

Full Length Research Paper

# Effects of Mn<sup>2+</sup> levels on the resistance properties of *Bacillus cereus* spores

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**In some *Bacillus* species, manganese levels influence the resistance properties of spores. To determine if this was true for *Bacillus cereus*, bacteria were sporulated with different MnCl<sub>2</sub> concentrations resulting in spores with 30-fold differences in core Mn<sup>2+</sup> levels. Spores with different Mn<sup>2+</sup> levels displayed no differences in resistance to dry heat, UV radiation,  $\gamma$ -radiation, or hydrogen peroxide. However, spores with the lowest Mn<sup>2+</sup> level were less resistant to wet heat. Overall, Mn<sup>2+</sup> levels were not a major factor in *B. cereus* spore resistance, and this suggests that this will also be true for the closely related *B. anthracis* spores.**

**Key words:** Manganese (Mn<sup>2+</sup>), spores, spore resistance,  $\gamma$ -radiation, *Bacillus*, *Deinococcus*.

## INTRODUCTION

Spores of *Bacillus* species are extremely resistant to a variety of stress factors including wet or dry heat, UV or  $\gamma$ -radiation, or toxic chemicals, including oxidizing agents such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Setlow and Johnson, 2012). Spore resistance to these agents is due to a number of factors including the proteinaceous spore coats, the low hydration level of the spore core, the high levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) in the spore core, the saturation of the spore genome with the  $\alpha/\beta$ -type small, acid-soluble proteins (SASP), and DNA repair during spore outgrowth. A number of reports indicate that some agents, in particular UVA radiation and  $\gamma$ -radiation, kill growing cells of many aerobic organisms by generating reactive oxygen species (ROS) that damage macromolecules, including proteins and nucleic acids (Avery, 2011; Daly, 2012). Interestingly, elevated Mn levels are associated with increased resistance of growing cells of many bacteria and archaea

to UVA and  $\gamma$ -radiation, perhaps due to non-enzymatic detoxification of ROS by Mn<sup>2+</sup> containing small molecules (McEwan, 2009; Daly, 2012).

Previous work has also shown that spore Mn levels have significant effects on the resistance properties of *Bacillus megaterium* spores, with elevated Mn levels associated with increased spore resistance to wet or dry heat, UVC radiation, and hydrogen peroxide, although, Mn levels had no effect on spore  $\gamma$ -radiation resistance (Donnellan and Stafford, 1968; Aoki and Slepecky, 1974; Ghosh et al., 2011). Elevated Mn levels are also associated with elevated resistance of *Bacillus fastidiosus* spores to wet heat (Aoki and Slepecky, 1973). In contrast, *Bacillus subtilis* spores with over a 200-fold range of protoplast Mn levels exhibited no significant differences in resistance to wet or dry heat,  $\gamma$ -radiation or hydrogen peroxide, although, spores with low Mn levels were less resistant to UVC radiation than high Mn spores (Ghosh et al., 2011; Granger et al., 2011). Given the increasing use of  $\gamma$ -radiation as a means for sterilization of foodstuffs and the importance of *Bacillus cereus* as a food-borne pathogen (Setlow and Johnson, 2012), it

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**Table 1.** Levels of Mn<sup>2+</sup> in spores prepared in media with different amounts of added MnCl<sub>2</sub>\*.

[MnCl <sub>2</sub> ] Added to sporulation medium (μmol l <sup>-1</sup> )	Mn <sup>2+</sup> content of spores (μg gm <sup>-1</sup> dry weight)
0.5	73
1	485
10	1709
100	2311

\**B. cereus* was sporulated at 30°C in Ellar's modified liquid sporulation medium with different MnCl<sub>2</sub> concentrations added (Stewart et al., 1981), and the spores were then purified and treated with EDTA (Ghosh et al., 2011; Granger et al., 2011). Spore Mn<sup>2+</sup> and DPA levels (data not shown) were determined as described (Yi and Setlow, 2010; Ghosh et al., 2011; Granger et al., 2011).

seemed worthwhile to examine the effects of *B. cereus* spore Mn levels on these spores' resistance properties. In addition, given the close relatedness between *B. cereus* and *Bacillus anthracis* (Priest et al., 2004), results with *B. cereus* spores will also likely be applicable to *B. anthracis* spores.

## RESULTS AND DISCUSSION

### Preparation of *B. cereus* spores with varying levels of Mn

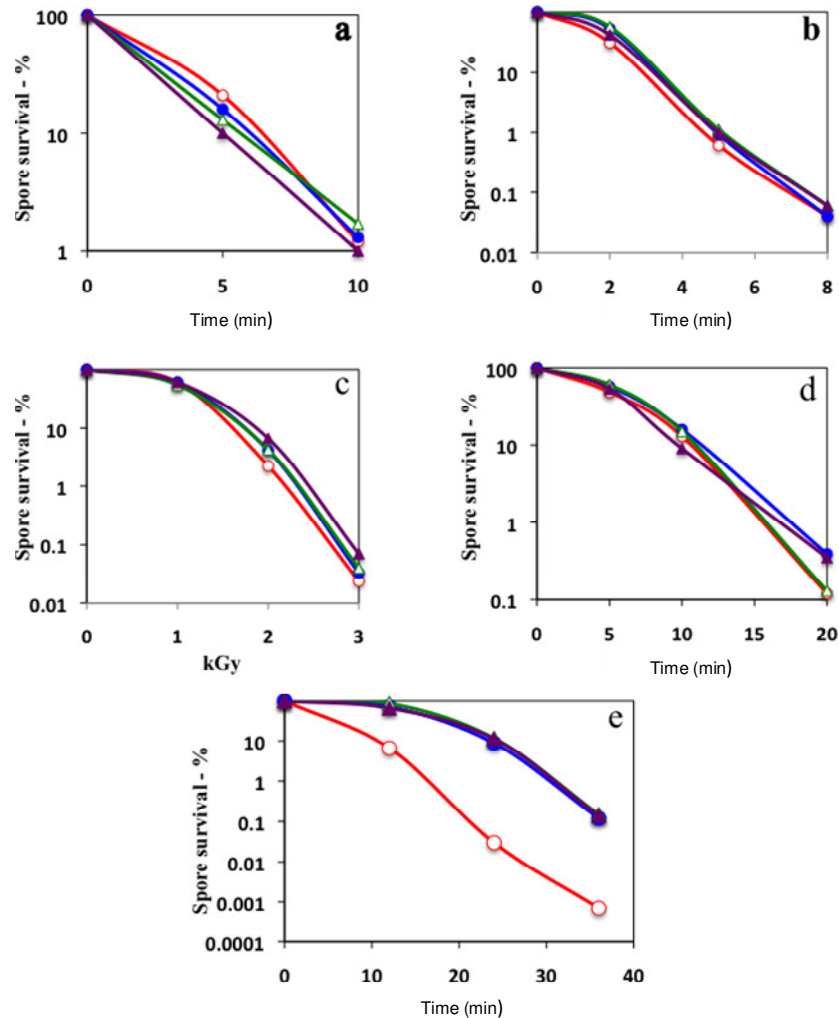
*B. cereus* was able to form spores in the presence of 0.5 to 100 μmol L<sup>-1</sup> added MnCl<sub>2</sub>, resulting in an ~30-fold increase in spore Mn levels in EDTA-washed spores at the highest MnCl<sub>2</sub> levels employed (Table 1). As found with spores of *B. megaterium* and *B. subtilis* (Ghosh et al., 2011; Granger et al., 2011), EDTA treatment removed < 2% of Mn from spores prepared with ≤ 10 μmol L<sup>-1</sup> Mn, and only ~ 10% from spores prepared with 100 μmol L<sup>-1</sup> Mn. These results suggest that ≥ 90% of Mn incorporated into spores prepared with ≤ 100 μM Mn is in the spore core, as EDTA should remove all Mn, except for that present in the spore core where most divalent cations are chelated with DPA. Note that even the maximal level of Mn found in spores will chelate < 5% of spore DPA, and DPA levels in spores made with various Mn levels were all within 7% of each other.

### Resistance properties of spores with different Mn levels

Analysis of the resistance of spores containing different Mn levels, with the exception of γ-radiation resistance, was carried out at least three times, each time using at least duplicate measurements of spore viability, and always with essentially identical results. Spore treatment with γ-radiation was carried out only once, but the irradiated samples were analyzed at least three times, each using at least duplicate measurements of spore viability, again with essentially identical results. One representative measurement of the resistance of spores

prepared with different Mn levels to various agents is shown in Figure 1. In these measurements, values for spore viability are ≤ ± 20%. These results indicate that over a 30-fold range, Mn levels play no notable role in *B. cereus* spore resistance to dry heat, UVC radiation, γ-radiation or H<sub>2</sub>O<sub>2</sub> (Figure 1a to d). However, spores with the lowest Mn levels did exhibit lower resistance to wet heat (Figure 1e).

Compared to other *Bacillus* species where the role of Mn on multiple resistance properties has been well-studied, *B. cereus* is more similar to *B. subtilis*, in that, only a single resistance property is affected by Mn (wet heat and UVC radiation, respectively). Why might these species differ in regard to the effects of Mn compared to *B. megaterium*, where multiple resistance properties are sensitive to Mn levels? One potential explanation is that the resistance properties of the different species are related to the number of chromosomes in the spores, since *B. subtilis* and *B. cereus* spores are monogenomic, while *B. megaterium* spores are digenomic (Hauser and Karamata, 1992). Multiple chromosomes in spores would allow for recombinational repair of DNA damage early in spore outgrowth, and it is possible that a component of this process is particularly sensitive to ROS. However, this scenario seems unlikely, as a number of reports indicate that wet heat and H<sub>2</sub>O<sub>2</sub> kill spores of *Bacillus* species by damage to proteins, and not by DNA damage (Palop et al., 1998; Coleman et al., 2007, 2010; Setlow and Johnson, 2012). Moreover, even in the case of γ-irradiation there is uncertainty in regard to the type, level and possible importance of DNA damage in spore killing. The level of γ-irradiation-induced DNA damage would be expected to be significantly less in *Bacillus* spores compared to vegetative cells, since the core water content of spores is extremely low (Setlow and Johnson, 2012). Indeed, because of spores' low core water content, spore DNA is predicted to be significantly more resistant to double strand break (DSB) formation by γ-radiation, and Mn-complexes that act as antioxidants may give only minimal protection against γ-radiation (Gaidamakova et al., 2012). As a result, DNA may not even be the lethal target for ROS generated by oxidative stress caused by γ-radiation and other agents (Daly,



**Figure 1.** Resistance properties of *B. cereus* spores with different Mn contents. Plots illustrating survival of *B. cereus* spores prepared with varying  $\text{MnCl}_2$  concentrations following treatment with (a) dry heat at  $120^\circ\text{C}$ , (b) UVC radiation at  $5 \times 10^{-4} \text{ J min}^{-1} \text{ cm}^{-2}$ , (c) varying doses of  $\gamma$ -radiation measured in kiloGrays (kGy), (d)  $\text{H}_2\text{O}_2$  (5% in 25 mM  $\text{KPO}_4$  buffer (pH 7.4) at  $23^\circ\text{C}$ ) and, (e) wet heat at  $87^\circ\text{C}$  are shown. All values were  $\leq \pm 20\%$ , and all resistance measurements were made at least twice, with at least duplicate determinations at each time point. The symbols denoting the  $\text{MnCl}_2$  added to the sporulation medium are: ( $\circ$ )  $0.5 \mu\text{mol l}^{-1}$ ; ( $\bullet$ )  $1 \mu\text{mol l}^{-1}$ ; ( $\triangle$ )  $10 \mu\text{mol l}^{-1}$ ; and ( $\blacktriangle$ )  $100 \mu\text{mol l}^{-1}$ . Spores of *B. cereus* T (originally obtained from H.O. Halvorson) were prepared at  $37^\circ\text{C}$  in liquid medium with different levels of added  $\text{MnCl}_2$ , and harvested and purified as described (Stewart et al., 1981; Ghosh and Setlow, 2010). These spores were free ( $> 98\%$ ) of growing or sporulating cells, germinated spores and cell debris as determined by phase contrast microscopy. Prior to analyses of spore resistance, spore preparations were incubated for 1 h with 10 mM EDTA at  $4^\circ\text{C}$ , and then washed thoroughly with water and stored in water at  $4^\circ\text{C}$  protected from light. Measurements of spore killing by wet heat, dry heat, UVC radiation,  $\gamma$ -radiation in liquid, or hydrogen peroxide were all carried out as described (Ghosh et al., 2011; Granger et al., 2011).

2012).

Given the above facts, it is not clear why changes in Mn levels would have such different effects on the resistance of spores of *B. cereus*, *B. megaterium* and *B. subtilis* to wet or dry heat, UVC radiation or hydrogen

peroxide. The differences in these effects is most striking in examining wet heat resistance, as a low Mn level has no effect on *B. subtilis* spore wet heat resistance, decreases *B. cereus* spore wet heat resistance slightly, and decreases *B. megaterium* wet heat resistance

markedly (Figure 1e; Ghosh et al., 2011; Granger et al., 2011). Why might Mn levels alter or not alter spores' wet heat resistance? While there is no definitive answer for this question, we suggest the following, and highly speculative, scenario. Since wet heat killing of spores of *Bacillus* species is most likely by protein damage (Coleman et al., 2007, 2010), perhaps in *B. megaterium* spores increasing Mn levels protect some key protein whose inactivation by wet heat results in spore death. In *B. subtilis* spores this same protein might be more resistant to wet heat, such that it is wet heat damage to another protein, and one whose stability is insensitive to Mn levels that results in spore death. In *B. cereus* spores, perhaps cumulative wet heat damage to several proteins causes spore death, and Mn levels affect the stability of only one of these proteins.

Two other points are also worth noting on the effects of Mn levels on spore resistance. First, given the close relatedness between *B. cereus* and *B. anthracis* (Priest et al., 2004), it seems likely that Mn levels will also have minimal effects on the resistance of *B. anthracis* spores. Given the interest in the killing and resistance of *B. anthracis* spores, this is a conclusion with significant applied importance. Second, usual media for spore preparation are invariably supplemented with  $\text{MnCl}_2$ , and with concentrations  $\geq 10 \mu\text{mol l}^{-1}$ . At least with spores of *B. cereus* and *B. subtilis*, this Mn concentration results in spores with maximal resistance to all agents examined. Consequently, one does not have to worry that sporulation in media with extremely high Mn levels will give spores of these species with abnormally elevated resistance.

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## REFERENCES

- Aoki H, Slepecky RA (1973). Inducement of a heat-shock requirement for germination and production of increased heat resistance in *Bacillus fastidiosus* spores by manganous ions. *J. Bacteriol.* 114:137-143.
- Aoki H, Slepecky RA (1974). The formation of *Bacillus megaterium* spores having increased heat and radiation resistance and variable heat shock requirements due to manganous ions. In *Spore Research 1973* ed. Barker AN, Gould, GW, Wolf J, Academic Press, London, pp. 93-102.
- Avery SV (2011). Molecular targets of oxidative stress. *Biochem. J.* 434:201-210.
- Coleman WH, Chen D, Li YQ, Setlow, P (2007). How moist heat kills spores of *Bacillus subtilis*. *J. Bacteriol.* 189:8458-8466.
- Coleman WH, Zhang P, Li YQ, Setlow P (2010). Mechanism of killing of spores of *Bacillus cereus* and *Bacillus megaterium* by wet heat. *Lett. Appl. Microbiol.* 50:507-514.
- Daly MJ (2012) Death by protein damage in irradiated cells. *DNA Repair* 11:12-21.
- Donnellan JE Jr, Stafford RS (1968). The ultraviolet photochemistry and photobiology of vegetative cells and spores of *Bacillus megaterium*. *Biophys. J.* 8:17-27.
- Gaidamakova EK, Myles IA, McDaniel DP, Fowler CJ, Valdez PA, Naik S, Gayen M, Gupta P, Sharma A, Glass PJ, Maheshwari RK, Datta SK, Daly MJ (2012). Preserving immunogenicity of lethally irradiated viral and bacterial vaccine epitopes using a radio-protective  $\text{Mn}^{2+}$ -peptide complex from *Deinococcus*. *Cell Host Microbe* 12:117-124.
- Ghosh S, Ramirez-Peralta A, Gaidamakova E, Zhang P, Li Y-Q, Daly MJ, Setlow P (2011). Effects of Mn levels on resistance of *Bacillus megaterium* spores to heat, radiation and hydrogen peroxide. *J. Appl. Microbiol.* 111:663-670.
- Ghosh S, Setlow P (2010) The preparation, germination properties and stability of superdormant spores of *Bacillus cereus*. *J. Appl. Microbiol.* 108:582-590.
- Granger AC, Gaidamakova EK, Matrosova VY, Daly MJ, Setlow P (2011). Effects of levels of Mn and Fe on *Bacillus subtilis* spore resistance, and effects of  $\text{Mn}^{2+}$ , other divalent cations, orthophosphate, and dipicolinic acid on resistance of a protein to ionizing radiation. *Appl. Environ. Microbiol.* 77:32-40.
- Hauser PM, Karamata D (1992). A method for the determination of bacterial spore DNA content based on isotopic labeling, spore germination and diphenylamine assay; ploidy of spores of several *Bacillus* species. *Biochimie* 74:723-733.
- McEwan AG (2009). New insights into the protective effect of manganese against oxidative stress. *Mol. Microbiol.* 72:812-814.
- Palop A, Rutherford GC, Marquis RE (1998). Inactivation of enzymes within spores of *Bacillus megaterium* ATCC 19213 by hydroperoxides. *Can. J. Microbiol.* 44:465-470.
- Priest FG, Barker M, Baillie LWJ, Holmes EC, Maiden MCJ (2004). Population structure and evolution of the *Bacillus cereus* group. *J. Bacteriol.* 186:7959-7970.
- Setlow P, Johnson EA (2012). Spores and their significance. In *Food Microbiology, Fundamentals and Frontiers*, 4<sup>th</sup> edition (Doyle MP, Buchanan R, eds.), p. 45-79. ASM Press, Washington, DC.
- Stewart GSAB, Johnstone K, Hagelberg E, Ellar DJ (1981). Commitment of bacterial spores to germinate. *Biochem. J.* 198:101-106.
- Yi X, Setlow P (2010). Studies of the commitment step in the germination of spores of *Bacillus* species. *J. Bacteriol.* 192:3424-3433.