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Mechanisms of *Bacillus subtilis* spore resistance to and killing by aqueous ozone

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ABSTRACT

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Aims: To determine the mechanisms of *Bacillus subtilis* spore killing by and resistance to aqueous ozone.
Methods and Results: Killing of *B. subtilis* spores by aqueous ozone was not due to damage to the spore's DNA, as wild-type spores were not mutagenized by ozone and wild-type and *recA* spores exhibited very similar ozone sensitivity. Spores (termed $\alpha^-\beta^-$) lacking the two major DNA protective α/β-type small, acid-soluble spore proteins exhibited decreased ozone resistance but were also not mutagenized by ozone, and $\alpha^-\beta^-$ and $\alpha^-\beta^-$ *recA* spores exhibited identical ozone sensitivity. Killing of spores by ozone was greatly increased if spores were chemically decoated or carried a mutation in a gene encoding a protein essential for assembly of the spore coat. Ozone killing did not cause release of the spore core's large depot of dipicolinic acid (DPA), but these killed spores released all of their DPA after a subsequent normally sublethal heat treatment and also released DPA much more readily when germinated in dodecylamine than did untreated spores. However, ozone-killed spores did not germinate with either nutrients or Ca^{2+}-DPA and could not be recovered by lysozyme treatment.
Conclusions: Ozone does not kill spores by DNA damage, and the major factor in spore resistance to this agent appears to be the spore coat. Spore killing by ozone seems to render the spores defective in germination, perhaps because of damage to the spore's inner membrane.
Significance and Impact of the Study: These results provide information on the mechanisms of spore killing by and resistance to ozone.

Keywords: *Bacillus subtilis*, ozone, spore coat, spore killing, spore resistance.

INTRODUCTION

When nutrients are limited, bacteria of the genus *Bacillus* form metabolically dormant spores that are extremely resistant to environmental insults and toxic chemicals that readily kill their cellular counterparts (Russell 1990; Bloomfield 1999; McDonnell and Russell 1999; Nicholson *et al.* 2000; Setlow 2000). Many factors contribute to the resistance of spores. The spore coat consisting of >25 proteins in *Bacillus subtilis* provides the first line of defence against enzymes and many chemicals (Driks 1999; McDonnell and Russell 1999; Riesenman and Nicholson 2000; Setlow 2000; Tennen *et al.* 2000; Loshon *et al.* 2001; Genest *et al.* 2002). The peptidoglycan cortex protects the spore's inner membrane, an important barrier to the entry of small hydrophilic molecules into the central spore core (Nicholson *et al.* 2000; Setlow 2000). The core contains a large depot of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] chelated with divalent cations, principally Ca^{2+} (Paidhungat *et al.* 2000; Paidhungat and Setlow 2002). DPA contributes to spore dormancy and is also important for the dehydration of the core; this dehydration is a major factor in spore resistance to wet heat (Gerhardt and Marquis 1989; Paidhungat *et al.* 2000; Setlow 2000). The spore core also contains the α/β-type small, acid-soluble spore proteins (SASP) that bind to

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and protect spore DNA from u.v. radiation, heat and some genotoxic chemicals (Setlow 2000; Tennen *et al.* 2000). Finally there is significant repair of damage to the spore's DNA in spore outgrowth by a number of repair systems (Setlow and Setlow 1996; Setlow 2000).

The high level of resistance of spores causes problems in the food industry, in medical settings and in the decontamination of buildings after spore-based bioterrorism attacks. Consequently, it is of significant interest to understand the mechanisms of spore resistance to and killing by particular agents and to devise new and efficient means for spore inactivation. One class of chemicals commonly used to kill spores is oxidizing agents, including compounds such as chlorine dioxide, hydrogen peroxide, hypochlorite, Oxone™ and Sterilox® (McDonnell and Russell 1999; Loshon *et al.* 2001; Melly *et al.* 2002; Young and Setlow 2003, 2004). An additional oxidizing agent that has significant potential as a sporicide is ozone, as aqueous ozone has a higher oxidizing potential than most oxidizing agents used to kill spores (Menzel 1971; Kim *et al.* 2003). Ozone has well-known antimicrobial and sporicidal properties (Rickloff 1987) and has been used since the late nineteenth century to purify water (Rice *et al.* 1981). Ozone is thought to kill bacteria by membrane damage followed by cell lysis (Komanapalli and Lau 1996; Thanomsub *et al.* 2002). Advantages in using ozone for sterilization are that ozone decomposes to diatomic oxygen leaving no toxic residues, and it also inactivates organisms other than bacteria, including endotoxins and viruses (http://www.gewater.com/library/tp/index.jsp). While ozone is sporicidal, the effects of ozone leading to spore killing are not well understood. Given the potential utility of ozone in spore inactivation, we examined the mechanisms of spore resistance to and killing by ozone.

MATERIALS AND METHODS

Strains and spore preparation

The *B. subtilis* strains used in this work are isogenic with strain PS832 that is a prototrophic derivative of strain 168. Strains PS533 (considered wild-type for this work) and PS578 (Setlow and Setlow 1996) contain plasmid pUB110 with a kanamycin resistance marker. Strain PS578 (termed $\alpha^-\beta^-$) also lacks the *sspA* and *sspB* genes encoding the two major α/β-type SASP of *B. subtilis* (Setlow and Setlow 1996). The *recA* gene, whose product is responsible for much DNA repair in *B. subtilis* (Yasbin *et al.* 1993), is inactivated by chloramphenicol and erythromycin resistance markers in strains PS2318 (*recA*) and PS2319 ($\alpha^-\beta^-$ *recA*) that are derivatives of strains PS533 and PS578 respectively (Setlow and Setlow 1996). Strains PS3394 (*cotE*) and PS3395 ($\alpha^-\beta^-$ *cotE*) are derived from strains PS3328 and PS3329, respectively (Paidhungat *et al.* 2001), and also contain plasmid pUB110. Strain PS3379 contains the *luxAB* genes from *Vibrio harveyi* under the control of the forespore-specific *sspB* promoter, and also carries plasmid pSB357 with an erythromycin resistance marker (Hill *et al.* 1994; Ciarciaglini *et al.* 2000; Setlow *et al.* 2001). Strain FB112 has a spectinomycin resistance marker replacing the coding sequence of the gene encoding the spore cortex lytic enzyme SleB (Paidhungat *et al.* 2001).

Spores of all strains were prepared at 37°C on 2X SG medium agar plates without antibiotics unless noted otherwise and were cleaned and stored as described (Nicholson and Setlow 1990; Paidhungat *et al.* 2000). All spore preparations were free (>98%) of growing cells, germinated spores and cell debris as determined by observation in the phase contrast microscope.

Treatment of spores with ozone

For analysis of the mutagenesis of spores by ozone, the ozone generated by a model WOZ4 Water Ozonator (SOTA Instruments Inc., Penticton, BC, Canada) was bubbled into 30 ml deionized water for 20 min at 24°C giving *ca* 0·5 μg l^{-1} ozone (5×10^{-4} ppm). The indigo method (Bader and Hoigne 1980) was used to determine ozone concentrations. Decoated spores (see below) at an optical density at 600 nm (O.D.$_{600nm}$) of 0·01 (*ca* $1{\cdot}5 \times 10^6$ CFU ml^{-1}) were incubated in aqueous ozone for 0·5–4 min, diluted in sterile phosphate-buffered saline (PBS) [50 mmol l^{-1} potassium phosphate (pH 7·4)-100 mmol l^{-1} NaCl], aliquots plated on Luria–Bertani (LB) medium (Sambrook *et al.* 1989) plates containing the appropriate antibiotic and plates incubated at 30°C for 24–48 h to determine spore killing – longer incubations gave no further survivors. To determine levels of auxotrophic or asporogenous mutations, untreated spores or spores that survived ozone treatment were transferred onto minimal medium or sporulation medium plates and examined for mutations as described (Fairhead *et al.* 1993).

Ozone concentrations generated by the KWOZ4 ozonator were too low to kill intact spores and larger quantities of spores, so further experiments used ozone produced by an electrochemical Ozone Generator Model LT 1 (Lynntech, Inc., College Station, TX, USA). Pressurized ozone was bubbled into 100 ml low ozone demand water (deionized, ozonated and autoclaved) for 10 s to 20 min resulting in ozone concentrations from 11 to 40 ppm (11–40 mg l^{-1}), determined as described above. The pH of the distilled deionized water fell slightly from pH 3·7 to 3·1 after ozonation. Spores at an O.D.$_{600nm}$ of 1 were incubated in 7–23 ppm aqueous ozone at 24°C and at various times samples were diluted 10-fold in 10 g l^{-1} sodium thiosulphate to inactivate the ozone, incubated for 10 min at 24°C and diluted further in PBS. To obtain larger quantities of ozone-killed spores, the spores were used at an O.D.$_{600nm}$ of 2 and

the reaction was terminated by centrifugation and resuspension in water. All experiments to measure spore killing were performed at least twice using two independent spore preparations with similar results, and the variation in the slopes of killing curves for individual spore preparations was ≤25% in duplicate experiments at identical ozone concentrations.

Cells of strain PS533 were grown at 37°C without antibiotics in 2X YT medium (Paidhungat *et al.* 2000) to an $O.D._{600nm}$ of 2·8 (late exponential growth phase). After centrifugation the cells were washed with PBS, resuspended in PBS at an $O.D._{600nm}$ of 2·4, treated with ozone, and viability determined as described above.

Decoating and recovery of decoated spores

Spores at an $O.D._{600nm}$ of 10 with or without prior ozone treatment were decoated in 100 mmol l^{-1} NaOH, 100 mmol l^{-1} NaCl, 100 mmol l^{-1} dithiothreitol and 5 g l^{-1} sodium dodecylsulphate for 30 min at 65°C, followed by extensive washing with water (Bagyan *et al.* 1998). Decoated spores were incubated in a hypertonic medium with 25 mg l^{-1} lysozyme as described (Popham *et al.* 1996), and the incubations were monitored in a phase contrast microscope to determine the extent of lysozyme hydrolysis of the spore cortex. Decoated untreated and ozone-treated spores were also applied to LB plates containing 5 mg l^{-1} lysozyme.

Assays of DPA and spore cortex fragments

The DPA content of untreated and ozone-treated dormant and germinated spores was determined as described (Rotman and Fields 1967; Setlow and Setlow 1993). DPA released from spores suspended in water and heated to 80 or 85°C for 30 min was measured in the supernatant fluid after centrifugation (Genest *et al.* 2002), and the total hexosamine content of treated and untreated dormant and germinated spores and of the supernatant fluid from germinated spores was determined as described (Popham *et al.* 1996; Tennen *et al.* 2000).

Spore germination

Spores were routinely germinated at an $O.D._{600nm}$ of 1 at: (i) 37°C in nutrients [either 2X YT medium (Paidhungat *et al.* 2000) with 4 mmol l^{-1} L-alanine or 50 mmol l^{-1} Tris–HCl (pH 8.4) with 8 mmol l^{-1} L-alanine); (ii) 24°C in 60 mmol l^{-1} Ca^{2+}-DPA in 50 mmol l^{-1} Tris–HCl (pH 8) (Paidhungat *et al.* 2000); or (iii) 50°C with 1 mmol l^{-1} dodecylamine and 20 mmol l^{-1} KPO_4 (pH 7·4). During dodecylamine germination DPA release was monitored by centrifugation of 1-ml samples of germinating spores and measuring the $O.D._{270nm}$ of the supernatant fluid as described (Setlow *et al.* 2003).

Measurement of light production by spores carrying the *luxAB* genes

Spores of strain PS3394 were germinated at 37°C in 2X YT medium plus 4 mmol l^{-1} L-alanine as described above. Aliquots (500 μl) of the germinating spores were mixed with 500 μl of germination medium containing 100 mg l^{-1} dodecanal, and light production was measured using a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale CA, USA) as described (Hill *et al.* 1994; Ciarciaglini *et al.* 2000; Loshon *et al.* 2001; Setlow *et al.* 2001).

Staining and microscopic analysis of spores

Spores were stained with 1 mg l^{-1} 4′,6′-diamino-2-phenylindole (DAPI), and spores examined by fluorescence microscopy as described (Melly *et al.* 2002). The percentage of spores that became dark in the phase contrast microscope during germination was determined as described (Wyatt and Waites 1975; Tennen *et al.* 2000).

RESULTS

Spore killing by ozone

Spores of various *Bacillus* strains are reportedly killed by aqueous ozone (Broadwater *et al.* 1973; Foegeding 1985; Rickloff 1987; Khadre and Yousef 2001), and this was also the case for wild-type *B. subtilis* spores (Fig. 1). Late exponential growth phase *B. subtilis* cells were ≥10-fold more ozone sensitive than spores (data not shown), as found previously for growing cells and spores of *B. cereus* and *B. megaterium* (Broadwater *et al.* 1973; Foegeding 1985). Ozone can be genotoxic for bacteria (Dillon *et al.* 1992; Victorin 1992), so it was possible that this was the mechanism whereby ozone kills spores. However, *B. subtilis* spores deficient in RecA-dependent DNA repair were no more ozone sensitive than wild-type spores (Fig. 1). Agents known to kill spores by DNA damage including alkylating agents, formaldehyde and nitrous acid kill *recA* spores two- to fourfold faster than wild-type spores (Setlow *et al.* 1998; Loshon *et al.* 1999; Tennen *et al.* 2000). Consequently, the similar ozone sensitivity of wild-type and *recA* spores strongly suggested that ozone is not killing spores by DNA damage. This suggestion was supported by analysis of the level of mutants in wild-type spores that survived ozone treatment. In contrast to levels of auxotrophic plus asporogenous mutants of 5–15% in survivors of wild-type spores treated with alkylating agents, formaldehyde or nitrous acid (Setlow *et al.* 1998; Loshon *et al.* 1999; Tennen *et al.* 2000), spores that survived ozone treatment exhibited

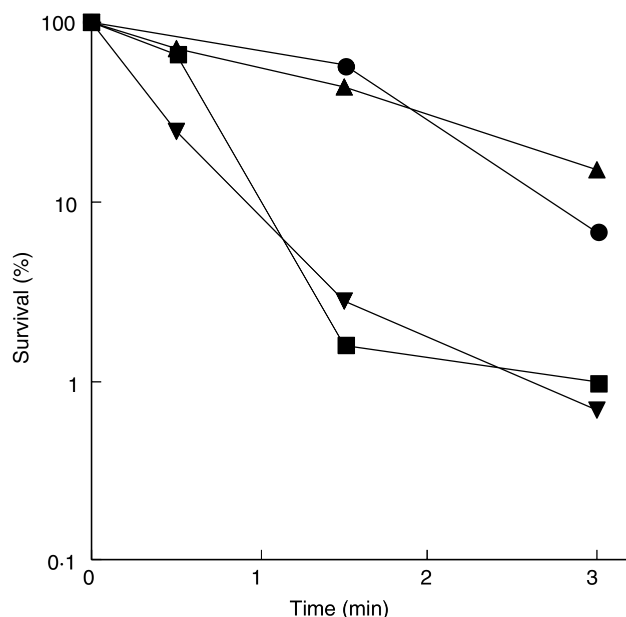

Fig. 1 Resistance of spores of various *B. subtilis* strains to aqueous ozone. Spores of strain PS533 (wild-type) (●), PS578 ($\alpha^-\beta^-$) (■), PS2318 (*recA*) (▲), and PS2319 ($\alpha^-\beta^-$ *recA*) (▼) were incubated in 11 ppm aqueous ozone at 24°C and spore survival determined as described in Materials and methods

no significant amount of auxotrophic or asporogenous mutations compared with untreated spores (Table 1). The absence of DNA damage in wild-type spores treated with many other oxidizing agents has been seen previously (Setlow and Setlow 1993; Tennen *et al.* 2000; Loshon *et al.* 2001; Genest *et al.* 2002; Young and Setlow 2003, 2004), so it is not surprising that ozone also does not kill spores by DNA damage.

The resistance of spore DNA to damage by genotoxic chemicals such as formaldehyde and nitrous acid as well as the oxidizing agent hydrogen peroxide is due in large part to the protection of spore DNA by its saturation with α/β-type SASP (Setlow 2000; Tennen *et al.* 2000). Indeed, while hydrogen peroxide does not kill wild-type spores by DNA damage, this agent does kill $\alpha^-\beta^-$ spores largely if not completely by DNA damage (Setlow and Setlow 1993). Thus it was of interest to examine the killing of $\alpha^-\beta^-$ spores by ozone. Strikingly, $\alpha^-\beta^-$ spores were killed approx. threefold faster by ozone than were wild-type spores (Fig. 1), suggesting that ozone may be killing $\alpha^-\beta^-$ spores by DNA damage. This suggestion was reasonable, as the relative sensitivity of wild-type and $\alpha^-\beta^-$ spores to alkylating agents, formaldehyde, hydrogen peroxide and nitrous acid, all of which kill at least $\alpha^-\beta^-$ spores by DNA damage, ranges from essentially identical (alkylating agents) to ≥10-fold higher sensitivity for $\alpha^-\beta^-$ spores (hydrogen peroxide) (Setlow and Setlow 1993; Setlow *et al.* 1998; Loshon *et al.* 1999; Tennen *et al.* 2000). However, the ozone sensitivity of $\alpha^-\beta^-$ spores was not increased by a *recA* mutation (Fig. 1), and $\alpha^-\beta^-$ spores were not mutagenized by ozone (Table 1). These latter two findings strongly suggest that ozone also does not kill $\alpha^-\beta^-$ spores by DNA damage, and that the greater ozone sensitivity of $\alpha^-\beta^-$ spores compared with wild-type spores is due to factors other than an absence of DNA protection in $\alpha^-\beta^-$ spores (see Discussion).

The spore coat and resistance to ozone

While the findings noted above indicated that DNA protection by the spore's α/β-type SASP is likely not involved in spore protection against ozone, there must be some spore-specific factor(s) involved in ozone resistance, as growing cells are much more ozone sensitive than spores. The spore coat is an obvious candidate for such a spore-specific factor, as this layer provides protection against many oxidizing agents (Bloomfield 1999; Setlow 2000; Young and Setlow 2003, 2004). Indeed, the spore coat has been reported to be extremely important in protecting *B. cereus* spores against ozone, as chemically decoated *B. cereus* spores exhibited ozone resistance equivalent to the low resistance of growing cells (Foegeding 1985). Spore coats were indeed important in *B. subtilis* spore ozone resistance, as chemically decoated spores and spores with a defective coat resulting

Table 1 Lack of mutagenesis of spores killed by ozone*

Strain	Treatment	Killing (%)	No. examined	Colonies with mutations (%)			
				aux	spo	Both	Total
PS533 (wt)	None	0	422	0	0	0	0
PS578 ($\alpha^-\beta^-$)	None	0	408	0	0·2	0·2	0·4
PS533 (wt)	Ozone	91	491	0·2	0	0	0·2
PS578 ($\alpha^-\beta^-$)	Ozone	91	405	0·2	0·2	0	0·4

*Decoated spores of various strains were treated with ozone as described in Materials and methods, and spore killing assessed and surviving colonies analysed for auxotrophic (aux) or asporogenous (spo) mutations or for both types of mutations.

from a mutation in *cotE*, a major coat morphogenic protein (Driks 1999), were killed much more rapidly by ozone than were spores with intact coats (Fig. 2a–c). Interestingly, there was much less difference in the ozone sensitivity of wild-type and $\alpha^-\beta^-$ spores, when these spores were first chemically decoated (Fig. 2b) or when the ozone sensitivity of *cotE* and $\alpha^-\beta^-$ *cotE* spores was compared (Fig. 2c).

Ozone treatment also results in changes in spores that are visible to the naked eye. As has been seen with other oxidizing agents (Young and Setlow 2003, 2004), pellets of ozone-treated spores that had been killed 90–99% were not the normal brown colour but were white (data not shown). This is probably due to bleaching of the CotA protein that gives purified spores their brown colour (Driks 1999) and indicates that ozone reacts with at least one component of the spore coat.

Effects of ozone on spore staining and DPA release

In addition to DNA damage, another mechanism for spore killing is the rupture of a spore permeability barrier leading to the release of the contents of the spore core. Both strong acid and ethanol at elevated temperatures appear to kill spores by this mechanism, as may wet heat to some degree (Setlow *et al.* 2002). Rupture of this spore permeability barrier can be examined using nucleic acid stains such as DAPI that are unable to enter the dormant spore core, the site of spore nucleic acids, as DAPI only faintly stains the periphery of undamaged dormant spores, resulting in a doughnut-like appearance (Melly *et al.* 2002; Setlow *et al.* 2002). While wild-type spores killed 90–99% by ozone exhibited somewhat brighter peripheral staining than untreated spores, likely indicative of some changes in the spore coat and/or cortex caused by ozone, the staining of the ozone-killed spore core was not bright as seen with germinated spores or spores with a ruptured inner membrane (data not shown; Setlow *et al.* 2002). That the spore's inner membrane was not ruptured in the process of ozone killing was further shown by the retention of >95% of the spore core's depot of DPA in spores killed >90% by ozone (data not shown). In contrast, loss of DPA accompanies spore killing by some agents, including acid and hot ethanol (Setlow *et al.* 2002).

While spores killed with ozone did not lose DPA, subsequent incubation of ozone-killed spores at 80 or 85°C, temperatures that are not lethal for untreated spores, resulted in almost total loss of DPA, while untreated spores lost little DPA upon incubation at these temperatures (Table 2). This facile release of DPA upon incubation at normally nonlethal temperatures has been seen with a number of other oxidizing agents (Genest *et al.* 2002; Young and Setlow 2003, 2004).

As another probe of damage to the spore's inner membrane, we examined the ability of ozone-killed spores to germinate with the cationic surfactant dodecylamine. This agent initiates spore germination utilizing neither the receptors for nutrient germinants nor the action of the spore's cortex lytic enzymes, and appears to trigger spore germination by promoting DPA release through the spore's inner membrane (Rode and Foster 1961; Setlow *et al.* 2003; Cortezzo *et al.* 2004). Strikingly, ozone-killed spores

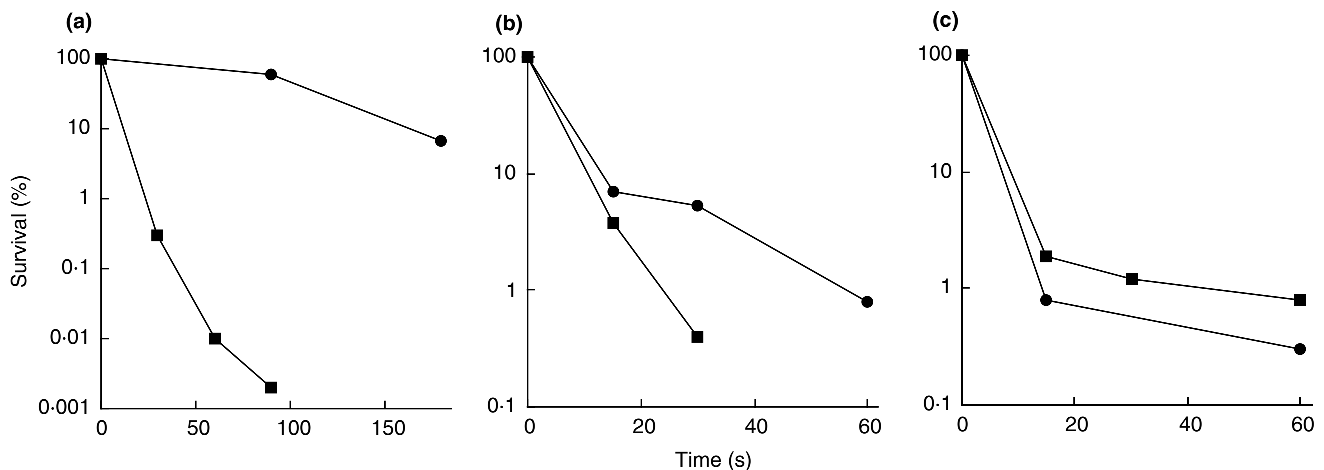

Fig. 2 Effects of coat defects on spore resistance to ozone. Spores of: (a) strain PS533 (wild-type) with intact coats (●) or chemically decoated (■); (b) strains PS533 (wild-type) (●) and PS578 ($\alpha^-\beta^-$) (■) (both chemically decoated); or (c) strains PS3394 (*cotE*) (●) and PS3394 (*cotE* $\alpha^-\beta^-$) (■) were incubated at 24°C in: (a) 11.5 ppm aqueous ozone; (b) 7 ppm aqueous ozone; or (c) 8 ppm aqueous ozone and spore survival determined as described in Materials and methods

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Table 2 DPA release from untreated or ozone-treated spores*

Treatment	Killing (%)	Heat treatment (°C)	DPA release (%)
None	0	24	<3
Ozone	98	24	<3
None	0	80	<3
Ozone	99	80	96
None	0	85	7
Ozone	98	85	99

*Spores of strain PS533 (wild type) were either not treated or treated with aqueous ozone. After the level of spore killing by ozone was determined, the spores were washed with water, incubated in water for 30 min at various temperatures, and the DPA released was determined as described in Materials and methods. The amount of DPA in untreated spores of strain PS533 was 114 mg g^{-1} of dry spores, and this value was set as 100%. Incubation of wild-type spores for 30 min at 85°C caused ≤10% spore killing.

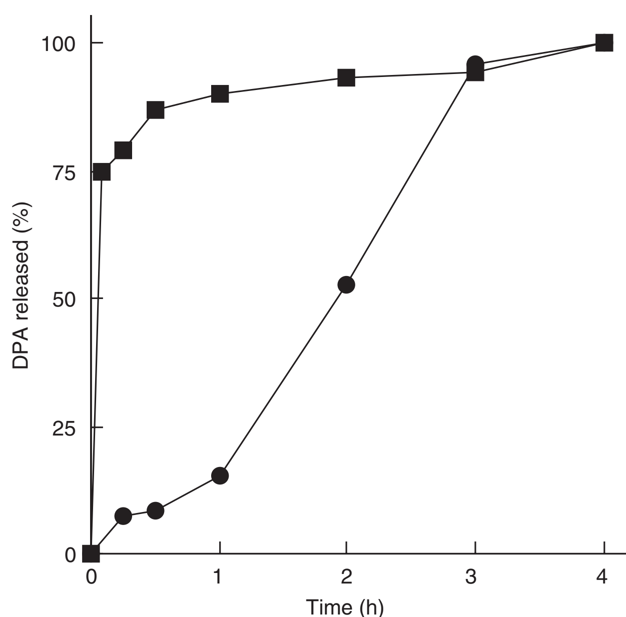

Fig. 3 Germination of untreated or ozone-treated spores with dodecylamine. Spores of strain PS533 (wild-type) either untreated or killed 93% with aqueous ozone were germinated with dodecylamine and spore germination monitored by measuring DPA release as described in Materials and methods. The symbols used are: ●, untreated spores; ■, spores killed by ozone

germinated much more rapidly than did untreated spores, when spore germination was measured by monitoring the release of DPA (Fig. 3), a result previously obtained with spores killed with the oxidizing agent Oxone™ (Young and Setlow 2004).

Germination of ozone-treated spores

Previous work has shown that spores killed by many oxidizing agents germinate poorly if at all with nutrients (Loshon *et al.* 2001; Genest *et al.* 2002; Melly *et al.* 2002; Young and Setlow 2003, 2004). This was also the case with ozone-killed spores, as these failed to turn dark in the phase contrast microscope upon incubation in either 2X YT medium plus L-alanine or Tris–HCl plus L-alanine (Table 3). As expected, ozone-killed spores incubated in nutrient germination media released neither DPA from the spore core nor hexosamine fragments of the spore cortex (Table 3), events that characterize stages I and II of spore germination respectively (Setlow *et al.* 2001). Ozone-killed spores that carried the *luxAB* genes from *V. harveyi* also exhibited no light production upon incubation in nutrient germination medium, in contrast to the large amount of light production from untreated germinated spores incubated similarly (data not shown); this light production indicates the resumption of metabolism by the untreated germinated spores (Hill *et al.* 1994; Ciarciaglini *et al.* 2000; Loshon *et al.* 2001; Genest *et al.* 2002; Young and Setlow 2003, 2004). Spores killed by hypochlorite and Oxone™ also exhibit no release of DPA or cortical fragments and no resumption of metabolism upon incubation in nutrient germination media (Young and Setlow 2003, 2004).

Table 3 Changes upon incubation of untreated or ozone-killed spores in germination media*

Treatment	Killing (%)	Phase dark (%)	Spore components released (%)	
			DPA	Hexosamine
None†	0	98	100	ND‡
Ozone†	96	0	16§	ND
None¶	0	98	ND	28§
Ozone¶	97	0	ND	4
None**	0	75	ND	ND
Ozone**	98	0	ND	ND

*Spores of strain PS533 (wild type) were either not treated or treated with ozone, and the level of killing was determined. The spores were then incubated for 100 min at 37 °C in either 50 mM Tris–HCl (pH 8·4) plus 8 mM L-alanine or in 2X YT medium plus 4 mM L-alanine, or for 6 h at 24°C in Ca^{2+}-DPA, and the percentage of spores that became phase dark, and the percentages of total spore DPA and hexosamine released into the supernatant fluid were determined as described in Materials and methods.
†Spores incubated in 2X YT medium with alanine.
‡Not determined.
§Incubation for ≥4 h gave no further release of DPA or hexosamine.
¶Spores incubated in Tris–alanine.
**Spores incubated in Ca^{2+}-DPA.

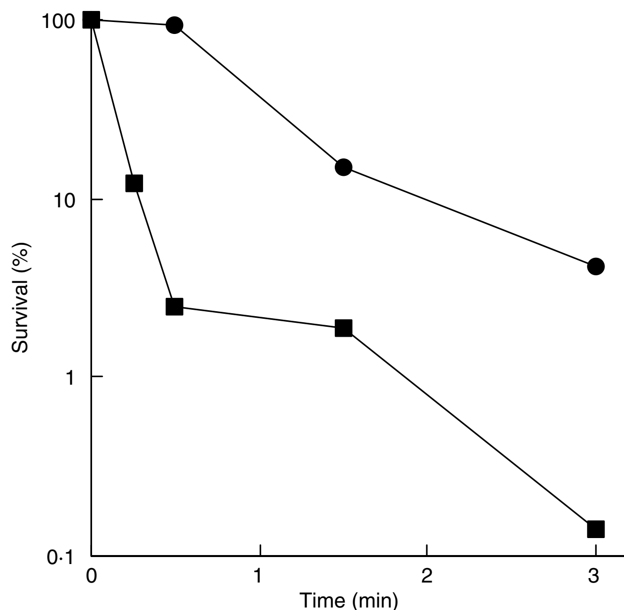

Fig. 4 Ozone resistance of spores with or without SleB. Spores of strains PS533 (wild-type) and FB112 (*sleB*) were incubated at 24°C in 20 ppm aqueous ozone and spore survival was measured as described in Materials and methods. The symbols used are: ●, PS533 spores; ■, FB112 spores

Ca^{2+}-DPA is an alternative germinant whose action requires only one of the two redundant cortex lytic enzymes (CwlJ) and does not require the spore's nutrient germinant receptors (Paidhungat and Setlow 2000; Paidhungat *et al.* 2001). Ozone-killed spores failed to become phase dark after incubation in Ca^{2+}-DPA (Table 3) and did not exhibit increased colony formation on plates after treatment with Ca^{2+}-DPA (data not shown). This could indicate specific damage by ozone to CwlJ, the cortex lytic enzyme required for Ca^{2+}-DPA germination. Indeed, spores lacking the second cortex lytic enzyme, SleB, were killed more rapidly by ozone than were wild-type spores (Fig. 4). Results similar to these have been obtained when spores were treated with chlorine dioxide (Young and Setlow 2003) and it has been suggested that CwlJ may be more sensitive to exogenous chemicals than SleB (Paidhungat *et al.* 2001). However, destruction of CwlJ cannot be the only cause of spore killing by ozone, as shown below.

If the mechanism of spore killing by ozone is the inactivation of both of the spore's cortex lytic enzymes, as is the case with spores apparently killed with alkali (Setlow *et al.* 2002), then ozone-killed spores should be recovered by removal of the spore coat followed by lysozyme degradation of the cortex in a hypertonic medium (Popham *et al.* 1996; Ishikawa *et al.* 1998; Setlow *et al.* 2002). Decoated ozone-killed spores incubated in a lysozyme-containing hypertonic medium did become dark in the phase contrast microscope, indicating that hydrolysis of the cortex had taken place. However, shortly thereafter these phase dark spores lysed and did not give colonies on nutrient plates; decoated ozone-killed spores also did not give colonies upon direct application to plates containing lysozyme (data not shown). Spores killed with a number of other oxidizing agents are also not revived by lysozyme treatment (Williams and Russell 1993; Genest *et al.* 2002; Young and Setlow 2003, 2004). In contrast, a high percentage (≥75%) of decoated untreated spores are recovered after lysozyme treatment in hypertonic medium, even when the spores are unable to degrade their own cortex (data not shown; Popham *et al.* 1996).

DISCUSSION

Although ozone can damage DNA and mutagenize bacteria (Dillon *et al.* 1992; Victorin 1992), it seems most likely that ozone does not kill spores by DNA damage. It might be argued that ozone kills spores by DNA damage that is neither repaired in a RecA-dependent fashion nor mutagenic. However, this seems unlikely, especially as spores killed 96–98% by ozone: (i) do not germinate with nutrients or Ca^{2+}-DPA, and (ii) when germinated artificially, they lyse rapidly. Perhaps ozone causes lethal damage to spores by reacting with compounds outside of the spore core before there can be significant damage to spore DNA.

While wild-type spores are almost certainly not killed by ozone through DNA damage, the mechanism(s) protecting spore DNA from ozone damage are not so clear. One obvious possibility is that this protection is provided by the α/β-type SASP that protect spore DNA from a number of other chemicals, including hydrogen peroxide (Setlow 2000; Tennen *et al.* 2000), and this idea was supported by the decreased ozone resistance of $\alpha^-\beta^-$ spores. However, even $\alpha^-\beta^-$ spores did not appear to be killed by ozone through DNA damage. Decreased resistance of $\alpha^-\beta^-$ spores to many chemicals that do not kill spores by DNA damage has been seen previously (Tennen *et al.* 2000; Loshon *et al.* 2001; Genest *et al.* 2002; Young and Setlow 2003, 2004). The major factor in spore resistance to this latter group of chemicals is the spore coat, with decoated and *cotE* spores exhibiting much lower levels of resistance. Thus it is possible that slight differences in the coats of wild-type and $\alpha^-\beta^-$ spores might result in differences in spore resistance to this group of chemicals and also ozone. Indeed, there are significant differences in the expression of genes encoding coat proteins during sporulation of wild-type and $\alpha^-\beta^-$ *B. subtilis* strains (Setlow *et al.* 2000). We also note that compared with intact spores, chemically decoated wild-type and $\alpha^-\beta^-$ spores exhibited similar ozone resistance, as did *cotE* and $\alpha^-\beta^-$ *cotE* spores. Presumably the chemical decoating or the *cotE* mutation has decreased levels of spore

coat protein sufficiently so as to minimize any differences in spore protection provided by the residual coat proteins in wild-type and $\alpha^-\beta^-$ spores. However, the identity of specific coat proteins whose precise level may modify spore ozone resistance is not known.

It has been suggested that spore coats are the major factor in the ozone resistance of *B. cereus* spores (Foegeding 1985), although a significant role in ozone resistance for one or more constituents of the large exosporium on the spores of this species was not ruled out. However, *B. subtilis* spores have a minimal, if any exosporium (Driks 1999), and the large decrease in the ozone resistance of decoated and *cotE B. subtilis* spores indicates that the spore coat is a major factor in *B. subtilis* spore resistance to ozone. The mechanism whereby the coats provide ozone resistance is not clear, but a simple hypothesis is that the large amount of protein and other constituents in the coats provides a reactive barrier that destroys much ozone before it can gain access to and react with more critical targets located further within the spore. That one or more constituents of the spore coats can react with ozone was shown in current work by the bleaching of *B. subtilis* spore coat pigment by ozone, and by previous work showing that *B. globigii* and *B. subtilis* spore coats are altered significantly by ozone treatment (Khadre and Yousef 2001; Cross *et al.* 2003). However, in the previous work, the spores with modified coats were from preparations killed to a very high degree by ozone. Thus it is not clear if the effects of ozone seen in these studies directly contribute to spore killing or are events that take place well after spores are already dead.

Examination of spores killed 96–99% by ozone in order to focus on initial events leading to spore killing indicated that ozone-killing of spores is accompanied by the loss of the ability to germinate in either nutrients or Ca^{2+}-DPA. The reasons for this loss of the ability to germinate are not clear, although at least the cortex lytic enzyme CwlJ is extremely sensitive to ozone. However, the loss of the capacity for spore germination in nutrients or Ca^{2+}-DPA cannot be the cause of spore killing by ozone, as ozone-killed spores are not recovered by artificial germination with lysozyme and actually lyse during this treatment. This finding suggests that spores killed by ozone may be so damaged, presumably in the inner membrane that becomes the plasma membrane of the germinated spores, that this membrane ruptures readily when the restraining spore cortex is removed by lysozyme digestion. Indeed, there are several reports that bacterial cells treated with ozone suffer membrane damage associated with cell killing (Doroszkiewicz *et al.* 1994; Komanapalli and Lau 1996; Thanomsub *et al.* 2002). Two other findings that support the concept of the spore's inner membrane as the site of lethal damage by ozone are that ozone -killed spores: (i) readily release their DPA upon a normally sublethal heat treatment, and (ii) germinate with dodecylamine much more readily than do untreated spores. Similar findings have been made for spores killed by a number of other oxidizing agents (Genest *et al.* 2002; Young and Setlow 2003, 2004). Presumably the damaged inner membrane in ozone-killed spores is: (i) unable to maintain its integrity upon a normally moderate heat treatment, and (ii) more readily made permeable by dodecylamine. However, the specific effects ozone might have on the inner membrane remain unclear. Ozone can react with unsaturated fatty acids forming a cyclic ozonide, and subsequent hydrolysis at the ozonide as well as the more hydrophilic nature of the hydrolysis products could significantly reduce the structural integrity of a membrane (Gunstone *et al.* 1994). Membrane proteins are another potential target for ozone, which can react with cysteine and methionine residues, and also with tryptophan, tyrosine and phenylalanine residues (Mudd *et al.* 1969). Biochemical analyses of the inner membrane of untreated and ozone-killed spores might shed some light on the effect of ozone on this membrane.

An alternative to ozone killing spores by damaging the spore's inner membrane is that ozone may enter the spore core and inactivate one or more critical core enzymes. Inactivation of core enzymes has been observed to accompany spore killing by peroxides (Palop *et al.* 1998), and ozone certainly has the potential to inactivate enzymes as noted above. However, it is not clear if inactivation of one or more core enzymes is the cause of spore killing by peroxides, or only a result of other already lethal damage to the spore. Indeed, damage to the spore's inner membrane might be expected to facilitate the entry of molecules such as ozone and peroxides into the spore core, just as ozone-killing facilitates the permeablization of the spore's inner membrane by dodecylamine. This in turn would be expected to speed up the inactivation of core enzymes by ozone and peroxides. It is also difficult to explain the lysis of ozone-killed spores that are germinated artificially by lysozyme, if spore death is caused only by inactivation of one or more core enzymes. Finally, it would seem likely that the great majority, if not all of a spore's complement of a particular enzyme would have to be inactivated by ozone in order to cause spore death, while only a small amount of damage to the spore's inner membrane might render it susceptible to rupture. Consequently, we favour the idea that spore killing by ozone is due to some type of damage to the spore's inner membrane, although the identity of this damage is yet unclear.

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