

Role of superoxide in the germination of *Bacillus anthracis* endospores

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Abstract

The spore forming Gram-positive bacterium *Bacillus anthracis*, the causative agent of anthrax, has achieved notoriety due to its use as a bioterror agent. In the environment, *B. anthracis* exists as a dormant endospore. Germination of endospores during their internalization within the myeloid phagocyte, and the ability of those endospores to survive exposure to antibacterial killing mechanisms such as superoxide ($O_2^{\cdot-}$), is a key initial event in the infective process. We report herein that endospores exposed to fluxes of $O_2^{\cdot-}$ typically found in stimulated phagocytes had no effect on viability. Further endospores of the Sterne strain of *B. anthracis* were found to scavenge $O_2^{\cdot-}$, which may enhance the ability of the bacterium to survive within the hostile environment of the phagolysosome. Most intriguing was the observation that endospore germination was stimulated by a flux of $O_2^{\cdot-}$ as low as 1 μ M/min. Data presented herein suggest that *B. anthracis* may co-opt $O_2^{\cdot-}$ which is produced by stimulated myeloid phagocytes and is an essential element of host immunity, as a necessary step in productive infection of the host.

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1. Introduction

The ability to form an impervious spore is the key survival strategy of members of the bacillus family [1]. Once formed the spore enables the organism to remain dormant until the local environment has improved to a level that will support active growth. The spore also confers considerable resistance to chemical and thermal insult. There is also evidence to suggest

that the spore may play a direct role in virulence. For instance, spore coat extract from two serovars of *Bacillus thuringiensis* have been reported to be toxic for the Indian meal moth [2]. *B. thuringiensis* belongs to a group of genetically related organisms which include *B. cereus* and the mammalian pathogen *Bacillus anthracis* [3]. All members of this group form endospores with a structurally similar outer layer called the exosporium. This layer is chemically complex, consisting of protein, amino and neutral polysaccharides and lipids [4]. More recently, it has been shown that glycoproteins, which appear to be specific to the outer surface layers of the

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spore, are synthesized by *B. thuringiensis* [5], *B. cereus* [6] and *B. anthracis* [7].

Preliminary studies have identified proteins in the exosporium that play a role in insect pathogenicity (i.e., immune inhibitor A, a zinc metalloprotease, which degrades components of the insects immune system), regulate germination (i.e., alanine racemase, inosine preferring nucleoside hydrolase) and control intracellular survival of *B. anthracis* (i.e., iron/manganese superoxide dismutase), the key early event in anthrax [8–11].

In their quiescent state, macrophages are metabolically subdued. Yet upon encountering a microorganism, these cells become stimulated, resulting in sequestration of the invading microbe into an enclosed vacuole, the phagosome, into which O_2^- is secreted and proteins and proteases are released following fusion of lysosomes to form the phagolysosome [12,13]. Studies with mouse alveolar macrophages have demonstrated that for anthrax spores germination occurs within the phagolysosome [14].

Thus to survive within this harsh environment the bacterium must possess mechanisms, such as superoxide dismutase (SOD) and catalase, to circumvent the toxic events initiated by O_2^- and hydrogen peroxide (H_2O_2), produced by and the result of the oxidative burst, by scavenging these oxidants.

Microorganisms have developed a number of strategies to deal with oxidants: avoidance, neutralization and prevention of production [15]. For example, the identification of a spore surface located SOD raises the possibility that endospores can control the availability of O_2^- , generated by stimulated macrophages, which would otherwise mediate cell killing [16]. Thus, prior to germination the spore represents a target against which antibacterial factors can expend their efforts without having any adverse effect on the dormant organism.

This study addresses two critical questions. First, can the spore protect the organism from physiologically relevant levels of O_2^- enabling germination to proceed? Second, what impact does the oxidant environment, typically found within the phagolysosome have on the rate of spore germination? To address these questions, endospores of the Sterne strain of *B. anthracis* were exposed to a continuous flux of O_2^- at a rate typically found in stimulated phagocytes [12,13]. The degree of O_2^- scavenging, spore survival, and subsequent rate of germination was determined.

2. Materials and methods

2.1. The bacterial strain and media

The Sterne, 34F2 strain of *B. anthracis* (Colorado Serum Company, Denver, CO, USA) an attenuated variant employed extensively as an animal vaccine, was

examined during this study. The organism was stored in 10% glycerol L broth at -20°C . Difco L agar and Difco L broth were obtained from Becton Dickinson and Company (Becton Drive, Franklin Lakes, NJ) and made up as per the manufacturers instructions. Isolation Agar was formulated as follows; 6 g Oxoid Nutrient broth No. 2 (Oxoid Ltd, NY), 12 g Oxoid Agar No. 3, 300 mg/L $MnSO_4$ (JT Baker, NJ) 0.25 g NaH_2PO_4 (Omnipur EM Science) and 1 L sterile distilled water. The pH was adjusted to 6.7 and the agar was sterilized by autoclaving at 121°C for 15 min. Brain Heart Infusion Broth (Difco) was used as a general growth media. To examine the role of specific germinants the following mixtures were used: L-alanine solution contained 100 mM L-alanine (Calbiochem), 100 mM NaCl (Omnipur EM Science) and 10 mM NaH_2PO_4 at pH 7.2 [17]. The inosine mixture contained 5 mM inosine (Calbiochem), 100 mM NaCl and 10 mM NaH_2PO_4 . The combined alanine/inosine mixture contained 100 mM L-alanine, 5 mM inosine, 100 mM NaCl and 10 mM NaH_2PO_4 at pH 7.2 [17].

2.2. Spore production

A single colony harvested from an overnight culture grown on L agar at 37°C was used to inoculate 100 mL of L-broth in a 250 mL Duran. The culture was incubated at 37°C on an orbital shaker (200 rpm) for 6 h. At the end of this period, 3 mL of culture was transferred to a 225 cm³ vented tissue flask (Corning Inc) containing 12 mL isolation agar. Following inoculation flasks were incubated at 30°C until 99–100% of the organisms had formed endospores (microscopic examination/phase contrast). The % spore yield was determined by comparing colony counts of heated (70°C for 20 min) and unheated samples. Endospores were harvested by adding 20 mL of sterile phosphate buffered saline (PBS) to the flask. The re-suspended endospores from 20 flasks were pooled and centrifuged at 4200 rpm for 10 min at 4°C . The resulting pellet was resuspended in 200 mL of sterile PBS and centrifuged again. To determine the effect of repeated washings on spore activity, endospores were washed either as total of 3 or 10 times with the endospores being resuspended in a final volume of 50 mL, which was refrigerated at 4°C until required. The final endospore concentration was determined to be 1×10^9 endospores/mL.

2.3. Spin trap experiments

Prior to the commencement of each experiment, endospores were heat activated at 56°C for 30 min. This relatively low temperature was employed throughout the study to minimize heat-induced inactivation of surface located enzymes such as SOD and alanine racemase.

Spin trapping of $O_2^{\cdot-}$ was conducted by mixing endospores, ranging from 0.5×10^4 to 0.5×10^7 endospores/mL, with 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO, 50 mM, see Fig. 1), in PBS (pH 7, containing diethylenetriaminepentaacetic acid, DTPA, 1 mM) and hypoxanthine (400 μ M). The generation of $O_2^{\cdot-}$ was initiated by the addition of xanthine oxidase to the reaction mixture such that the rate of $O_2^{\cdot-}$ was 1 μ M/min, as determined by the SOD-inhibitive reduction of ferricytochrome *c* (80 μ M) at 550 nm using an extinction coefficient of 21 mM⁻¹ cm⁻¹ [18]. Electron paramagnetic resonance (EPR) spectra were recorded (Varian Associates model E-109 spectrometer) at room temperature, 3 min after the addition of xanthine oxidase. Instrumental settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; sweep time, 12.5 G/min; response time, 0.5 s; and the receiver gain, 10×10^4 . As a control experiment, we monitor the formation of uric acid at 290 nm in the presence of the endospore. We found that there was no inhibition in xanthine oxidase activity. 5-*tert*-Butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide was prepared according to the method described by Stolze et al. [19].

2.4. Viability and germination studies

To determine if exposure to a low flux of $O_2^{\cdot-}$ had any effect on endospore viability and subsequent germination, heat shocked endospores were exposed to $O_2^{\cdot-}$, at a rate of 1 μ M/min, generated from xanthine/xanthine oxidase, for either 1 or 10 min at which point SOD (30 U/mL) was added to eliminate $O_2^{\cdot-}$ from the reaction mixture. Following exposure, counts were performed to determine the number of viable organisms that survived. As a control, endospores were placed in the same reaction mixture in the absence of $O_2^{\cdot-}$.

To determine if $O_2^{\cdot-}$ stimulated germination, endospores were incubated in the following media, BHI broth, L-alanine and inosine. A total of 0.5 mL of endospore suspension was added to 2.5 mL of germination mixture (final spore concentration 1.8×10^5 spore/mL) and incubated at room temperature (21 °C) the opti-

mum temperature for *B. anthracis* spore germination [11]. Samples (100 μ L) were taken at times 0 and 40 min and counts were performed. The percentage germination was calculated as follows; $(1 - (\text{heat resistant count} / \text{heat sensitive count})) \times 100$.

2.5. Bacterial counts

The concentration of vegetative phase organisms and endospores were determined as follows: samples were serially diluted in sterile PBS and 100 μ L of each dilution was plated onto a previously dried L-agar plate. The inoculated plate was incubated overnight at 37 °C and the resulting colonies at each dilution were determined. Each count was repeated at least twice. To determine the number of endospores present, the samples were first incubated at 56 °C for 30 min, conditions which had previously been shown to inactive vegetative *B. anthracis*.

3. Results

3.1. Superoxide scavenging by endospores

In an initial series of experiments, we used spin trapping/EPR spectroscopy to measure the inhibitory properties of endospores (Fig. 1). Endospores of the Sterne vaccine strain of *B. anthracis* were exposed to $O_2^{\cdot-}$ flux, from xanthine/xanthine oxidase, at 1 μ M/min for

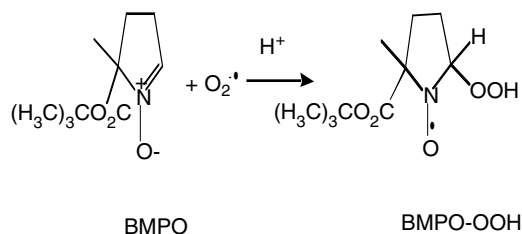


Fig. 1. The nitron, BMPO, reacts with $O_2^{\cdot-}$, affording BMPO-OOH that is measurable by EPR spectroscopy at ambient temperatures. This analytical method, spin trapping allows real time detection of $O_2^{\cdot-}$ in the presence of spores.

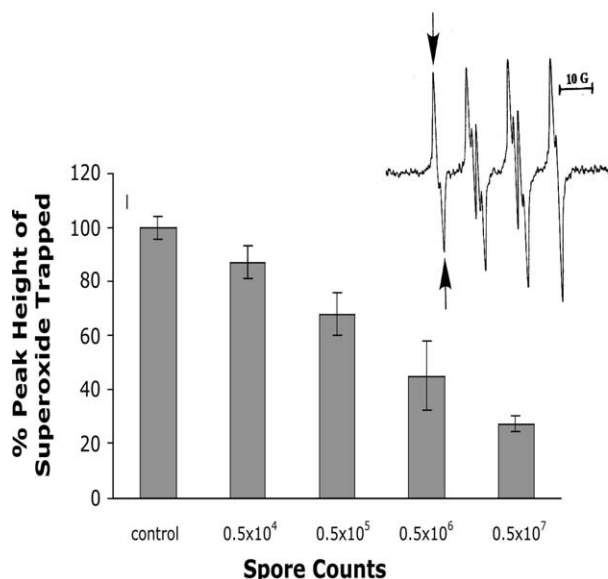


Fig. 2. A representative plot of $O_2^{\cdot-}$, spin trapped by BMPO, in the presence of *B. anthracis* endospores, washed 10 times. Data show a decrease in the first low-field peak height, arrows in the inset, of the EPR spectrum of BMPO-OOH as a function of spore concentration. Data are the average of three independent experiments.

3 min, typically found in stimulated phagocytes (4). The degree of $O_2^{\cdot-}$ scavenging is shown in Fig. 2.

Two observations are worth noting from data presented in Fig. 2. First, even at a very low concentration of endospores, e.g., 5000 spores, the spin trapping of $O_2^{\cdot-}$ by BMPO was modestly inhibited as compared to control. As we increased the number of spores in the experiment exposed to $O_2^{\cdot-}$, the percentage inhibition increased, reaching nearly 60% at 500,000 spores. Second, there was no significant difference in the $O_2^{\cdot-}$ scavenging properties of these endospores, whether they were washed three times (data not shown) or 10 times. These findings suggest that there is a SOD or a SOD mimic that is tightly bound to or associated with the endospore, as extensive washing did not alter the ability of the endospore to scavenge $O_2^{\cdot-}$.

3.2. Effect of $O_2^{\cdot-}$ on endospore viability

Endospores of the Sterne strain of *B. anthracis* ($0.5 \times 10^6/\text{mL}$) were exposed to $O_2^{\cdot-}$ fluxes of either 1 or 10 $\mu\text{M}/\text{min}$ for 10 min ($n = 4$ for each group). Endospore survival was assessed as the number of viable colony forming units (cfu)/mL following exposure to $O_2^{\cdot-}$. As shown in Fig. 3, there was no measurable decrease in endospore viability in the presence of $O_2^{\cdot-}$ as compared to endospores that were not exposed to this free radical.

3.3. Effect of $O_2^{\cdot-}$ on germination

While exposure to $O_2^{\cdot-}$ had no discernable effect on endospore viability, we hypothesized that low fluxes of $O_2^{\cdot-}$ may actually promote spore germination. The critical role of macrophages in promoting *B. anthracis* propagation during infection suggests that the organism may co-opt host defenses as a necessary part of the organism's pathogenesis.

Percentage of endospores, which germinated following exposure to $O_2^{\cdot-}$ flux of 1 $\mu\text{M}/\text{min}$ for 1 min, was compared to that of unexposed controls. While there was no evidence of germination at time 0, however, by 40 min between 25% and 80% of the spores had germinated, the

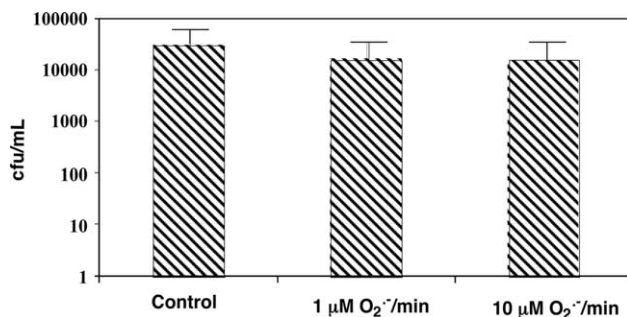


Fig. 3. The number of viable organisms follows exposure of endospores of *B. anthracis* to $O_2^{\cdot-}$ at two different rates of $O_2^{\cdot-}$ production.

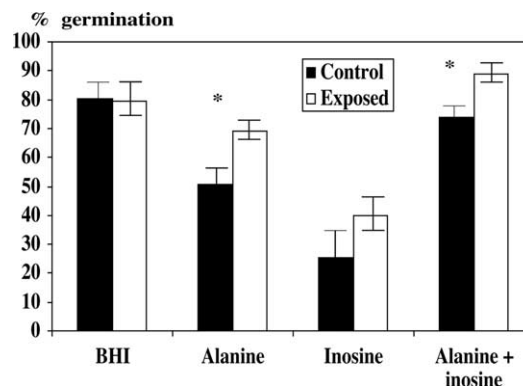


Fig. 4. The percent germination of washed (3 $\times$) *B. anthracis* endospores exposed to $O_2^{\cdot-}$ at 1 $\mu\text{M}/\text{min}$ for 1 min following 40 min incubation in germination medium at room temperature (21 °C). Each assay was repeated three times. * Statistically different results ($p > 0.05$).

range of levels reflecting differences in spore treatment and germination medium as can be seen in Fig. 4.

The degree of spore washing was found to have little effect on the rate of $O_2^{\cdot-}$ induced germination, with spores washed three times giving similar results to those washed 10 times (data not shown). These data suggest that the phenomenon was not due to contaminating vegetative cell debris.

The most pronounced germination (80%) was seen when spores were incubated in BHI. That there was no difference between $O_2^{\cdot-}$ treated and control spores ($p = 0.76$) was not particularly surprising given that BHI contains a range of germination triggers.

In the case of L-alanine, a strong, independent germinant of *B. anthracis*, germination was significantly greater by 69% (paired student *t*-test) after treatment with $O_2^{\cdot-}$ as compared to untreated endospores (50%, $p = 0.038$) after 40 min. While these data were similar to those reported by Titball and Manchee [11], they differed markedly to those reported by Ireland and Hanna, who found that spores of the Δ Sterne variant strain of *B. anthracis* germinated extremely rapidly in L-alanine, >90% in 10 min at room temperature [17].

The ability of inosine to enhance the activity of alanine has been reported previously [17,20]. In this study, we found that germination levels of 73% (control) and 89% ($O_2^{\cdot-}$ treated) were achieved and that significantly greater germination was observed in the $O_2^{\cdot-}$ exposed group ($p = 0.0007$). Finally, the ability of inosine alone to stimulate germination in these studies, all be it at low levels compared to the other groups, again contradicted the results of Ireland and Hanna [17].

4. Discussion

It was not unexpected that endospores of *B. anthracis* survived exposure to physiological relevant levels of

O_2^- , given that the endospore is designed to provide protection under much harsher environments. However, at some point endospores must germinate into vegetative bacilli in order to replicate. Oxidants, proteases, and other macrophage products are more likely to be successful in targeting the metabolically active vegetative bacilli. One source of potential protection for germinating *B. anthracis* may be endospore remnants that act as scavengers of biologic oxidants, such as O_2^- and H_2O_2 . In the case of the *Leishmania donovani*, LPG (lipophosphoglycan), a surface expressed glycoprotein, protects the parasite from cytotoxic effects of the macrophage by scavenging O_2^- generated during the respiratory burst [21]. The presence of SOD in the exosporium, which surrounds the endospore, may play a role in protecting the germinating bacilli from oxidative injury during a sensitive period until the bacillus fully matures.

The observation that low fluxes of O_2^- can promote germination may explain why endospores need to be phagocytosed by macrophages before germination can proceed [22,23]. In this theory, once the endospore is inside the phagolysosome, NADPH-oxidase secretes O_2^- which triggers germinations. This hypothesis is consistent with the studies of Weiner and Hanna who reported that germination of *B. anthracis* endospores was dramatically enhanced as the result of spore/macrophage interaction [23]. While these authors did not offer a mechanism by which macrophages increased the rate of spore germination, the possibility that O_2^- is the triggering event cannot be discounted.

The rates of germination observed in these studies mirrored those reported by Titball and Manchee but differed from those of Ireland and Hanna who reported significantly faster germination with >90% of endospore germinating within 10 min [17]. These conflicting results may reflect differences in assay conditions and the strains used, unlike the Sterne strain employed in this study, and the fully virulent strains examined by Titball and Manchee [11], the Δ Sterne strain used by Ireland and Hanna lacked pXO1, a plasmid known to encode at least one germination operon, *gerX*, which has been shown to play a role in germination within macrophages [17,24].

Interestingly, Ireland and Hanna also reported that germination in L-alanine did not require the presence of OCDS, an inhibitor of alanine racemase, an enzyme which inhibits germination of *B. anthracis* and *B. cereus* endospores by converting L-alanine to D-alanine, an isomer that is not recognized by germination receptors [11,20,25]. This mechanism is thought to enable the organism to control germination when large numbers of spores are present in the same environment [11].

Recent proteomic studies have demonstrated the presence of alanine racemase and an inosine preferring nucleoside hydrolase in the endospore exosporium, the outer most layer of the spore [8–11,26]. Germination is

thought to occur when germinants such as alanine and inosine traverse the outer layers of the endospore and interact with spore-germination receptor proteins of the GerA family, which are located at the inner membrane of the endospore [20]. Studies with *B. cereus*, a close relative of *B. anthracis*, which is also reported to germinate in the presence of alanine and inosine, have to date identified three germination operons, the *gerL* operon, which recognizes alanine, the *gerQ* operon which responds to inosine and *gerI* operon which responds primarily to inosine but it also has some activity in the presence of alanine [20].

It is fascinating to speculate that these enzymes may play a role in regulating germination by preventing the access of alanine and inosine and that their activities are influenced by the conditions found inside the macrophage, principally radical production. Further studies will be required to define the roles of these surface located enzymes.

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