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Atomic force microscopy study of germination and killing of *Bacillus atrophaeus* spores†

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Bacterial spores such as *Bacillus atrophaeus* are one of the most resistant life forms known and are extremely resistant to chemical and environmental factors in the dormant state. During germination, as bacterial spores progress towards the vegetative state, they become susceptible to anti-sporal agents. *B. atrophaeus* spores were exposed to the non-nutritive germinant dodecylamine (DDA), a cationic surfactant that can also be used as a killing agent, for up to 60 min, or to the nutrient germinant L-alanine. In kinetic studies, 99% of the spores were killed within 5 min of exposure to DDA. Atomic force microscopy (AFM) can be used as a sensitive tool to assess how the structure of the spore coat changes upon exposure to germinants or killing agents. Changes in cell height and roughness over time of exposure to DDA were examined using AFM. DDA caused the spore height to decrease by >50%, which may have been due to a partial breakdown of the spore coat. Treatment of *B. atrophaeus* with the nutrient germinant resulted in a decrease in height of spores after 2 h of incubation, from 0.7 ± 0.1 μm to 0.3 ± 0.2 μm. However, treatment with L-alanine did not change the surface roughness of the spores, indicating that the changes that occur during germination take place underneath the spore coat. We propose that exposure to DDA at high concentrations causes pores to form in the coat layer, killing *B. atrophaeus* without the need to fully germinate spores. Published 2009 by John Wiley & Sons, Ltd.

Keywords: RMS roughness; spore; L-alanine; dodecylamine

INTRODUCTION

Under nutrient deprivation, vegetative cells of *Bacillus* spp. and *Clostridium* spp. are able to undergo a restructuring and differentiation process known as sporulation (Atrih and Foster, 2002; Chada *et al*., 2003; Giorno *et al*., 2007). Bacterial spores are metabolically dormant and are the most resistant life forms known. Their inner cell membrane surrounds and protects the core of the spore, which contains its chromosome and other cellular contents. This membrane is protected from external environmental factors by a nearly 100 nm barrier consisting of a polymer layer and protein coat (Figure 1) (Henriques and Moran, 2000). Because of their unique structure and morphology, spores can overcome environmental and chemical factors such as radiation, desiccation, heat, changes in pH and exposure to toxic chemicals (Slieman and Nicholson, 2001; Setlow, 2003; Plomp *et al*., 2007). Dormant spores are able to constantly monitor their surrounding environment so that when nutrients become available, they can return to a vegetative state, after passing through the stages of germination and outgrowth (Setlow, 2003). Because of their virulent pathogenic nature, *B. anthracis* and other spores are problematic since they can be used as biowarfare and bioterrorism agents causing severe and frequently lethal foodborne and airborne diseases, such as pulmonary anthrax (Atrih and Foster, 2002; Akoachere *et al*., 2007).

The current theory of spore inactivation assumes that in most deactivation technologies germination must occur before spores can be killed by anti-sporal agents (Atrih and Foster, 2002), while some examples of spore killing, even in the absence of germination, also exist (Tennen *et al*., 2000). Germination can be triggered in response to nutrients, such as amino acids, sugars and purine nucleosides, or by non-nutrient factors such as lysozyme, Ca^{2+}-DPA, cationic surfactants, high pressures or salts (Setlow, 2003). During the first phase of germination, the spore releases H^{+}, monovalent cations and Zn^{2+}, which causes an elevation of pH in the core (Jedrzejas and Setlow, 2001; Setlow, 2003). This is followed by the release of Ca^{2+} and pyridine-2 6-dicarboxylic acid (dipicolinic acid (DPA)), accounting for approximately10% of the spores dry weight (Setlow, 2003). As DPA is released, water molecules enter and hydrate the core, causing a loss of heat resistance (Slieman and Nicholson, 2001). During the second stage, further water uptake allows for hydrolysis of the spore cortex and swelling of the core and germ cell wall. After expansion of the core, metabolism begins and macromolecular synthesis converts the spore into a germinated cell, by breaking of the spore coat and final release of a vegetative cell (outgrowth) (Atrih and Foster, 2002).

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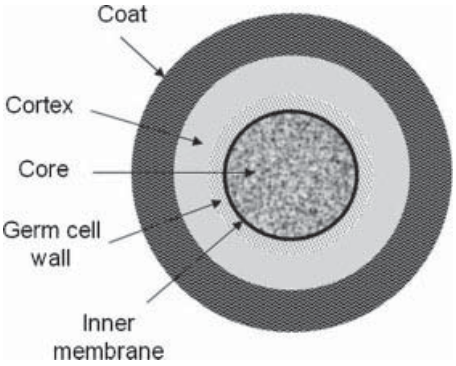

Figure 1. Schematic representation of *B. atrophaeus* spore structure. Common dimension of the different layers of spore structure are: Spore coat (60–100 nm), spore cortex (50–100 nm), germ cell wall (2–10 nm), inner membrane (3–4 nm) and spore core (0.7 to 1 μm).

Dodecylamine (DDA) is a cationic surfactant that has been used as a chemical agent to stimulate spore germination (Rode and Foster, 1961; Setlow *et al*., 2003). Previous work with *B. subtilis* indicated that the mechanism by which DDA triggers germination is different from how other nutrients and non-nutrient factors, such as Ca^{2+}-DPA (Setlow *et al*., 2003). Rather than binding to nutrient germinant receptors or to cortex lytic enzymes, DDA may act against and compromise the spore's inner membrane (Setlow *et al*., 2003). *B. megaterium* spores exposed to 6×10^{-5} M DDA for more than 3 min could be killed as germination was taking place, suggesting that DDA both germinates and deactivates *B. megaterium* (Rode and Foster, 1961).

Traditional methods to study spore germination rely on bulk or indirect measurements. For example, spore germination is monitored by quantifying the amount of DPA released, through optical density measurements (Setlow *et al*., 2003). Spore activity can also be monitored, such as assessment of the percentage of mammalian cells killed due to germination of spores (Akoachere *et al*., 2007). One direct method to study spore germination is to use electron microscopy to examine how the morphology of the spores changes during germination (Zaman *et al*., 2005). However, this technique has limitations since sample preparation dehydrates cells and can affect morphology, which would lead to misinterpretation of the images.

Atomic force microscopy (AFM) is a technique that can be used to examine individual spores with minimal sample preparation and it has been widely used to study epithelial cells, bacteria, viruses and fungi in their native conditions (Dufrene, 2002). Changes in morphology and ultrastructure of several *Bacillus* spp. before and after exposure to different nutrient germinants were studied using AFM, such as determining how mutations in particular coat proteins affect germination (Chada *et al*., 2003). Most of these studies focused on a qualitative understanding of how the spore coat changes during germination and how these changes affect spore size (Plomp *et al*., 2005b; Zaman *et al*., 2005; Zolock *et al*., 2006; Plomp *et al*., 2007). To our knowledge, no study has assessed the morphological changes that spores undergo when treated with non-nutrient germinants, such as DDA, and how different treatments affect the roughness of the spore surface as it is being germinated.

In this study, AFM was used to quantify spore surface roughness as a function of incubation time in nutrient and non-nutrient germinants, and to measure changes in the heights of the spores, during the process of germination with DDA and L-alanine. Our results suggest that DDA kills *B. atrophaeus* spores before outgrowth occurs. Therefore, DDA may serve as a deactivation agent against a broad spectrum of normal and mutant *Bacillus* spp., since complete germination is not required in order for deactivation to occur.

MATERIALS AND METHODS

Bacterial strains and spore preparation

Bacillus atrophaeus, previously classified as *Bacillus subtilis* var. *niger, Bacillus niger,* or *Bacillus globigii* (Fritze and Pukall, 2001), is a Gram-positive, aerobic, spore-forming bacterium that has been widely used as a nonpathogenic surrogate for *B. anthracis* and as a biological indicator for decontamination and sterilization processes and environmental biotracers (Burke *et al*., 2004; Plomp *et al*., 2005b). *B. atrophaeus* NRRL B-4418 was purchased (American Tissue Culture Collection, ATCC 6455). *B. atrophaeus* cultures were grown on plates of sporulation media, consisting of 8 g nutrient broth, 4 g yeast extract, 0.001 g $MnCl_2.4H_2O$, 5 g peptone and 15 g agar in 1 L of ultrapure water (Milli-Q water, Millipore Corp., Bedford, MA) and maintained at a pH of 7.2.

Plates were incubated at 37°C for 4 days. Spores were collected by centrifugation at 5000 RPM for 20 min and resuspended in ultrapure water. The cells were washed eight times to separate the spores from vegetative and partially sporulated cells and stored at 4°C. The spores were allowed to remain in water overnight and then washed two more times to remove any remaining vegetative cells or semi-sporulated cells.

Kinetics studies of spore killing by DDA

A solution of *B. atrophaeus* at 10^7 spores/mL was centrifuged and resuspended in 1 mM DDA (Sigma-Aldrich, St. Louis, MO). Spores were incubated at 37°C for 0, 1, 5, 10, 20, 25, 30, 40, 50 and 60 min and kept in an ultrasonication bath (Bronson, 1510, 40 kHz, 130 W, Branson Ultrasonics Corp., Danbury, CT) to prevent settling. The action of DDA on the spores at the end of the incubation period was terminated by immersion of spore suspension into an ice bath.

To determine the effects of DDA on spore viability, the treated spore solution was serially diluted and aliquots of spore solution were inoculated onto sporulation agar plates and cultured in an incubator at 37°C. The number of surviving spores or colony forming units (cfu) that became vegetative cells on the agar plates was determined after 18 h.

Monitoring the germination of *B. atrophaeus* spores

Germination of *B. atrophaeus* was monitored by determining the amount of DPA released from the core of the spore, using time resolved fluorescence intensity measurements. A spore solution of approximately 10^7 cfu/mL was incubated at 37°C in the presence of L-alanine or DDA at various concentrations, for 0, 2, 10, 20, 30, 40, 50, 60 and 100 min. The spore solution was combined with a stock solution of terbium chloride ($TbCl_3$; Sigma-Aldrich, St. Louis, MO) to yield a 1 mM $TbCl_3$. Terbium chloride reacts with DPA and forms the chelate, terbium dipicolinate, $(Tb(DPA)_3)^{3-}$, which luminesces with UV excitation (Rosen *et al*., 1997). After addition of $TbCl_3$ in a microtiter 96-well plate, 200 μL of spore solution were placed in each well and the

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mixed system was excited at 270 nm. Photoluminescence excitation and emission spectra were measured from each sample with a Gemini XPS microplate Spectrofluorometer (Molecular Devices, now part of MDS Analytical Technologies Inc., Toronto, Canada).

Imaging of *B. atrophaeus* spores with AFM

AFM was used to study the morphological changes of *B. atrophaeus* spores after exposure to 1 mM DDA for 0, 1, 5, 15, 20, 25, 30, 40, 50 and 60 min, or after exposure to 25 mM L-alanine for 120 min. Droplets of treated *B. atrophaeus* spores (5 μL) were deposited directly onto freshly cleaved mica and allowed to air dry for imaging under ambient conditions.

Images were collected using an atomic force microscope (Digital Instruments Dimension 3100 with Nanoscope IIIa controller; Veeco Metrology; Santa Barbara, CA) that was operated in intermittent contact mode to minimize lateral forces on the sample during imaging. Rectangular cantilevers with conical silicon tips having force constants of approximately 40 N/m and resonance frequencies of approximately 300 kHz were used (Applied Nanostructures; Santa Clara, CA). Images were captured with scan areas of 0.5, 1, 5, 10 and 20 μm^2. Images of larger areas (10 and 20 μm^2) were acquired using hard tapping, where the proportional and integral gains were 1.2–1.5 and 0.8–1.0, respectively, and low amplitude setpoints. Smaller scanned areas (0.5, 1 and 5 μm^2) were probed using light tapping, where the proportional and integral gains were decreased to 0.5–0.8 and 0.2–0.4, respectively, and the amplitude setpoint was moderately increased to avoid missing important surface structures. All images were captured at a scan rate of 1 Hz and with a resolution of 512×512 points.

Off-line image analysis

AFM height and amplitude images were collected simultaneously. At least 10 images were obtained per time period and condition. Height images were used for quantitative analysis of the root-mean-squared roughness (R_{rms}), as well as height and length profiles of the spores. Amplitude images were used to obtain qualitative information. Height images were flattened using a zero order filter to remove the Z offset between scan lines before calculating R_{rms} values. The R_{rms} values were acquired on areas ranging from 0.05 to 0.5 μm^2.

Between 10 and 20 spores were analyzed per image and the calculated height and roughness values of the spores were analyzed using SigmaStat 2.03 statistical software. Statistical analysis was performed by one-way analysis of variance (ANOVA) for repeated measurements. Tukey's test was used for multiple comparisons among treatment groups, while Dunnett and Duncan's test was used for comparisons between treatment and control groups. A difference was considered significant if $p < 0.05$.

RESULTS

Anti-sporal activity of DDA against *B. atrophaeus*

Exposure of *B. atrophaeus* to 1 mM DDA resulted in a significant decrease in surviving spores with increasing exposure time to the anti-sporal agent (Figure 2). After 1 min of treatment with DDA, the number of cfu decreased from approximately 5×10^7 cfu to approximately 2×10^6 cfu, corresponding to 90% of the spores being killed. After 5 min of exposure, 99% of the spores had been killed and only 10^3 cfu remained after exposure to DDA for 60 min.

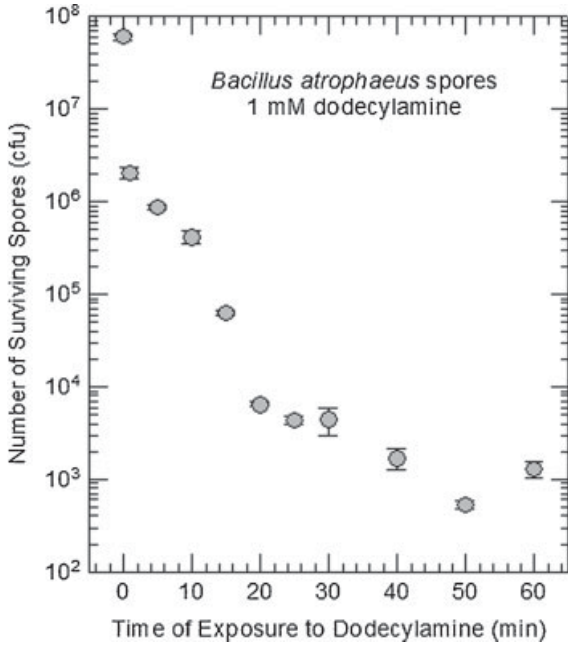

Figure 2. Kinetics of *B. atrophaeus* spore killing by DDA. Number of spore cfu as a function of time of exposure to 1 mM DDA. Error bars represent the standard deviation. Original spore inoculum was at 1×10^7 spores/mL.

Since germinated spores are more susceptible to anti-sporal agents, the germination of *B. atrophaeus* spores was monitored in terms of DPA release from the core. Exposure of *B. atrophaeus* to different concentration of L-alanine caused DPA to be released within 30 min (Figure 3A). The maximum concentration of DPA released from the core was obtained by exposing spores to a concentration of 25 mM L-alanine. Exposure of *B. atrophaeus* to various concentration of DDA resulted in a slower DPA release when compared to the effects of L-alanine. Total release of DPA took 100 min when bacteria were exposed to DDA (Figure 3B). The effect of DDA concentration on germination is complex and is affected by the self-assembly behaviour of DDA in solution (the critical concentration for aggregation is between 0.5 and 1 mM) and also by the promotion of clustering of spores with increasing DDA concentration.

B. atrophaeus size and surface morphology

The morphology of *B. atrophaeus* was characterized via AFM and images showed rodlet structures typically observed for these spores (Figure 4A). Drying of the *B. atrophaeus* on mica surfaces resulted in some clumping. Even when the spore concentration was decreased, spores always aggregated during the drying process on mica, thus it was difficult to observe isolated spores. Spore morphology changed after treatment with 1 mM DDA for 15 or 30 min (Figure 4B and 4C). Spores treated for 15 min appeared dehydrated and the images showed more creases or folds on the spore surface (Figure 4B). The spores treated with DDA for 30 min did not show as much evidence of dehydration,

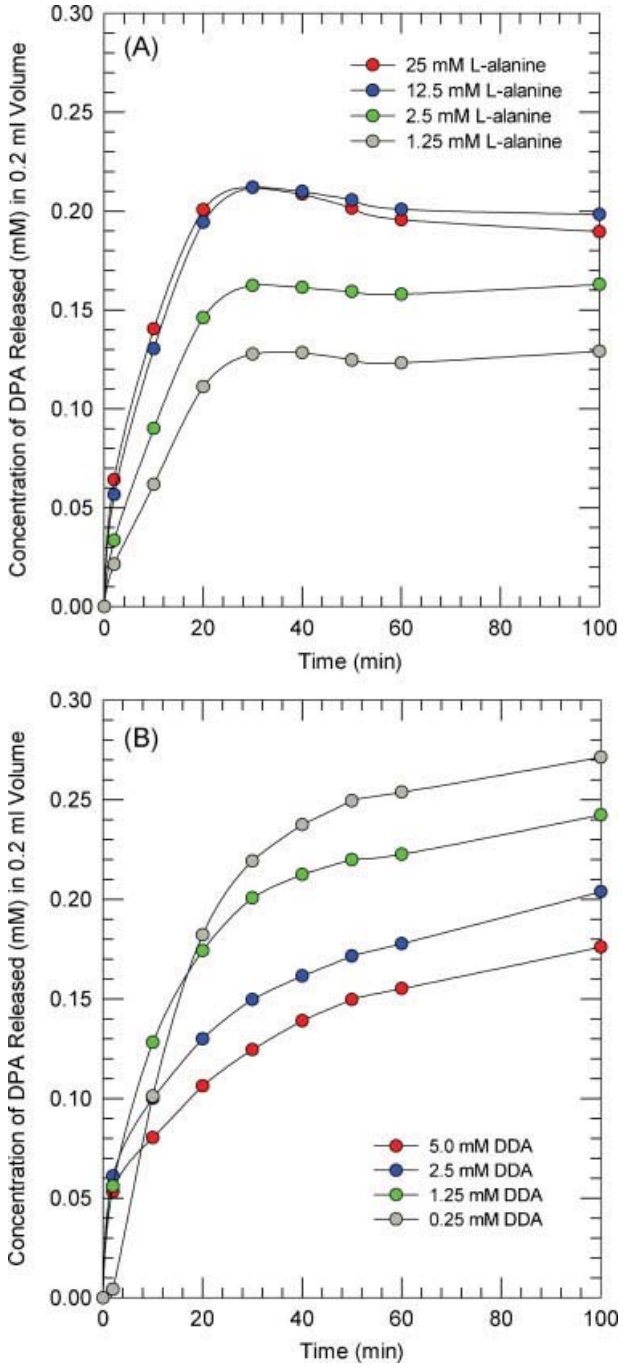

Figure 3. Germination of *B. atrophaeus* spores with various concentrations of (A) L-alanine or (B) DDA. The amount of DPA released from the core was measured by terbium enhanced fluorescence lifetime measurements. Original spore inoculum was at 1×10^7 spores/mL.

but we noticed that no rodlets were present on the surface, compared to the control case.

R_{rms} values were measured for *B. atrophaeus* exposed to DDA for several time points. Spores that were not treated with DDA had an average R_{rms} value of 25.0 ± 2.3 nm when areas of 500 nm^2 covering the surface of the spores were analyzed (Figure 5A). After treating the spores for 1 min with DDA, the average R_{rms} value increased to 28.2 ± 4.1 nm. The average roughness of the spores continued to increase with time, reaching a local maximum R_{rms} corresponding to 1 mM DDA

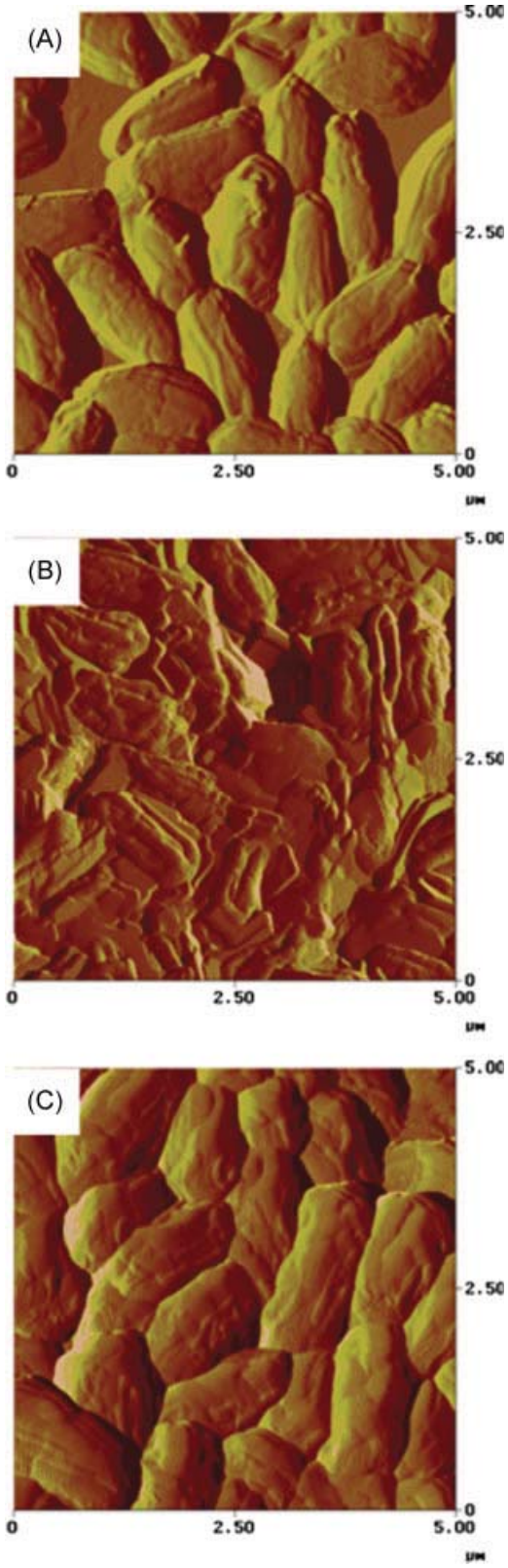

Figure 4. Representative amplitude AFM images of *B. atrophaeus* after exposure to 1 mM DDA for (A) 0 min, (B) 15 min and (C) 30 min. Images collected in intermittent contact mode.

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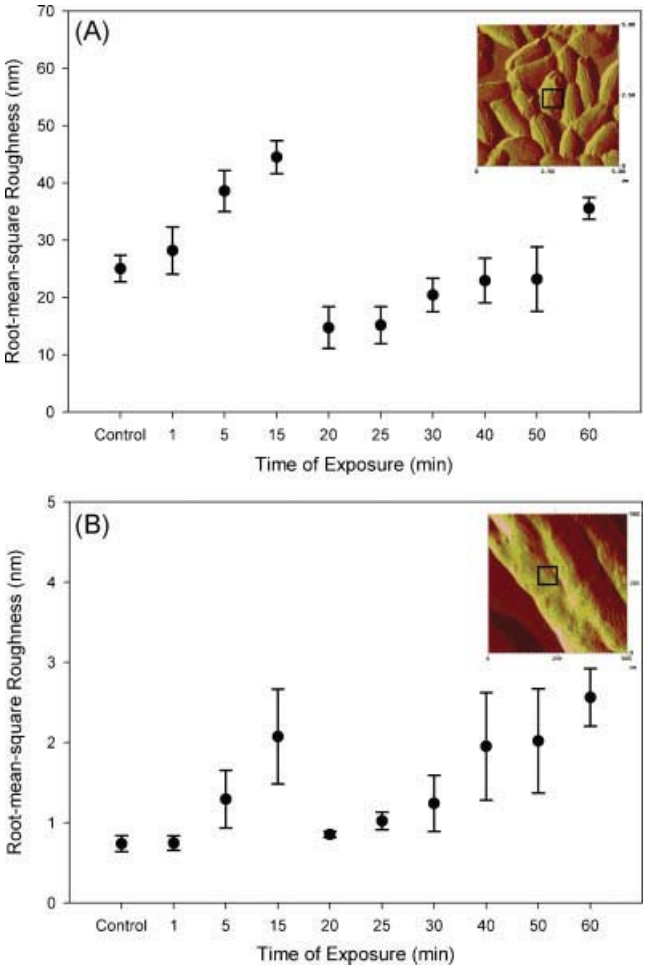

Figure 5. R_{rms} of *B. atrophaeus* as a function of exposure time to 1 mM DDA, for scan areas of (A) 5 μm^2 and (B) 0.5 μm^2, Data are expressed as the average R_{rms} with standard deviation. Inset AFM image represents how roughness analysis was carried out: for scan areas of 5 μm^2, a 500 nm^2 area box covering the surface of the spore was analyzed. For scan areas of 0.5 μm^2, the roughness within a 50 nm^2 area box of the spore surface was analyzed.

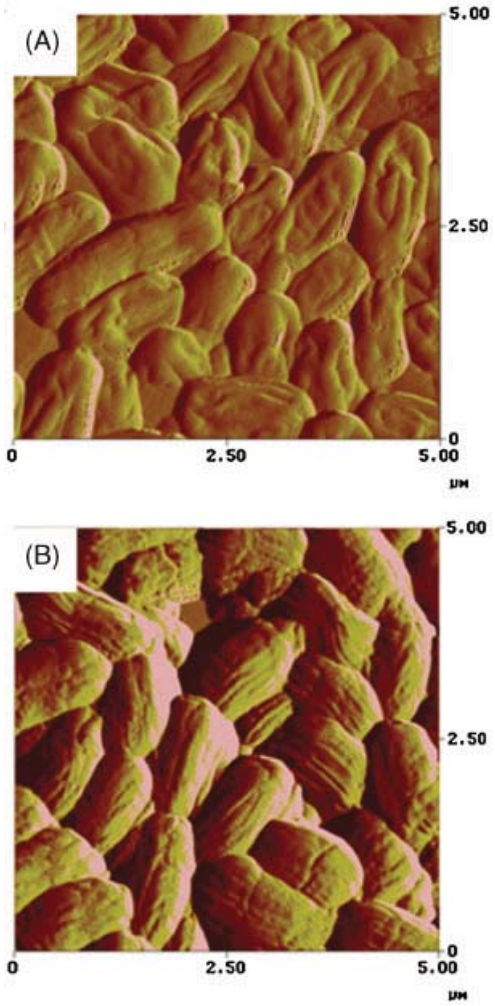

Figure 6. Representative amplitude AFM images of *B. atrophaeus* before and after exposure to L-alanine or DDA for (A) dead spores after 1 mM DDA treatment and (B) germinated spores after 25 mM L-alanine treatment. Images collected in intermittent contact mode.

treatment for 15 min, which was 44.5 ± 2.9 nm. At 20 min, R_{rms} decreased to an average of 14.7 ± 3.6 nm, but began to increase again after 25 min, and continued increasing until 60 min. Statistical analysis showed that R_{rms} values from times 5, 15, 20, 25, 30 and 60 min were significantly different when compared to the control group (untreated spores; $p < 0.001$).

Roughness values were also obtained using smaller scan areas of 50 nm^2 on a 0.5 μm^2 image. Sampled at a different scale, the roughness values decreased compared to the 5 μm^2 scanned image (Figure 5B). The roughness measured at this scale showed the same trend we observed for the larger scale, with roughness increasing until 15 min, then dropping and increasing again. Statistical analysis showed that the R_{rms} values at times 15, 40, 50 and 60 min were significantly different compared to the control group ($p < 0.001$).

Morphology changes were observed for spores exposed to L-alanine, as quantified by height measurements. Chemical treatments affected spore morphology (Figure 6A and 6B) and the height of spores decreased after treatment with DDA or L-alanine. After exposure to L-alanine, the height of the spores decreased from an average of 0.7 ± 0.1 μm to 0.3 ± 0.2 μm. Treatment with DDA resulted in a similar decrease of spore height, from an average of 0.7 ± 0.1 μm to 0.26 ± 0.1 μm. The differences in height were significant against the control group ($p < 0.05$). However, the roughness of the L-alanine treated spores did not increase (data not shown), in contrast with the spores exposed to DDA.

DISCUSSION

Previous studies have addressed the effects that nutrient and non-nutrient germinants have on *Bacillus* and *Clostridium* cells (Powell and Strange, 1953; Ando, 1980). Current theory suggests that germination of spores must occur before killing, since the peptidoglycan layer of vegetative cells is easier to penetrate than the thick multilayered structure of the spore coat (Figure 1). There are several studies that examined how nutrient and non-nutrient germinants affect spores (Rode and Foster, 1961; Barlass *et al.*, 2002; Akoachere *et al.*, 2007). While most studies focused on germination of spores using nutrients, such as L-alanine and inosine (Barlass *et al.*, 2002; Zaman *et al.*, 2005; Plomp *et al.*, 2007),

the use of non-nutrient germinants, such as DDA, have not been well studied. The mechanism of action of nutrient germinants such as L-alanine has been investigated and it is known that nutrient germinants bind to receptors located in the spore's inner membrane, which causes the opening of ion/DPA channels (Setlow, 2003). In this study, we exposed *B. atrophaeus* spores to the nutrient germinant L-alanine and to the non-nutrient germinant DDA. We propose the mechanism of action of DDA against bacterial spores and discuss how it is different from germination with L-alanine.

Germination mechanisms of *B. atrophaeus* after exposure to L-alanine

L-alanine has been widely used to study changes in morphology as well as biochemical alterations during germination (Rode and Foster, 1961; Barlass *et al*., 2002). In *B. cereus* the major receptor for alanine germination was identified as GerL, from the GerA family at the inner membrane (Barlass *et al*., 2002). Using L-alanine as the germinant, we observed that there is a rapid release of DPA and by 30 min all DPA was released (Figure 3A).

Treatment with L-alanine caused a >50% decrease in the height of the spores after 2 h of treatment, which is due in part to DPA release from the core. Prior work showed that the rapid release of DPA from the core due to L-alanine germination caused an estimated 32% loss of dry weight in bacterial spores (Rode and Foster, 1961). A flattening of *B. subtilis* spores and collapse around spore center was observed after exposure to L-alanine for 1 h, according to a previous optical microscopy study (Leuschner *et al*., 2003). Although these spores were considered to be germinating, as observed qualitatively by the decrease in phase brightness, no swelling of the spore could be detected.

Although DPA release supports that germination began in our study with L-alanine, we did not observe outgrowth within the 2 h period, as no vegetative bacteria were observed in the AFM images. This may be because a longer time would be necessary to transform the spore to the vegetative state, which could vary within *Bacillus* spp. For instance, Zaman *et al*., found *B. anthracis* became vegetative after treatment with L-alanine for 3 h (Zaman *et al*., 2005) since the length of the spores increased from 0.8–0.9 to 3.4–3.8 μm. However, another study showed that approximately 30% of a *B. atrophaeus* population did not proceed to outgrowth after exposure to L-alanine for 3 to 7 h (Plomp *et al*., 2007). The percentage of outgrowing cells was even lower in a different study where vegetative cells were only observed in a few cases, suggesting that L-alanine initiates the process of germination and metabolic activity but not the synthesis of macromolecules, such as cell wall peptidoglycan, for all the spore population (Leuschner *et al*., 2003).

The roughness of L-alanine-treated spores was similar to the roughness of untreated spores, suggesting that the effects of L-alanine on the spore occur underneath the coat layer. This was confirmed by the AFM images, showing similar morphology for treated and control spores (Figures 4A and 6B). These findings suggest that during the first stages of germination, the spore coat remains intact and L-alanine causes internal restructuring of the spore. Since there is no degradation of the coat, this layer is a limiting barrier for outgrowing spores, which may explain why vegetative cells were not observed and why outgrowth did not occur consistently in previous studies. One recent study suggested that the spore coat has to crack laterally on one or both sides to allow the vegetative cell to expand (Leuschner *et al*., 2003). L-alanine may start germination by changing the internal structure of the spore and this effect may work its way to the outside of the spore after all internal changes, such as DPA release and hydrolysis of the cortex, have occurred.

Germination mechanisms of *B. atrophaeus* after exposure to DDA

Although some studies have investigated the process of spore germination using a non-nutrient germinant, such as DDA (Rode and Foster, 1961; Setlow *et al*., 2003; Paredes-Sabja *et al*., 2008), the efficacy of this cationic surfactant as a sporicide has not been well studied. In the early 1960s, Rode and Foster reported that exposing *B. megaterium* spores to 10^{-5} M DDA for more than three min resulted in loss of heat resistance for 97% of the spore population and exposure to DDA for 10 min caused the killing of approximately 96% of the spores (Rode and Foster, 1961). In our studies, we have observed that approximately 95% of killed spores can be obtained under 1 min and 99% after 5 min if they are exposed to 1 mM DDA (Figure 2).

Even though spore killing occurs with exposure to 1 mM DDA, we studied the germination of spores by measuring the amount of DPA released from the core as a function of time (Figure 3B). Exposing spores to 1.25 mM DDA resulted in complete DPA release within 100 min. In a previous study, researchers using 1 mM DDA to germinate *B. subtilis* spores showed that it took approximately 3 h for the DPA to be released from the core (Setlow *et al*., 2003). This difference in DPA release rate may be related to variability within the *Bacillus* spp., growth conditions, or treatment. For instance, Setlow *et al*. boiled spore cultures for 30 min to determine DPA release, while we did not (Setlow *et al*., 2003).

The spore heights were significantly lower for DDA-treated spores, compared to untreated spores. We suggest that these height changes are due to decreased hydration of the core and a breakdown of the spore coat. A previous study showed that exposure of *Bacillus* spores to DDA results in a 45 to 55% loss of dry weight, which could have contributed to the reduced height of spores (Rode and Foster, 1961). In another study, it was observed that spores germinated with DDA did not rehydrate well and core water content in these spores was low, which prevented them from expanding normally (Setlow *et al*., 2003). From our roughness analysis we also observed that the outer coat of the spore was affected by DDA treatments since there was an initial increase in roughness values. This increase in roughness indicates that the coat of the spore is rupturing and disintegrating. The inner and outer coat of spores can be up to 130 nm thick (Henriques and Moran, 2000), which could lead to a diameter decrease of 260 nm. When spore coat breakdown and loss of hydration simultaneously occurred, we observed a decrease in height and increase in roughness of the spores.

Based on our roughness, height and DPA release measurements, we considered a mechanism of action of DDA against bacterial spores. DDA does not interact with any receptors in the inner membrane of spores, as reported previously (Setlow *et al*., 2003). From our roughness measurements we can state that DDA starts acting from the outside of the spore and works its way in by causing the coat to rupture and form pores on the surface. The drop in roughness observed after 15 min of treatment with DDA indicates that the outer coat layer was completely removed; leaving behind another smoother coat that will be subsequently

ruptured or disintegrated after prolonged treatment with DDA. With prolonged DDA treatment, the protective coat layers of the spore were compromised, decreasing the overall cell height. While most of the cells were killed within 1 min of exposure to DDA, this happened during the first stages of germination since there was a continuous release of DPA from the core. However, germination was interrupted and spores were killed without outgrowing. Using DDA as a germinant or sporicide may be a more effective mechanism to deactivate *B. atrophaeus* and perhaps other hazardous species such as *B. anthracis*, since DDA can act against spores that have not progressed to the fully vegetative state.

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REFERENCES

Akoachere M, Squires RC, Nour AM, Angelov L, Brojatsch J, Abel-Santos E. 2007. Identification of an *in vivo* inhibitor of *Bacillus anthracis* spore germination. *J. Biol. Chem.* **282**(16): 12112–12118.

Ando Y. 1980. Mechanism of nitrite-induced germination of Clostridium perfringens spores. *J. Appl. Bacteriol.* **49**(3): 527–535.

Atrih A, Foster SJ. 2002. Bacterial endospores the ultimate survivors. *Int. Dairy J.* **12**(2–3): 217–223.

Barlass PJ, Houston CW, Clements MO, Moir A. 2002. Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of gerL and gerQ operons. *Microbiology.* **148**(Pt 7): 2089–2095.

Burke SA, Wright JD, Robinson MK, Bronk BV, Warren RL. 2004. Detection of molecular diversity in *Bacillus atrophaeus* by amplified fragment length polymorphism analysis. *Appl Environ Microbiol.* **70**(5): 2786–2790.

Chada VG, Sanstad EA, Wang R, Driks A. 2003. Morphogenesis of *Bacillus* spore surfaces. *J. Bacteriol.* **185**(21): 6255–6261.

Dufrene YF. 2002. Atomic force microscopy, a powerful tool in microbiology. *J. Bacteriol.* **184**(19): 5205–5213.

Fritze D, Pukall R. 2001. Reclassification of bioindicator strains *Bacillus subtilis* DSM 675 and *Bacillus subtilis* DSM 2277 as *Bacillus atrophaeus*. *Int. J. Syst. Evol. Microbiol.* **51**(Pt 1): 35–37.

Giorno R, Bozue J, Cote C, Wenzel T, Moody KS, Mallozzi M, Ryan M, Wang R, Zielke R, Maddock JR, Friedlander A, Welkos S, Driks A. 2007. Morphogenesis of the *Bacillus anthracis* spore. *J. Bacteriol.* **189**(3): 691–705.

Henriques AO, Moran CP Jr. 2000. Structure and assembly of the bacterial endospore coat. *Methods.* **20**(1): 95–110.

Jedrzejas MJ, Setlow P. 2001. Comparison of the binuclear metalloenzymes diphosphoglycerate-independent phosphoglycerate mutase and alkaline phosphatase: Their mechanism of catalysis via a phosphoserine intermediate. *Chem. Rev.* **101**(3): 607–618.

Leuschner RGK, Ferdinando DP, Lillford PJ. 2003. Structural analysis of spores of *Bacillus subtilis* during germination and outgrowth. *Colloids Surf. B.* **19**: 31–41.

Paredes-Sabja D, Torres JA, Setlow P, Sarker MR. 2008. Clostridium perfringens spore germination: characterization of germinants and their receptors. *J. Bacteriol.* **190**(4): 1190–1201.

Plomp M, Leighton TJ, Wheeler KE, Hill HD, Malkin AJ. 2007. *In vitro* high-resolution structural dynamics of single germinating bacterial spores. *Proc. Natl. Acad. Sci. USA.* **104**(23): 9644–9649.

Plomp M, Leighton TJ, Wheeler KE, Malkin AJ. 2005a. The high-resolution architecture and structural dynamics of *Bacillus* spores. *Biophys. J.* **88**(1): 603–608.

Plomp M, Leighton TJ, Wheeler KE, Pitesky ME, Malkin AJ. 2005b. *Bacillus atrophaeus* outer spore coat assembly and ultrastructure. *Langmuir* **21**(23): 10710–10716.

Powell JF, Strange RE. 1953. Biochemical changes occurring during the germination of bacterial spores. *Biochem. J.* **54**(2): 205–209.

Rode LJ, Foster JW. 1961. Germination of bacterial spores with alkyl primary amines. *J. Bacteriol.* **81**: 768–779.

Rosen DL, Sharpless C, McGown. LB. 1997. Bacterial spore detection and determination by use of terbium dipicolinate photoluminescence. *Anal. Chem.* **69**(6): 1082–1085.

Setlow B, Cowan AE, Setlow P. 2003. Germination of spores of *Bacillus subtilis* with dodecylamine. *J. Appl. Microbiol.* **95**(3): 637–648.

Setlow P. 2003. Spore germination. *Curr. Opin. Microbiol.* **6**(6): 550–556.

Slieman TA, Nicholson WL. 2001. Role of dipicolinic acid in survival of *Bacillus subtilis* spores exposed to artificial and solar UV radiation. *Appl. Environ. Microbiol.* **67**(3): 1274–1279.

Tennen R, Setlow B, Davis KL, Loshon CA, Setlow P. 2000. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *J. Appl. Microbiol.* **89**(2): 330–338.

Zaman MS, Goyal A, Dubey GP, Gupta PK, Chandra H, Das TK, Ganguli M, Singh Y. 2005. Imaging and analysis of *Bacillus anthracis* spore germination. *Microsc. Res. Tech.* **66**(6): 307–311.

Zolock RA, Li G, Bleckmann C, Burggraf L, Fuller DC. 2006. Atomic force microscopy of *Bacillus* spore surface morphology. *Micron.* **37**(4): 363–369.