, L., Crow, J. P. & Beckman, J. S. (1995). The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. *Arch Biochem Biophys* **316**, 327–334.
- Bryk, R., Griffin, P. & Nathan, C. (2000). Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* **407**, 205–211.
- Buchanan, C. E. & Neyman, S. L. (1986). Correlation of penicillin-binding protein composition with different functions of two membranes in *Bacillus subtilis* forespores. *J Bacteriol* **165**, 498–503.
- Casillas-Martinez, L. & Setlow, P. (1997). Alkyl hydroperoxide reductase, catalase, MrgA, and superoxide dismutase are not involved in resistance of *Bacillus subtilis* spores to heat or oxidizing agents. *J Bacteriol* **179**, 7420–7425.
- Chen, L., Xie, Q. & Nathan, C. (1998). Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol Cell* **1**, 795–805.
- Ciarciagli, G., Hill, P. J., Davies, K., McClure, P. J., Kilsby, D., Brown, M. H. & Coote, P. J. (2000). Germination-induced bioluminescence, a route to determine the inhibitory effect of a combination preservation treatment on bacterial spores. *Appl Environ Microbiol* **66**, 3735–3742.
- Driks, A. (1999). The *Bacillus subtilis* spore coat. *Microbiol Mol Biol Rev* **63**, 1–20.
- Driks, A. & Setlow, P. (1999). Morphogenesis and properties of the bacterial spore. In *Prokaryotic Development*, pp. 191–218. Edited by Y. V. Brun & L. J. Shimkets. Washington, DC: American Society for Microbiology.
- Fairhead, H., Setlow, B. & Setlow, P. (1993). Prevention of DNA damage in spores and *in vitro* by small, acid-soluble proteins from *Bacillus* species. *J Bacteriol* **175**, 1367–1374.
- Flowers, R. S., Martin, S. E. & Ordal, Z. J. (1977). Catalase and enumeration of stressed *Staphylococcus aureus* cells. *Appl Environ Microbiol* **33**, 1112–1117.
- Gadelha, F. R., Thomson, L., Fagian, M. M., Costa, A. D., Radi, R. & Vercesi, A. E. (1997). Ca²⁺-independent permeabilization of the inner mitochondrial membrane by peroxynitrite is mediated by membrane protein thiol cross-linking and lipid peroxidation. *Arch Biochem Biophys* **345**, 243–250.
- Gerhardt, P., Scherrer, R. & Black, S. H. (1972). Molecular sieving by dormant spore structures. In *Spores V*, pp. 68–74. Edited by H. O. Halvorson, R. Hanson & L. L. Campbell. Washington, DC: American Society for Microbiology.
- Guidi-Rontani, C., Weber-Levy, M., Labruyere, E. & Mock, M. (1999). Germination of *Bacillus anthracis* spores within alveolar macrophages. *Mol Microbiol* **31**, 17–19.
- Hill, P. J., Hall, L., Vinicombe, D. A., Soper, C. J., Setlow, P., Waites, W. M., Denyer, S. & Stewart, G. S. A. B. (1994). Bioluminescence and spores as biological indicators of inimical processes. *J Appl Bacteriol* **76**, 129S–134S.
- Hood, A. M., Tuck, A. & Dane, C. R. (1990). A medium for the isolation, enumeration and rapid presumptive identification of injured *Clostridium perfringens* and *Bacillus cereus*. *J Appl Bacteriol* **69**, 359–372.
- Hozz, N., Darley-Usmar, V. M., Wilson, M. T. & Moncada, S. (1992). Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem J* **281**, 419–424.
- Hurst, J. K. & Lyman, S. V. (1997). Toxicity of peroxynitrite and related reactive nitrogen species toward *Escherichia coli*. *Chem Res Toxicol* **10**, 802–810.

- Karp, M. (1989). Expression of bacterial luciferase genes from *Vibrio harveyi* in *Bacillus subtilis* and in *Escherichia coli*. *Biochim Biophys Acta* **1007**, 84–90.
- Keyer, K. & Imlay, J. A. (1997). Inactivation of dehydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J Biol Chem* **272**, 27652–27659.
- Khairutdinov, R. F., Coddington, J. W. & Hurst, J. K. (2000). Permeation of phospholipid membranes by peroxynitrite. *Biochemistry* **39**, 14238–14249.
- Kuwahara, H., Miyamoto, Y., Akaike, T., Kubota, T., Sawa, T., Okamoto, S. & Maeda, H. (2000). *Helicobacter pylori* suppresses bactericidal activity of peroxynitrite via carbon dioxide production. *Infect Immun* **68**, 4378–4383.
- Loshon, C. A., Melly, E., Setlow, B. & Setlow, P. (2001). Analysis of the killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox®. *J Appl Microbiol* **91**, 1051–1058.
- McDonnell, G. & Russell, A. D. (1999). Antiseptics and disinfectants: activity, action and resistance development. *Clin Microbiol Rev* **12**, 147–179.
- Mallozzi, C., Stasi, A. M. & Minetti, M. (1997). Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. *FASEB J* **11**, 1281–1290.
- Middleton, A. M., Chadwick, M. V., Sanderson, J. & Gaya, H. (2000). Comparison of a solution of super-oxidized water (Sterilox) with glutaraldehyde for the disinfection of bronchoscopes, contaminated *in vivo* with *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* in sputum. *J Hosp Infect* **45**, 278–282.
- Murray, T., Popham, D. L., Pearson, C. B., Hand, A. R. & Setlow, P. (1998). Analysis of the outgrowth of *Bacillus subtilis* spores lacking penicillin-binding protein 2a. *J Bacteriol* **180**, 6493–6502.
- Nicholson, W. L. & Setlow, P. (1990). Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus*, pp. 391–450. Edited by C. R. Harwood & S. M. Cutting. Chichester: Wiley.
- Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* **64**, 548–572.
- Paidhungat, M. & Setlow, P. (2001). Spore germination and outgrowth. In *Bacillus subtilis and its Relatives: From Genes to Cells*. Edited by J. A. Hoch, R. Losick & A. L. Sonenshein. Washington, DC: American Society for Microbiology.
- Paidhungat, M., Setlow, B., Driks, A. & Setlow, P. (2000). Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. *J Bacteriol* **182**, 5505–5512.
- Perrin, D. & Koppenol, W. H. (2000). The quantitative oxidation of methionine to methionine sulfoxide by peroxynitrite. *Arch Biochem Biophys* **377**, 266–272.
- Popham, D. L., Helin, J., Costello, C. E. & Setlow, P. (1996). Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. *Proc Natl Acad Sci USA* **93**, 15405–15410.
- Rotman, Y. & Fields, M. L. (1967). A modified reagent for dipicolinic acid analysis. *Anal Biochem* **22**, 168.
- Routledge, M. N. (2000). Mutations induced by reactive nitrogen oxide species in the *supF* forward mutation assay. *Mutat Res* **450**, 95–105.
- Russell, A. D. (1982). *The Destruction of Bacterial Spores*, pp. 62–64. London: Academic Press.
- Russell, A. D. (1990). Bacterial spores and chemical sporicidal agents. *Clin Microbiol Rev* **3**, 99–119.
- Selkon, J. B., Babb, R. B. & Morris, R. (1999). Evaluation of the antimicrobial activity of a new super-oxidized water, Sterilox®, for the disinfection of endoscopes. *J Hosp Infect* **41**, 59–70.
- Setlow, B. & Setlow, P. (1980). Measurements of the pH within dormant and germinated bacterial spores. *Proc Natl Acad Sci USA* **77**, 2474–2476.
- Setlow, B. & Setlow, P. (1996). Role of DNA repair in *Bacillus subtilis* spore resistance. *J Bacteriol* **178**, 3486–3495.
- Setlow, B., McGinnis, K. A., Ragkousi, K. & Setlow, P. (2000). Effects of major spore-specific DNA binding proteins on *Bacillus subtilis* sporulation and spore properties. *J Bacteriol* **182**, 6906–6912.
- Setlow, B., Melly, E. & Setlow, P. (2001). Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. *J Bacteriol* **183**, 4894–4899.
- Setlow, P. (1994). Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. *J Appl Bacteriol* **23**, 49S–60S.
- Setlow, P. (2000). Resistance of bacterial spores. In *Bacterial Stress Responses*, pp. 217–230. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.
- Shetty, N., Srinivasan, S., Holton, J. & Ridgway, C. L. (1999). Evaluation of microbiocidal activity of a new disinfectant: Sterilox® 2500 against *Clostridium difficile* spores, *Helicobacter pylori*, vancomycin resistant *Enterococcus* species, *Candida albicans* and several mycobacterial species. *J Hosp Infect* **41**, 101–105.
- Soszynski, M. & Bartosz, G. (1996). Effect of peroxynitrite on erythrocytes. *Biochim Biophys Acta* **1291**, 107–114.
- Spek, E. J., Wright, T. L., Stitt, H. S., Taghizadeh, N. R., Tannenbaum, S. R., Marinus, M. G. & Engelward, B. P. (2001). Recombinational repair is critical for survival of *Escherichia coli* exposed to nitric oxide. *J Bacteriol* **183**, 131–138.
- Tennen, R., Setlow, B., Davis, K. L., Loshon, C. A. & Setlow, P. (2000). Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *J Appl Microbiol* **89**, 330–338.
- Williams, N. D. & Russell, A. D. (1993a). Revival of biocide-treated spores of *Bacillus subtilis*. *J Appl Bacteriol* **75**, 69–75.
- Williams, N. D. & Russell, A. D. (1993b). Revival of *Bacillus subtilis* spores from biocide-induced injury in the germination process. *J Appl Bacteriol* **75**, 76–81.
- Yasbin, R. E., Cheo, D. & Bol, D. (1993). DNA repair systems. In *Bacillus subtilis and Other Gram Positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 529–538. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

Received 18 June 2001; revised 7 September 2001; accepted 24 September 2001.