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

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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 peroxynitrite (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 & Inlay, 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 α−β−) 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, peroxynitrite; SASP, small acid-soluble protein.
PS3394 (cotE pUB110) and PS3395 (βp cotE pUB110). Strains PS2318 and PS2319 are isogenic derivatives of PS3393 and PS378, 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 PS378 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; Ciarcia et al., 2000, Setlow et al., 2001). Spores of all these strains were prepared by growth at 37 °C on 2×5G 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 OD_{600} of 1, washed with 25 mmol potassium phosphate pH 7 (pH 7), and resuspended in this buffer at an OD_{600} 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 & Koppenol, 1996). PON concentrations were determined immediately prior to use by measurement of the OD_{600} of appropriate dilutions in 1.2 M NaOH (Beckman et al., 1994).

Spores and growing cells were incubated with PON at an OD_{600} of 1 in 200 mmol potassium phosphate pH 7-4 (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 further at 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, 4.4 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 OD_{600} 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 OD_{600} of 1 in either 2× YT medium (Setlow & Setlow, 1996) plus 4 mmol l-alanine l⁻¹ or 10 mmol Tris/HCl 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 swollen 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 1⁻¹ as described (Hill et al., 1994; LoShon et al., 2001). Light production was measured in a Turner TD/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; Ciarcia 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-morpholinosydnonimine-N-ethylcarbamide (SIN-1) 1⁻¹, 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 1⁻¹ 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 (PS333) or αp βp (PS378) strains (Fig. 1a). In contrast, germinated wild-type and αp βp spores were readily killed by PON and growing wild-type and αp βp 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 αp βp 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.
Survival (%) Total tested α β α−β α−β− cotE α−β− recA α−β− spo aux spo aux spo %

Wild-type (PS533) None 100 475 0 0 0 0
Wild-type (PS533) 8 mmol PON l−1 22 689 0 0 0 0
α−β− (PS578) None 100 727 4 3 0 1
α−β− (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 α−β− strains (Fig. 1b) and even this slight difference was not observed with intact or decoated cotE or α−β− 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 α−β− 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 α−β− spores, although the untreated α−β− 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 α−β− 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 α−β− strains causing differences in the properties of the

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**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.

<table>
<thead>
<tr>
<th>Spores</th>
<th>Treatment</th>
<th>Survival (%)</th>
<th>Total tested</th>
<th>aux</th>
<th>spo</th>
<th>aux spo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>None</td>
<td>100</td>
<td>475</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wild-type</td>
<td>8 mmol PON l−1</td>
<td>22</td>
<td>689</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>α−β− (PS578)</td>
<td>None</td>
<td>100</td>
<td>727</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>α−β− (PS578)</td>
<td>8 mmol PON l−1</td>
<td>6</td>
<td>533</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (%)</th>
<th>DPA release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No further</td>
</tr>
<tr>
<td>Decoated*</td>
<td>&gt;90</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PON*</td>
<td>1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>None†</td>
<td>100</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Sterilox†</td>
<td>5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* PS578 spores.
† PS533 spores.

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

Spores of strain PS578 (αβ−) 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.

<table>
<thead>
<tr>
<th></th>
<th>PON-treated ( % killing)</th>
<th>Control-treated*</th>
<th>Decoated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPA release upon spore germination (% of total)†</td>
<td>89 (96)</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>Hexosamine release upon spore germination (% of total)‡</td>
<td>3 (95)</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Viability of germinated spores (%)§</td>
<td>8 (94)</td>
<td>97</td>
<td>96</td>
</tr>
</tbody>
</table>

* There was <10% spore killing by decoating with or without the control treatment.
† Spores were germinated for 2 h in 2x 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 membrane. 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
Spore killing by peroxynitrite

Fig. 2. Germination of and light production from PON- and control PON-treated spores. (a) Decoated spores of strain PS578 (α β) 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 PS3388 (α β luxAB), either PON-treated or control PON-treated, or untreated intact spores of strain PS33 were germinated in 2x 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) ○, control PON-treated α β spores (PS578); □, α β spores (PS578) treated with 8 mmol PON l⁻¹ giving 94% killing; (b) ●, untreated wild-type spores (PS533); ○, control PON-treated α β luxAB spores (PS3388); □, α β luxAB spores (PS3388) treated with 5 mmol PON l⁻¹ giving 64% killing; and △, α β luxAB spores (PS3388) 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 %) α β 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). While 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 α/β− 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, peroxynitrous 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 peroxynitrous 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 peroxynitrous 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 peroxynitrous acid relative to PON (Hurst & Lymar, 1997; Khairutdinov et al., 2000). It is also worth noting that enzymatic 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 twofold 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 (Loison 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 quantify this membrane damage.

ACKNOWLEDGEMENTS

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

REFERENCES


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