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ORIGINAL ARTICLE

Effects of Mn levels on resistance of *Bacillus megaterium* spores to heat, radiation and hydrogen peroxide

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Abstract

Aims: To determine the effects of Mn levels in *Bacillus megaterium* sporulation and spores on spore resistance.
Methods and Results: *Bacillus megaterium* was sporulated with no added $MnCl_2$ and up to 1 mmol l^{-1} $MnCl_2$. The resultant spores were purified and loosely bound Mn removed, and spore Mn levels were found to vary *c.* 100-fold. The Mn level had no effect on spore γ-radiation resistance, but *B. megaterium* spores with elevated Mn levels had higher resistance to UVC radiation (as did *Bacillus subtilis* spores), wet and dry heat and H_2O_2. However, levels of dipicolinic acid and the DNA-protective α/β-type small, acid-soluble spore proteins were the same in spores with high and low Mn levels.
Conclusions: Mn levels either in sporulation or in spores are important factors in determining levels of *B. megaterium* spore resistance to many agents, with the exception of γ-radiation.
Significance and Impact of the Study: The Mn level in sporulation is an important factor to consider when resistance properties of *B. megaterium* spores are examined, and will influence the UV resistance of *B. subtilis* spores, some of which are used as biological dosimeters.

Introduction

Spores of *Bacillus* species are extremely resistant to a variety of harsh treatments including heat, radiation and oxidizing agents (Setlow 2006). While there are multiple factors that contribute to spore resistance, the prevention and repair of damage to the spore's genome are of prime importance. A major mechanism preventing DNA damage in spores is the saturation of spore DNA with specific DNA-binding proteins, the α/β-type small, acid-soluble spore proteins (SASP), which protect DNA against damage by wet heat, dry heat, UV and γ-radiation and some oxidizing agents, including hydrogen peroxide (H_2O_2) (Setlow 2006; Moeller *et al.* 2008). The DNA protection given by the α/β-type SASP in spores is so great that a number of agents that might be expected to cause DNA damage, such as wet heat and H_2O_2, do not kill spores by damage to DNA. However, agents such as dry heat, UV radiation and γ-radiation do kill spores by DNA damage. Consequently, the other major factor in spore DNA resistance is the repair of DNA damage when spores return to life in germination followed by outgrowth, with this repair catalysed by a number of different enzymes, some of which are spore-specific (Setlow 2006; Wang *et al.* 2006; Moeller *et al.* 2007, 2008; Ibarra *et al.* 2008; Barraza-Salas *et al.* 2010).

Recent work in several biological systems has indicated that prevention of cell killing by agents like γ-radiation that can cause lethal damage by generation of reactive oxygen species (ROS) is in large part owing to the protection of proteins against such agents, in particular proteins involved in DNA repair (Daly *et al.* 2004, 2007, 2010; Daly 2009; Križko and Radman 2010; Slade and Radman 2011). One agent protecting proteins against ROS is Mn^{2+} ions complexed with low molecular weight species such as phosphate, amino acids, peptides or nucleosides,

and cells of several types of organisms exhibit heightened ROS resistance when their Mn content is increased (Daly *et al.* 2007, 2010; Barnese *et al.* 2008; McEwan 2009; McNaughton *et al.* 2010; Slade and Radman 2011). This work suggested that spores of *Bacillus* species might also exhibit increased ROS resistance if their Mn content was increased. However, a recent study found that *Bacillus subtilis* spore resistance to wet heat, dry heat, H_2O_2 or γ-radiation was unchanged over a ≥200-fold range of cytoplasmic Mn content (Granger *et al.* 2011). While this finding suggests that detoxification of ROS by Mn-containing complexes might not be important in bacterial spore resistance, *B. subtilis* spores are monogenomic. Consequently, haploidy might have masked potential gains in these spores' resistance owing to ROS scavenging by Mn-containing complexes, because recombination repair cannot be carried out efficiently in a haploid outgrowing spore. In contrast to *B. subtilis* spores, *Bacillus megaterium* spores are digenomic and can exhibit significant shoulders in their inactivation curves with both UVC and γ-radiation (Donnellan and Stafford 1968; Aoki and Slepecky 1974; Hauser and Karamata 1992), a general characteristic of radiation-resistant polyploid organisms. Consequently, in this work, we have examined the resistance of *B. megaterium* spores with very different Mn contents to wet and dry heat, H_2O_2 and UV and γ-radiation.

Materials and methods

Spores of *B. megaterium* QM B1551 (ATCC #12872; originally obtained from H.S. Levinson) were prepared at 30°C in liquid-supplemented nutrient broth (Goldrick and Setlow 1983) (125 ml in a 1 l flask) with no additional $MnCl_2$ added (supplemented nutrient broth with no $MnCl_2$ added has *c.* 0·3 μmol l^{-1} Mn) and with $MnCl_2$ added up to 1 mmol l^{-1}, and the spores were harvested and purified, including washing twice for 1 h with 10 mmol l^{-1} EDTA at 4°C to remove loosely bound Mn. The EDTA-treated spores were washed thoroughly with water, stored in water at 4°C and protected from light, and spores' Mn levels were determined as described (Nicholson and Setlow 1990; Granger *et al.* 2011). Spores of *B. subtilis* PS533 (Setlow and Setlow 1996) were prepared at 37°C in liquid 2xSG medium with different levels of $MnCl_2$ added, and the spores were isolated, purified and EDTA treated, and Mn levels were determined as described (Nicholson and Setlow 1990; Granger *et al.* 2011). All spores used in this work were free (>98%) of growing or sporulating cells and germinated spores.

Resistance of *B. megaterium* spores to wet heat [85°C in water with spores at an optical density at 600 nm (OD_{600}) of 1], dry heat (105°C with the lyophilized material from 1 ml of spores at an OD_{600} of 1) and H_2O_2 [5% in 50 mmol l^{-1} KPO_4 buffer (pH 7·4) with spores at an OD_{600} of 1] was measured as described (Popham *et al.* 1995; Granger *et al.* 2011). Spore resistance to γ-radiation in liquid (spores in water at an OD_{600} of 1) was measured using a ^{60}Co source with an output of 2·9 kGy h^{-1} as described (Granger *et al.* 2011). Spore resistance to γ-radiation in the dry state was measured by irradiation as for spores in liquid, but 1 ml of spores at an OD_{600} of 1 was centrifuged in a 1·5-ml microcentrifuge tube and the pelleted spores were freeze dried prior to irradiation. For measurements of spore viability following dry heat treatment or γ-irradiation of dry spores, samples were rehydrated with 1 ml water and treated briefly in a bath sonicator to disperse the spores, the OD_{600} was measured to quantitate spore recovery and spore viability was measured. UV resistance of *B. megaterium* and *B. subtilis* spores was measured at 23°C with spores at an OD_{600} of 1 in 2 ml water in a rotating 35-mm-diameter Petri dish exposed to 254 nm radiation (UVC) from a UVG-1 short wave UV lamp (UVP Inc., San Gabriel, CA, USA). The output of the lamp at the surface of the liquid was measured as 2×10^{-3} J min^{-1} cm^{-2} using a J-225 BLAK-RAY Ultraviolet Meter (UVP Inc.). All resistance properties were measured on two independent spore preparations with similar results.

Levels of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] that normally comprises *c.* 20% of the dry weight of the central core of spores of *Bacillus* species and is present in spores as a 1 : 1 chelate with divalent metal ions, generally Ca^{2+}, were determined in spores by laser tweezers Raman spectroscopy as described (Huang *et al.* 2007). Levels of the DNA-protective α/β-type SASP in spores were determined by dry rupture of 5 mg of purified dry spores, extraction of the ruptured spores twice with 0·75 ml of cold 3% acetic acid-30 mmol l^{-1} HCl, dialysis of the pooled extracts against cold 1% acetic acid for 18 h with one change and then lyophilization of the dialysates (Nicholson and Setlow 1990). The dry residue was dissolved in 30 μl of 8 mol l^{-1} urea plus 15 μl acid-gel diluent, aliquots run on polyacrylamide gel electrophoresis at low pH, the proteins on the gel were stained with Coomassie Blue, and the staining intensity of the bands because of SASP-α and SASP-β was compared (Nicholson and Setlow 1990).

Results

Sporulation of *B. megaterium* in supplemented nutrient broth with no $MnCl_2$ added and up to 1 mmol l^{-1} additional $MnCl_2$ was indistinguishable (data not shown), even though the Mn levels increased *c.* 100-fold in spores prepared with high $MnCl_2$ added to the sporulation

Table 1 Mn levels in *Bacillus megaterium* and *Bacillus subtilis* spores sporulated with different $MnCl_2$ concentrations*

[$MnCl_2$] added to sporulation (μmol l^{-1})	Mn (μg gm^{-1} dry wt) and DPA (attomol per spore) levels in spores		
	B. megaterium		*B. subtilis*
	Mn	DPA	Mn
0	52	505 ± 54	nd
0·3	–		28
1	–		261
3	757	547 ± 78	–
10	2922	533 ± 64	1811
100	5687	472 ± 56	2613
1000	6926	464 ± 39	3903

DPA, dipicolinic acid.

**B. megaterium* and *B. subtilis* were sporulated with different $MnCl_2$ concentrations added, spores were purified and EDTA treated twice, and the spores' Mn contents and DPA contents (±standard deviations for the 50 individual spores analysed) were determined as described in Materials and methods. The EDTA treatment only removed a significant amount of Mn (*c.* 60%) from the spores prepared with 1 mmol l^{-1} $MnCl_2$, as found previously (Granger *et al.* 2011).

medium (Table 1). This was seen with two independent preparations of *B. megaterium* spores (data not shown). The great majority of this spore Mn was tightly bound, because it was only with spores prepared with 1 mmol l^{-1} $MnCl_2$ that EDTA treatment removed any large percentage of the spores Mn (Table 1). While two EDTA treatments were routinely used to remove loosely bound Mn from spores, >95% of the Mn was removed in the first EDTA treatment (data not shown), as was found when *B. subtilis* spores were prepared in medium with up to 1 mmol l^{-1} $MnCl_2$ (Granger *et al.* 2011). The Mn levels in the EDTA-treated *B. megaterium* spores prepared with different $MnCl_2$ concentrations were also relatively similar to levels in *B. subtilis* spores prepared with the same added $MnCl_2$ (Table 1). It is most likely that the Mn remaining in spores after the EDTA washes is in the spore core and chelated to the spore's huge DPA pool that comprises *c.* 20% of the core's dry weight (Setlow 2006). However, even at the highest spore Mn levels obtained in this work, <5% of the spores' DPA was chelated with Mn, based on the amount of Mn in spores, and using 10% as the percentage of *B. megaterium* spore dry weight as DPA (Setlow 2006).

Previous work showed that increasing the Mn content *c.* 300-fold had no notable effect on *B. subtilis* spore resistance to wet heat, dry heat or hydrogen peroxide (Granger *et al.* 2011). However, with *B. megaterium* spores, increasing their Mn levels resulted in significantly elevated resistance to both wet heat and dry heat, and H_2O_2 was also increased when these spores' Mn levels were ≥750 μg g^{-1} (Fig. 1a–c). Increased Mn levels also greatly increased *B. megaterium* spores' resistance to UVC radiation, and this was also the case for *B. subtilis* spores but not as dramatically as with *B. megaterium* spores (Fig. 1d; Fig. 2). Note further that *B. megaterium* spores were significantly more resistant to UVC radiation than *B. subtilis* spores, perhaps because *B. subtilis* spores are monogenomic while *B. megaterium* spores are digenomic (Hauser and Karamata 1992). Previous work showed that *B. subtilis*

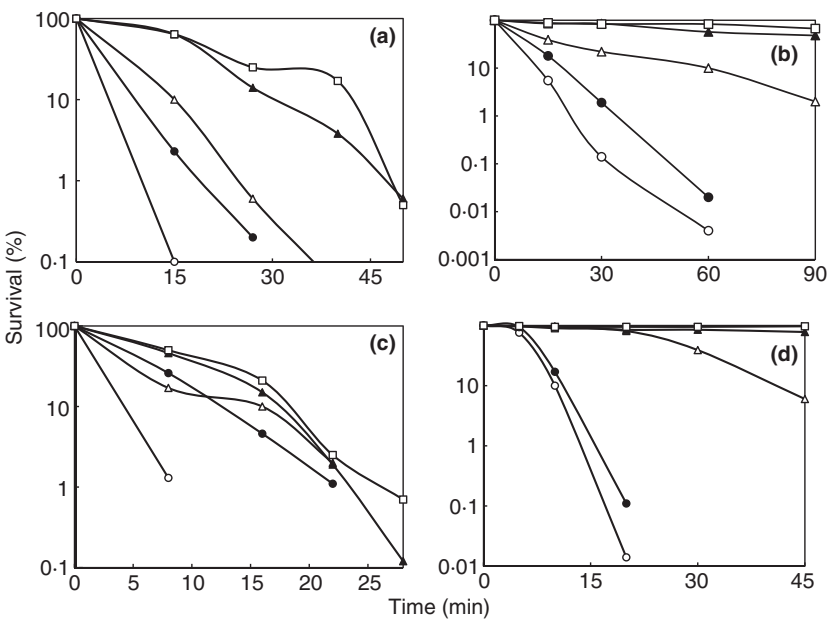

Figure 1 (a–d) Resistance of *Bacillus megaterium* spores with different Mn contents to heat, UVC radiation and H_2O_2. The resistance of *B. megaterium* spores with different Mn contents (Table 1) to (a) wet heat, (b) dry heat, (c) H_2O_2 and (d) UVC radiation were determined as described in Materials and methods. The symbols used to denote the concentrations of $MnCl_2$ added to the sporulation medium (Table 1) are (○) 0; (●) 3 μmol l^{-1}; (△) 10 μmol l^{-1}; (▲) 100 μmol l^{-1} and (□) 1 mmol l^{-1}.

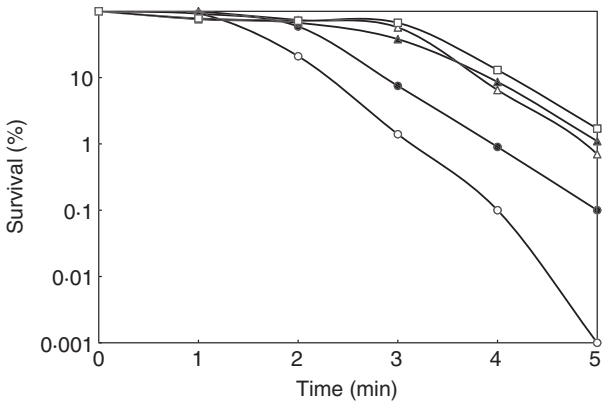

Figure 2 UVC radiation resistance of *Bacillus subtilis* spores with different Mn contents. The UVC resistance of *B. subtilis* spores with different Mn contents (Table 1) was determined as described in Materials and methods. The symbols used to denote the $MnCl_2$ concentrations added to the sporulation medium are (○) 0·3 μmol l^{-1}; (●) 1 μmol l^{-1}; (△) 10 μmol l^{-1}; (▲) 100 μmol l^{-1} and (□) 1 mmol l^{-1}.

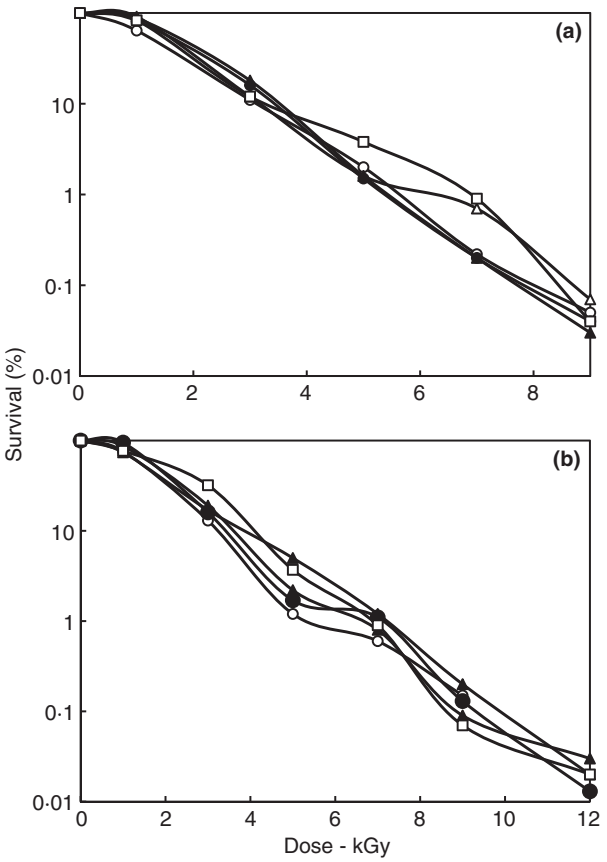

Figure 3 (a, b) The γ-radiation resistance of *Bacillus megaterium* spores with different Mn contents. The γ-radiation resistance of *B. megaterium* spores with different Mn contents (Table 1) was determined either (a) in liquid or (b) dry as described in Materials and methods. The symbols used to denote the $MnCl_2$ concentrations added to the sporulation medium are (○) 0; (●) 3 μmol l^{-1}; (△), 10 μmol l^{-1}; (▲), 100 μmol l^{-1} and (□) 1 mmol l^{-1}.

spore resistance to γ-radiation is independent of these spore's Mn content (Granger *et al.* 2011), and this was also the case with *B. megaterium* spores when irradiated either wet or dry (Fig. 3a,b). However, *B. megaterium* spores in liquid were slightly more resistant to γ-radiation than *B. subtilis* spores (Fig. 3a; and Granger *et al.* 2011).

While it seemed likely that the increased resistance of spores with high Mn levels was directly owing to the spores' high levels of Mn, it was certainly possible that elevated Mn levels during spore formation might have altered levels of some other component that protects spore proteins or DNA. One factor that certainly protects spore DNA from damage, and perhaps also spore protein, is DPA (Setlow 2006; Setlow *et al.* 2006; Magge *et al.* 2008). At least greatly elevated Ca^{2+} levels during sporulation have been shown to significantly alter the sporulating cell's transcriptome (Oomes *et al.* 2009). Interestingly, among the genes upregulated by elevated Ca^{2+} are the genes encoding the two subunits of DPA synthase. However, DPA levels in 50 individual *B. megaterium* spores prepared with $MnCl_2$ concentrations of 0–1 mmol l^{-1} were all essentially the same (Table 1).

A second major factor in spore DNA resistance is the α/β-type SASP, and spores with decreased α/β-type SASP levels are significantly less resistant to wet and dry heat, H_2O_2, and UVC and γ-radiation (Setlow 2006; Moeller *et al.* 2008). However, again, levels of α/β-type SASP were essentially identical in *B. megaterium* spores prepared with $MnCl_2$ concentrations of 0–1 mmol l^{-1} (data not shown). Another factor that could affect spore wet heat resistance is core water content (Setlow 2006). While we did not measure core water contents for *B. megaterium* spores, *B. subtilis* spores with large differences in Mn levels have identical core water contents (Granger *et al.* 2011).

Discussion

A number of observations made in the current work confirm reports made a number of years ago concerning the effects of preparation of spores of *Bacillus* species in media with elevated Mn concentrations. Thus, spores of *Bacillus fastidiosus* exhibit increased wet heat resistance when prepared in high-Mn media, and spores of *B. megaterium* prepared with high Mn exhibit significantly higher resistance to wet heat and UVC radiation (Donnellan and Stafford 1968; Aoki and Slepecky 1973, 1974). There is also one report that sporulation in medium with high Mn results in spores with very slightly increased

γ-radiation resistance (Aoki and Slepecky 1974), although this was not seen in the current work. In most of the work noted above, sporulation in media with high Mn resulted in increased spore Mn, although precautions were generally not taken to eliminate surface-bound Mn from spores. There have also been several studies in which the great majority of spores' divalent metal ions were replaced with Mn, by either sporulation with high Mn^{2+} concentrations and minimal levels of other divalent metal ions (Slepecky and Foster 1959) or removal of almost all divalent metal ions from spores by titration with acid and then back titration with Mn^{2+} in a basic solution (Marquis *et al.* 1981; Bender and Marquis 1985). In the latter experiments, almost all spore Ca and Mg were replaced by Mn, resulting in spore Mn levels 15–30 times higher than obtained with *B. megaterium* spores in this work. In contrast to the effects of increases in spore Mn levels seen by sporulation in normal media supplemented with up to 1 mmol l^{-1} $MnCl_2$, both in the current work and previously (Donnellan and Stafford 1968; Aoki and Slepecky 1973, 1974), replacement of almost all *B. megaterium* spore divalent cations with Mn generally resulted in slightly decreased wet heat resistance in the resultant spores (Slepecky and Foster 1959; Bender and Marquis 1985).

The current work showed clearly that increased Mn levels in sporulation and spores had major effects on the resistance properties of *B. megaterium* spores. For spore UV resistance, levels of two agents known to protect spore DNA against UVC radiation, DPA and α/β-type SASP, were essentially identical in spores with high and low Mn levels. Consequently, differences in DNA protection in spores with low and high Mn levels seem unlikely. Both UVC and γ-radiation can certainly kill spores by DNA damage, and dry heat kills at least *B. subtilis* spores by DNA damage (Setlow 2006; Moeller *et al.* 2007, 2008). In contrast, wet heat and H_2O_2 do not kill *B. megaterium* spores by DNA damage, but rather likely by damage to one or more spore proteins (Palop *et al.* 1998; Setlow 2006; Coleman *et al.* 2007, 2010), and at least *B. subtilis* spore inactivation by wet heat is independent of oxygen (Setlow and Setlow 1998). *Bacillus megaterium* spores prepared with high Mn levels exhibited elevated resistance to some but not all agents that kill spores by DNA damage, as well as to wet heat and H_2O_2. Consequently, it is difficult to ascribe the elevated resistance of spores with high Mn levels solely to either a general increase in DNA repair capacity, such as recombination repair to take advantage of the two complete genomes in *B. megaterium* spores or an increased ability to detoxify ROS. However, it is a logical possibility that the effects of high Mn are because of the ability of Mn complexes to inactivate specific ROS generated by some agents but not others. Indeed, Mn complexes from the radiation-resistant bacterium *Deinococcus radiodurans* strongly protect proteins against γ-rays, but protect DNA only poorly (Daly *et al.* 2007, 2010).

DNA in radiation-resistant and radiation-sensitive bacteria exhibits similar levels of DNA damage with the same dose of ionizing radiation (Daly *et al.* 2004; Daly 2009). In contrast, levels of protein damage in irradiated bacteria are dependent on their antioxidant status, and yields of radiation-induced protein oxidation can be >100-fold higher in radiation-sensitive bacteria (Daly 2009). Indeed in prokaryotes, the lethal effects of ionizing radiation appear to be mediated by oxidative protein damage, and for many oxidative stress conditions, including even UVA radiation, DNA may not be the major target of ROS (Daly *et al.* 2007; Leichert *et al.* 2008; Bosshard *et al.* 2010; Križko and Radman 2010; Avery 2011; Espirito Santo *et al.* 2011; Sobota and Imlay 2011). In addition, levels of protein damage in γ-irradiated bacteria are linked to the accumulation of Mn^{2+}, such that as bacteria's Mn^{2+} concentrations decline, cells become more sensitive to protein oxidation, but with no effects on DNA damage levels (Daly *et al.* 2004, 2007). These findings led to the conclusion that proteins are the principal targets of γ-radiation in bacteria and that Mn^{2+} prevents γ-radiation toxicity by protecting protein function (Daly 2009).

Mn^{2+} protection of proteins from ROS appears to occur at two levels: (i) by replacing Fe^{2+} with Mn^{2+} in enzymes, active sites are protected from oxidative damage (Anjem *et al.* 2009; Sobota and Imlay 2011); and (ii) surplus Mn^{2+} forms complexes with metabolites which can scavenge superoxide, H_2O_2 and hydroxyl radicals (Daly *et al.* 2010). Therefore, when spores are prepared with elevated $MnCl_2$, spore enzymes that normally bind Fe^{2+} but are also capable of binding Mn^{2+} might be more resistant to oxidative stress (Sobota and Imlay 2011). In addition, DPA is present in spores at very high levels and can form potent ROS-scavenging complexes with Mn^{2+} (Granger *et al.* 2011).

UVC causes substantial direct (ROS-independent) damage to cellular macromolecules *in vivo*. However, antioxidants can still increase the survival of cells exposed to UVC (Chan *et al.* 2006). Consequently, antioxidants in cells help avert ROS-mediated toxicity that is secondary to the UVC itself. For example, UVC disrupts certain types of disulfide bonds and causes protein aggregation, which could uncouple metabolism from electron transport and lead to ROS production. In turn, metabolism-induced ROS production would be expected to damage other cellular processes including DNA repair (Križko and Radman 2010; Slade and Radman 2011). Wet heat and exposure to H_2O_2 also can damage proteins

directly in spores and again could conceivably increase ROS production during spore outgrowth. A striking finding in this work was that UVC resistance but not γ-radiation resistance of *B. megaterium* spores was highly dependent on the spores' Mn content. This could be due at least in part to different modes of action of UVC and γ-radiation in cells. In contrast to UVC, most γ-radiation-induced lesions in cells are caused by ROS formed in the radiolysis of water during irradiation (von Sonntag 1987). As the water content of spores is lower than in vegetative cells (Setlow 2006), ROS production in γ-irradiated spores may be limited, and thus, ROS-scavenging Mn complexes might have less of an impact on spore survival. In addition, the limited molecular mobility of molecules in the spore core (Cowan *et al.* 2003) may decrease the ability of Mn complexes to scavenge long-lived ROS. Perhaps Mn complexes in outgrowing spores prevent oxidative inactivation of enzymes involved in the repair of UVC-damaged spore DNA. Alternatively, if enzymes that specifically repair UVC-induced DNA lesions in spores bind Mn^{2+} (note that such enzymes could not repair all the different lesions generated by γ-radiation), then elevated Mn levels during sporulation might selectively lead to the protection of the resultant spores against UVC but not γ-radiation. Because spore DNA lesions caused by wet heat, dry heat and H_2O_2 vary widely, and again most likely require different enzymes for their repair (Huesca-Espitia *et al.* 2002; Setlow 2006), the argument made above can also be made for differences observed in the effects of Mn accumulation on spore resistance to H_2O_2, dry and wet heat.

Another potential explanation for some results in the current work is that high Mn levels during sporulation selectively induce the synthesis of enzymes that can only repair DNA lesions generated in spores by UVC radiation (the spore photoproduct generated between adjacent thymine residues) and dry heat (abasic sites), but not the most dangerous lesions generated by γ-radiation (double-strand breaks). There are certainly repair enzymes that exhibit the appropriate specificity, although there is no information on the induction of synthesis of such enzymes by Mn and the packaging of such enzymes into spores. While induction of specific DNA repair enzymes by Mn could explain at least the elevated resistance to UVC radiation and dry heat of *B. megaterium* spores prepared with high Mn levels, such enzyme induction cannot explain such spores' elevated H_2O_2 and wet heat resistance, as these agents kill *B. megaterium* spores by protein damage (Palop *et al.* 1998; Coleman *et al.* 2010). However, we note that Mn^{2+} can form catalytic H_2O_2-decomposing complexes with amino acids and peptides, and Mn^{2+} can also selectively protect the function of metabolic pathways (e.g. pentose phosphate pathway), which could favour recovery from some stress conditions but not others (Berlett *et al.* 1990; Sobota and Imlay 2011).

Whatever the explanation for the increased resistance of *B. megaterium* spores with high Mn levels, this phenomenon will be important to consider when examining the resistance of spores of this species and perhaps spores of other species that form digenomic spores as well. In addition, the significant effects of Mn levels in sporulation and spores on the UVC radiation resistance of not only *B. megaterium* spores, but also *B. subtilis* spores and likely spores of all *Bacillus* species, will certainly need to be considered when preparing spores as indicators for UV inactivation regimens or UV dosimeters.

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