Effects of L-Alanine and Inosine Germinants on the Elasticity of *Bacillus anthracis* Spores

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The surface of dormant *Bacillus anthracis* spores consists of a multilayer of protein coats and a thick peptidoglycan layer that allow the cells to resist chemical and environmental insults. During germination, the spore coat is degraded, making the spore susceptible to chemical inactivation by antisporal agents as well as to mechanical inactivation by high-pressure or mechanical abrasion processes. While chemical changes during germination, especially the release of the germination marker, dipicolinic acid (DPA), have been extensively studied, there is as yet no investigation of the corresponding changes in the mechanical properties of the spore. In this work, we use atomic force microscopy (AFM) to characterize the mechanical properties of the surface of *Bacillus anthracis* spores during germination. The Hertz model of continuum mechanics of contact was used to evaluate the Young's moduli of the spores before and after germination by applying the model to load−indentation curves. The highest modulus was observed for dormant spores, with average elasticity values of 197 ± 81 MPa. The elasticity decreased significantly after incubation of the spores with the germinants L-alanine or inosine (47.5 ± 41.7 and 35.4 ± 15.8 MPa, respectively). Exposure of *B. anthracis* spores to a mixture of both germinants resulted in a synergistic effect with even lower elasticity, with a Young's modulus of 23.5 ± 14.8 MPa. The elasticity of the vegetative *B. anthracis* cells was nearly 15 times lower than that of the dormant spores (12.4 ± 6.3 MPa vs 197.0 ± 80.5 MPa, respectively). Indeed from a mechanical strength point of view, the germinated spores were closer to the vegetative cells than to the dormant spores. Further, the decrease in the elasticity of the cells was accompanied by increasing AFM tip indentation depths on the cell surfaces. Indentation depths of up to 246.2 nm were observed for vegetative *B. anthracis* compared to 20.5 nm for the dormant spores. These results provide quantitative information on how the mechanical properties of the cell wall change during germination, which may explain how spores become susceptible to inactivation processes based on mechanical forces during germination and outgrowth. The study of spore elasticity may be a valuable tool in the design of improved antisporal treatments.

Introduction

Bacillus and *Clostridium* cells are some of the most resistant life forms known due to their ability to undergo the restructuring and differentiation process of sporulation under nutrient deprivation.[1] During this process, vegetative cells synthesize a series of polymer and protein layers that encase the cellular contents and genetic information in a ~100−200 nm barrier, which protects the cell from external environmental stressors.[2,3] Spores are metabolically dormant, but they maintain the ability to germinate and transform into vegetative cells once nutrients are available.[4,5] In their vegetative state, organisms such as *Bacillus anthracis* (*B. anthracis*) are highly virulent, and they are considered as potent biological threat agents since they cause critical or fatal airborne diseases, such as pulmonary anthrax.[6,7]

The first step of the process in which the spores return to a fully vegetative, pathogenic state is known as germination. Germination is usually initiated in response to availability of nutrients, such as amino acids and sugars, non-nutrient chemicals such as dodecylamine and dipicolinic acid (DPA), or by other factors such as high pressure and temperature.[5,8] Germination involves a series of steps where the release of monovalent cations, dipicolinic acid (DPA), calcium, and the intake of water molecules result in the loss of heat resistance and initiation of metabolic activity, converting the spore into a vegetative, fully virulent bacterium.[5,9]

The mechanism of germination triggered by nutrients involves the presence of spore-germination receptors on the surface or inner membrane of the spore, which stimulate the cell to germinate and to achieve vegetative growth.[10] L-Alanine is an amino acid that interacts with a receptor on the inner membrane of the spores[11] and is a well-studied molecule for the germination of several *Bacillus* species, such as *B. subtilis*, *B. cereus*, *B. anthracis*, and *B. atrophaeus*.[6,8,12] Inosine is a purine ribonucleoside that has been shown to be a strong germinant of *B. cereus* spores.[13] While either germinant, L-alanine or inosine, alone can cause the germination of *B. cereus*, for *B. anthacis* spores, a binary mixture of the germinants has been shown to be more effective.[10,11]

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Report Documentation Page

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1. REPORT DATE **05 JAN 2010**	2. REPORT TYPE	3. DATES COVERED **00-00-2010 to 00-00-2010**

4. TITLE AND SUBTITLE **Effects of L-Alanine and Inosine Germinants on the Elasticity of Bacillus anthracis Spores**	5a. CONTRACT NUMBER
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Natick Soldier Research, Development & Engineering Center,Molecular Sciences and Engineering Team,Natick,MA,01760**	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**7**	

In contrast to the considerable attention paid to investigating the effects of nutrients on germination of spores, there are fewer studies in the literature on the effects of physical insults, such as pressure.[14,15] While it is widely known that the microbial cell wall has a structural role in maintaining the cellular shape and resisting turgor pressure,[16] changes in the mechanical properties of the cell wall during germination remains essentially unknown. *B. subtilis* spores exposed to 100 and 550 MPa resulted in the killing of < 25% and < 50% spores, respectively.[14] Treatment pressures at 550 MPa were able to induce the germination of spores without the need of activation of germinant receptors, as was confirmed by the number of viable colonies in Luria broth medium agar plates. However, spores treated at 100 MPa produced very few colonies on agar plates, suggesting that there may be a critical mechanical force necessary to initiate germination in the absence of nutrients.[14] Understanding the mechanical properties of *B. anthracis* spores, such as cell wall elasticity, may help us understand how germination affects the spore surface and ultimately how the cell resists chemical and physical insults.

Germination of spores is usually monitored by changes in optical densities that occur as DPA is released from the spore core after addition of germinants.[4] Electron microscopy (EM) has also been used to study changes in morphology of the spores during germination.[17] While EM explicitly demonstrates changes in cell structure, sample preparation necessitates dehydration and coating with a reflective material, which can cause artifacts.

Atomic force microscopy (AFM) is a technique that has proven practical for the physical characterization of individual spores, since it provides high-resolution imaging of the sample and the ability to obtain the adhesion forces present between the AFM probe and the cell surface under native conditions.[18] By studying the interaction of the AFM tip as it approaches or retracts from the spore surface, it is possible to study specific physical and chemical properties of the spore, such as elasticity, adhesion, and length of surface polymers. A few AFM studies available in the literature have addressed how nutrient germinants, such as L-alanine, affect the morphology of *Bacillus* species.[17,19−21] AFM has been used to obtain high-resolution images of the spore coat of *Bacillus* spores and understand how protein coat layers change during germination.[19,21]

Quantitative elasticity measurement with AFM is a new way to characterize how germinants affect the mechanical properties of the spore surface. Using the AFM probe as a nanoindentation tool, AFM deflection data can be converted into load vs indentation depth plots and analyzed using theoretical models that provide quantitative information on the elasticity of the sample (represented by the Young's modulus).[22] These models have been applied toward bacteria, such as *E. coli*, where it was suggested that the elasticity of the cells can change significantly depending on the solvent in which the cells are exposed.[23] The elasticity for *E. coli* in water was ∼12.8 MPa, while the elasticity of the same bacterium in a more polar solvent, such as formamide, decreased to ∼0.8 MPa.[23] The same technique has been applied with other types of cells, such as the yeast *Saccharomyces cerevisiae*, where the Young's modulus was ∼0.6 MPa at the cell wall but ∼6.1 MPa at the site of cellular division.[22] While these elasticity studies help characterize the physical properties of some bacterial strains, none of the cells studied possess the complex protein layers and coat structure that is common in sporulated *B. anthracis*.

Therefore, our objective was to use AFM to characterize how the nutrient germinants L-alanine and/or inosine affected the Young's modulus of *B. anthracis* spores. These results were compared with elasticity measurements on the dormant spores and also on the vegetative *B. anthracis* cells, these being the two limiting states of the spore subjected to germinants.

Experimental Section

Bacterial Strain and Preparation of Spores. *Bacillus anthracis* Sterne (referred to hereafter as "*B. anthracis*") was kindly provided by the U.S. Army Natick Soldier Research, Development & Engineering Center, Natick, MA. The Sterne strain is a low virulence strain that lacks the pXO2 extra chromosomal plasmid, which forms and develops the bacterial capsule; however, it contains the pXO1 extra chromosomal plasmid, which is responsible for the expression of virulent factors.[17]

Bacterial strains were grown in sporulation media as described previously.[3] A solution of sporulation media consisting of 8 g of nutrient broth, 4 g of yeast extract, 0.001 g of MnCl$_2$·4H$_2$O, 5 g of peptone, and 15 g of agar in 1 L of ultrapure water (Milli-Q water, Millipore Corp., Bedford, MA) was maintained at a pH of 7.2 and sterilized for 60 min, followed by preparation in Petri dishes.

Fresh cultures of *B. anthracis* Sterne were obtained by adding 100 μL of spores from a glycerol stock solution into a flask with 25 mL of sterilized nutrient broth (Himedia, Mumbai, India). The flask was agitated at 200 rpm at 37 °C for ∼20 h. This culture was used to inoculate plates of sporulation media using 50 μL aliquots, and the plates were incubated at 37 °C for 4 days to allow vegetative cells to sporulate. Harvesting of spores was done in autoclaved ultrapure water, and spores were collected by centrifugation at 5000 rpm for 20 min and resuspended in ultrapure water. The cells were washed eight times to separate spores from vegetative and partially sporulated cells since there is a significant difference in density between these two cell types.[24] The spores were stored overnight at 4 °C. After ∼18 h, the cells were washed two more times to remove any remaining vegetative *B. anthracis* cells.

Spore Germination Assays and Atomic Force Microscopy (AFM). The surface elasticity of dormant and germinated spores and of vegetative cells of *B. anthracis* was investigated. To germinate the spores, *B. anthracis* were exposed to 50 mM L-alanine (Sigma-Aldrich, St. Louis, MO), 5 mM inosine (Sigma-Aldrich), or both germinants together, in 50 mM Tris/HCl buffer (pH 8.0), and incubated at 37 °C for 2 and 4 h. The final concentration of the spore solution was ∼10^7 cells/mL. As a control condition, dormant *B. anthracis* spores were incubated for 4 h in 50 mM Tris/HCl buffer at 37 °C. To study the elasticity of vegetative *B. anthracis* cells, a 1 mL sample of dormant spore solution was suspended in tryptic soy broth (30 g/L, Sigma-Aldrich), supplemented with 1% yeast extract, and grown for 6 h at 37 °C with rotation at 18 rpm.

To test the effect of germinant concentration on the elasticity of the spores, additional experiments were carried out where *B. anthracis* spores were germinated with 25 mM L-alanine and/ or 2.5 mM inosine for 2 h. The elasticity values obtained were

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compared with those obtained at higher concentrations of germinants (50 mM L-alanine and/or 5 mM inosine).

AFM was used to study changes in the elasticity of *B. anthracis* spores induced by exposure to different germinants. After incubation with L-alanine and/or inosine, the spore solution was centrifuged at 5000 rpm for 5 min and resuspended in ultrapure water to stop treatment. Droplets of treated *B. anthracis* spores (5 μL) were deposited onto freshly cleaved mica and dried for 18 h.

AFM experiments were carried out with a Dimension 3100 with Nanoscope IIIa controller (Veeco Metrology, Santa Barbara, CA) that was operated in tapping mode in air to represent conditions of airborne spores. *B. anthracis* are commonly found in soil at concentrations of up to 3000 spores per gram of soil.[25,26] Since germination can be affected by humidity,[27] AFM measurements were taken at 30−35% relative humidity in air. A single rectangular cantilever with a conical silicon tip having a spring constant of 14 N/m and a resonant frequency of 315 kHz was used (Mikromasch, San Jose, CA). Samples were scanned at a rate of 1 Hz, and once a spore was located, it was centered so that force measurements could be performed on the cell away from the edges. Forces were measured on five spores, and ten force cycles were recorded per spore for a total of 50 force curves obtained for each treatment. The ten force cycles obtained within each spore were taken at the same location of the cell (center of the spore away from edges) to ensure that no deformation of the surface was taking place. The force cycle was set so the tip stayed in contact with the sample for 1 ms before retracting and the tip would travel at 2 μm/s with a loading rate of 3×10^{-5} N/s during the force cycle. Data in ASCII format were exported to a spreadsheet and converted from deflection to force.

Elasticity of *B. anthracis* Spores Obtained from Force−Indentation Depth Curves. The conversion of cantilever deflection data to force data allows for the assessment of the elasticity of the surface of the spores in response to exposure to germinants. AFM data were converted to force−indentation depth curves following procedures described previously.[28−30] Briefly, the deflection of the cantilever, d, as it approaches and retracts from the surface is plotted as a function of tip−sample separation distance, z. Since there was some scanner hysteresis and drift in the detection system, we often observed that the deflection of the free cantilever was not equal to zero. To correct this, a deflection offset, d_0, was subtracted from all deflection values. This deflection offset was determined from the cantilever deflection−sample separation distance plot by calculating the average cantilever deflection when the AFM probe was far away from the sample surface. This was observed as a horizontal line, where no repulsive or attractive forces were experienced by the AFM tip. The cantilever deflection was converted to force or load (F) using Hooke's law

$$F = k_c(d - d_0) \qquad (1)$$

where k_c is the spring constant.

Similar to the deflection offset, a separation distance offset, z_0, was defined. The value of this offset was the initial point where the tip makes contact with the sample surface or more specifically, when the deflection of the cantilever begins to increase from the horizontal line that represents the zero cantilever deflection (deflection offset). The distance offset was subtracted from all distance values.

Next, the force−indentation curves were created by taking into account the compliance of the sample surface. On a hard surface, such as mica, the slope of the compliance region of the cantilever deflection−separation distance curve is equal to 1, but this slope is expected to decrease for biological samples, such as bacteria.[22,23] The difference between the separation distance on a hard surface and the separation distance on the surface of the spore is the indentation depth of the sample, δ, and is defined by

$$\delta = (z - z_0) - (d - d_0) \qquad (2)$$

From eqs 1 and 2, the cantilever deflection−separation distance curves were converted into load, F, versus indentation depth, δ. Force curves were made on freshly cleaved mica free of spores before any measurements were taken on cells in order to calibrate the sensitivity of the photodetector.

Young's Modulus of *B. anthracis*. The tensile elastic modulus or Young's modulus was obtained by applying the Hertz model of continuum mechanics of contact to the load−indentation depth data.[22,23] The Hertz model can be used for samples that are elastic, isotropic, homogeneous, and semi-infinite although it does not take into account tip−surface adhesion.[22,31] Since there were not significant adhesion forces observed during the force cycle, this model was applicable.

From Hertzian theory, the model describes the indentation (without permanent deformation) of an indenter (the silicon AFM probe) into an infinitely deformable elastic half-space (spore surface). The geometry of the indenter was taken into account, and it was related to the load−indentation depth curves by

$$F_{cone} = \frac{2}{\pi} \tan \alpha \frac{E}{1 - v^2} \delta^2 \qquad (3)$$

where α is the half-opening angle of the conical tip used, taken as 40° as specified by the manufacturer, E is the Young's modulus or tensile elastic modulus of the spore, and v is the Poisson ratio of the spore, taken as 0.5.[22]

A MatLab script was written to analyze all raw data obtained with the AFM and calculate the elastic modulus of the spores for all treatments (MatLab Works Inc., Natick, MA).

Statistical Analysis. Young's modulus and indentation depth values were analyzed using the Kruskal−Wallis one-way analysis of variance (ANOVA) on ranks for repeated measurements since the samples did not have equal variances. The null hypothesis tested was that there were no differences in the distribution of values between different groups. Tukey's test was used for multiple comparisons among treatment groups. A difference was considered significant if $P < 0.05$.

Results and Discussion

Rationale for Germination Conditions. Germination of *Bacillus* spores has been extensively studied over the past few decades. L-Alanine has been shown to influence the germination of spores of many *Bacillus* species.[6,8,12] For *Bacillus anthracis,* it has been shown that L-alanine is a strongly independent germinant at concentrations > 10 mM and that rapid germination responses are observed at high L-alanine concentrations (100 mM).[11] In the same study, it was observed that lower L-alanine concentrations could germinate *B. anthracis* spores as long as there was another germinant present, such as inosine.[11] L-Alanine at a concentration of 10 mM acting together with inosine at a concentration of 1 mM were able to germinate 73% of *B. anthracis* spores in less than 15 min.[11] In another study, optimal germination conditions for *B. anthracis* were observed when spores were exposed to

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15−150 mM ʟ-alanine, and over 99% of the spore population had germinated within 24 h.[12] Clements and Moir observed that 50 mM ʟ-alanine and 5 mM inosine were concentrations at which the germination rates were the maximum for *B. cereus*, which is a close genetic relative of *B. anthracis*.[13] In another study, inosine concentrations that ranged from 1 to 10 mM resulted in comparable germination processes as measured by the fall in optical density.[8] While inosine has been shown to be a weak germinant while acting alone compared to ʟ-alanine, it has been suggested that inosine as a cogerminant is able to induce strong germination responses in *B. anthracis*. Germination with ʟ-alanine as the sole germinant has been shown to involve a single receptor while inosine as a sole germinant has been shown to require the interaction of two different receptors in *B. cereus*.[10] On the basis of these findings, and knowing that germinants at high enough concentrations resulted in optimal germination responses, we chose high concentrations of ʟ-alanine and inosine for our elasticity studies. To assess whether germinant concentration had an effect on elasticity, AFM experiments were also carried out on spores that were exposed to different concentrations of ʟ-alanine and/or inosine.

Elasticity of *B. anthracis* after Exposure to Germinants. Cantilever deflection vs tip−sample separation curves were converted to load vs indentation depth plots to quantify the elasticity of *B. anthracis* spores after exposure to ʟ-alanine and/or inosine (Figure 1A). A distinct nonlinear region of the curve was observed for all samples, which allowed for the detection of deflection and separation distance offsets. Once these offsets were defined, the plot was converted to loading force vs indentation depth (Figure 1B). When we probed a hard surface, such as mica, the nonlinear region of the curve was absent, so the deflection and separation offsets were defined as the point where a sudden jump to contact was observed in the approach curve.[22] The Young's modulus of the spores was obtained by fitting the nonlinear portion of the loading force vs indentation depth curves to the Hertz model. A quasi-quadratic relation was observed between the loading force and the indentation depth (Figure 1B), which was predicted by the model for a conical AFM probe. There was a good agreement between the Hertz model and the experimental data obtained, which suggests that this model can be used for the measurement of elasticity of cells with harder surfaces such as *Bacilllus* and *Clostridium* species. Furthermore, for all treatments, no adhesion forces were observed during approach or retraction of the AFM tip, allowing for the analysis of the data using the Hertz model. The lack of adhesive interactions during AFM force cycles has been associated with the crystalline nature of the surface of microorganisms, such as the one present in *Lactobacilli*.[32] A crystalline cell surface protein, S-layer, has been observed in the structure of *Bacillus* species,[33] which may consist of compactly folded proteins that prevent the AFM tip from experiencing adhesion during force measurements.

The slope of the load vs indentation depth curve was higher when taking AFM measurements on the dormant *B. anthracis* spores that were not exposed to any germinants or yeast extract and decreased significantly after incubation with either ʟ-alanine or inosine (Figure 2). Based on the shape of the loading force vs indentation depth curve, fewer data points were available to fit the Hertz model for the cells that showed harder surfaces (dormant, untreated spores), since the nonlinear portion of the curve that describes the indentation of the AFM probe into the untreated
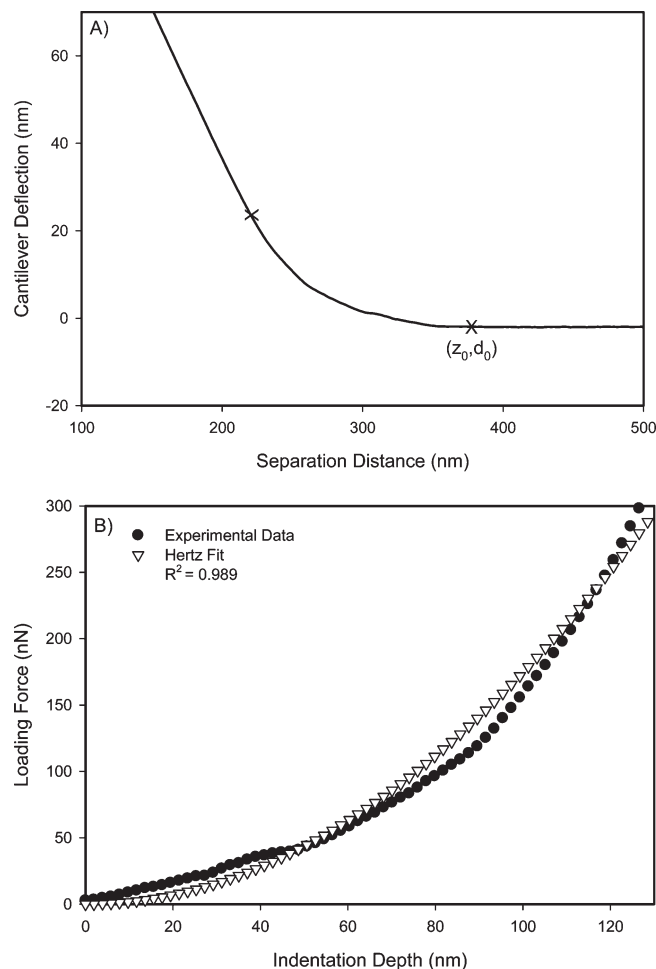
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Figure 1. (A) Representative AFM approach curve obtained when a silicon AFM tip approaches and indents a *B. anthracis* Sterne spore. The deflection offset d_0 and the offset in the separation distance z_0 used to calibrate the deflection vs distance plot are shown. The nonlinear portion of the curve is depicted within the two X marks. (B) Loading force−indentation depth curve corresponding to the deflection−distance curve in (A). The open triangles correspond to the best fit obtained after applying the Hertz model to the nonlinear portion of the approach curves (eq 3).

B. anthracis cell was significantly smaller. This may be due to the multiple protein layers that surround the cell, making the spore surface harder than the cell wall of vegetative bacteria.

Treatment of spores with TSB supplemented with yeast extract resulted in vegetative cells with weaker surfaces since very low loads were required to substantially indent the sample with the AFM tip. The slope of the load vs indentation depth curve for the vegetative *B. anthracis* resulting from the TSB and yeast extract treatment was the lowest of all conditions (Figure 2). Since untreated *B. anthracis* spores are covered by multiple layers of protein coats, higher loading forces were required to cause indentation of the spore. This suggests that the protein coat layers of dormant spores strengthen the surface and resist applied pressure, such as the one created by the AFM probe during force cycles. Similar changes in the slope of the load vs indentation plot have been observed for other microorganisms, such as *S. cerevisiae*.[22] During the division of *S. cerevisiae*, a scar is formed on the cell wall where the bud emerges, resulting in a steeper slope in the AFM data, which suggests strengthening of the surface and higher loading forces being required to indent the cell with an AFM probe.
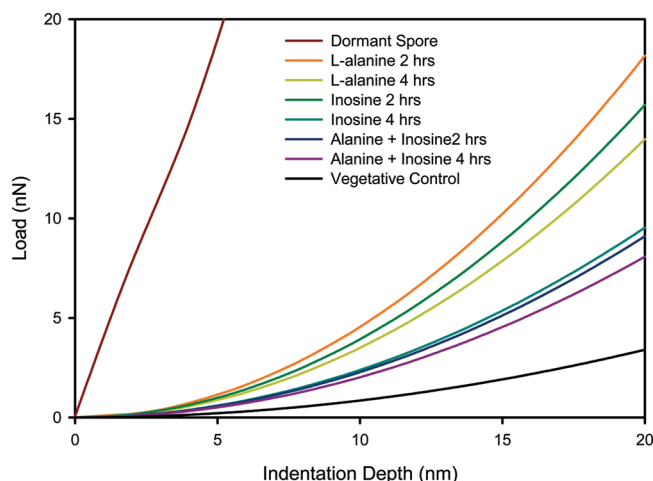
Figure 2. Representative load vs indentation depth curves of *B. anthracis* Sterne before and after incubation with 50 mM L-alanine and/or 5 mM inosine or tryptic soy broth supplemented with 1% yeast extract (vegetative control). Each line is the average of 50 approach curves measured between *B. anthracis* Sterne and a silicon AFM probe.

The effects of pressure on the germination of spores have been previously studied.[14,15,26] Exposure of spores to high pressure can initiate the process of germination.[14] Exposure of *Bacillus* spores to pressures of 550 MPa resulted in initiation of germination without the involvement of any germinant receptors.[14] However, treatment at lower pressures (100 MPa) did not initiate germination, unless one of the spore's germinant receptors was activated by nutrients. Since the pressure applied by the AFM tip to the spores was much lower than 100 MPa, a pressure-induced germination of untreated spores probably did not occur in our study.

The highest Young's moduli were obtained with dormant spores that were not exposed to germinants or yeast extract (197.02 ± 80.48 MPa; Figure 3). The high elasticity value may be due to the thickness of the inner and outer coats of the sporulated cells, which can be up to 130 nm.[2] This thickness may result in a hardening of the spore surface, protecting it from environmental insults. Young's moduli of *B. anthracis* spores decreased significantly after incubation for 4 h with L-alanine, inosine, or both germinants (47.54 ± 41.79, 35.41 ± 15.88, and 23.50 ± 14.87 MPa, respectively; $P < 0.05$). Incubation of *B. anthracis* with both germinants resulted in >90% of the spores having an elastic modulus lower than 50 MPa. There was no statistically significant difference between the Young's modulus of spores treated with L-alanine for 2 or 4 h; in the case of inosine, the elasticity decreased from 66.42 ± 25.90 MPa after 2 h of incubation to 35.41 ± 15.88 MPa after 4 h of incubation ($P > 0.05$). The rather large standard deviation values obtained for all conditions suggest that there may be heterogeneity of the distribution of proteins on the spore surface, especially in the dormant spores. The germination of spores using only inosine as the triggering molecule has been shown to require the interaction of the germinant with two different receptors in *B. cereus* spores, GerI and GerQ, since the activation of only one of them does not provide sufficient activation of the spore to carry on with the germination process.[10] *B. cereus* is a close relative of *B. anthracis*, and the *gerQ* and *gerI* operons are also present in *B. anthracis* and recognize inosine as a germinant.[34] Based on our

(34) Baillie, L.; Hibbs, S.; Tsai, P.; Cao, G. L.; Rosen, G. M. *FEMS Microbiol. Lett.* **2005**, *245* (1), 33−8.
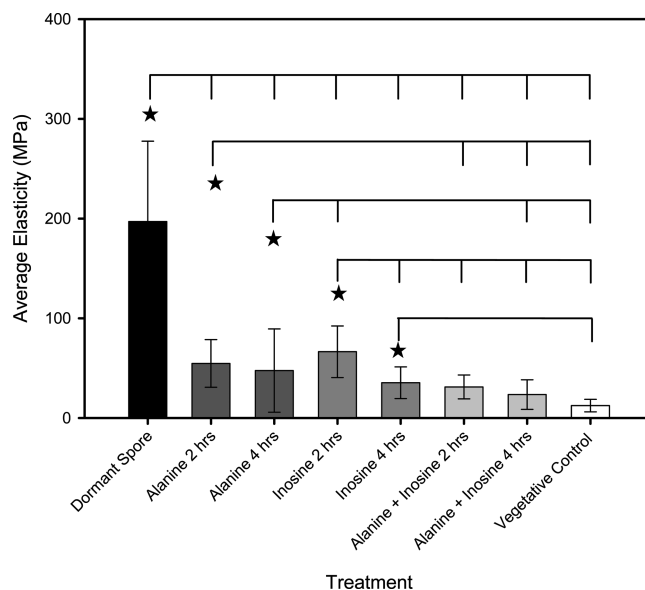

Figure 3. Elasticity of *B. anthracis* Sterne spores as a function of treatment. Spores germinated with 50 mM L-alanine and/or 5 mM inosine for 2 or 4 h. Bars depict the average elasticity of 50 spores per condition. Error bars show the standard deviation. The top lines connect statistically distinct points ($P < 0.05$) calculated by Kruskal−Wallis one-way ANOVA on ranks with Tukey's test to make pairwise comparisons The star symbol indicates the condition that is being compared to other treatments.

results, germination with inosine may require longer exposure time of the cells to the ribonucleoside to allow for the interaction with both receptors. Our investigation also suggests that while other studies have shown that inosine acting alone is a weak germinant for *B. anthracis* spores as measured by the rate of optical density change, it does display a stronger impact on the mechanical properties of the cells since the surface elasticity decreases significantly compared to the dormant spore.

The elasticity values of *B. anthracis* incubated with both germinants were significantly lower compared to the spores incubated with each of the individual nutrients ($P < 0.05$). It has been suggested in the literature that *B. anthracis* may not germinate in the presence of inosine alone but may require coupling with another germinant, such as an amino acid.[6,11] Ireland and Hanna showed that high concentrations of inosine (up to 50 mM) did not trigger a germination response from *B. anthracis* even when incubated for up to 16 h.[11] In the same study, it was concluded that L-alanine is a strong germinant only at high concentrations (>10 mM), but the presence of a second cogerminant, such as inosine, will result in a stronger germination response.[11] Our results suggest that a weakening of the spore surface is taking place after incubation of *B. anthracis* with L-alanine and inosine, since the elasticity values decreased significantly ($P < 0.05$). The use of two germinants may not only weaken the surface of the spore but speed up the degradation of the spore coat and the peptidoglycan cortex that protects the spore core, which would change the mechanical properties of the cell.

Spores treated with lower concentrations of L-alanine and/or inosine (25 and/or 2.5 mM, respectively) did not show significantly different Young's moduli when compared to the values obtained with higher concentrations of L-alanine and/or inosine (50 and/or 5 mM, respectively; Table 1). Clements and Moir have shown that comparable germination rates of *Bacillus* spores were achieved over inosine concentrations ranging from 1 to 30 mM and over L-alanine concentrations ranging from 10 to 100 mM.[13]

Table 1. Effects of Germinant Concentration on Young's Modulus and Indentation Depths of *Bacillus anthracis*

germinant concentration	average Young's modulus (MPa)	P value[a,b]	average indentation depth (nm)	P value[a,c]
25 mM L-alanine	55.7 ± 31.9	0.846	137.7 ± 31.6	0.612
50 mM L-alanine	54.7 ± 23.9		135.0 ± 41.6	
2.5 mM inosine	69.5 ± 16.6	0.608	121.8 ± 19.7	0.458
5 mM inosine	66.4 ± 25.9		117.8 ± 25.1	
25 mM L-alanine + 2.5 mM inosine	32.3 ± 14.2	0.669	224.7 ± 35.1	0.123
50 mM L-alanine + 5 mM inosine	31.15 ± 11.9		223.9 ± 82.1	

[a]Comparisons of Young's modulus and indentation depth data between germinant concentrations were done using one-way analysis of variance (ANOVA) for repeated measurements. Tukey's test was used for multiple comparisons among treatment groups. A difference was considered significant if $P < 0.05$. [b]P value obtained when comparing average Young's moduli. [c]P value obtained when comparing average indentation depths.

In another study, spore germination was measured by studying the decrease of optical density of spore suspensions after addition of germinants, and it was determined that inosine concentrations of 1 or 10 mM and L-alanine concentrations of 10 or 100 mM caused the same decrease of optical density of spore suspensions.[8] Based on these studies, the concentrations of L-alanine and inosine chosen for our elasticity experiments fall within the range where optimal spore germination rates occur. In this study we observe surface elasticity values for *B. anthracis* that are unaffected by the change in germinant concentration when the spores are exposed to high concentrations of L-alanine (25 and 50 mM) or inosine (2.5 and 5 mM). Similarly, mixtures of both germinants at concentrations of 50 mM L-alanine and 5 mM inosine or 25 mM L-alanine and 2.5 mM inosine yielded comparable Young's moduli. These results suggest that once the spores have been exposed to high enough concentrations of the germinants, the changes in elasticity will be the same independent of the amount of L-alanine and/or inosine in the spore suspension. The elasticity of vegetative *B. anthracis* showed the lowest Young's modulus with an average of 12.39 ± 6.32 MPa, and the values were significantly different when compared to other treatments ($P < 0.05$), except when the cells were treated with L-alanine and inosine for 4 h ($P > 0.05$). This elasticity value is close to the elastic modulus of other vegetative bacteria. For *E. coli*, a Young's modulus of ~12.8 MPa was estimated.[23] Once a spore has completely germinated, there is an outgrowth process leading to the vegetative cell where the spore coat is lost, leaving the cell with a membrane that is much thinner and allows for the transport of nutrients and release of waste products.[5,35] The degradation of the spore coat resulted in a cell that could be more easily indented by the AFM probe, as was observed on vegetative bacteria (Figure 4).

Indentation Depths of *B. anthracis* as a Function of Treatment. Indentation depths were significantly lower for the untreated spores than for those treated with L-alanine, inosine, or TSB supplemented with yeast extract ($P < 0.05$). For the dormant spores (untreated spores), the depth indented by the AFM probe was 20.45 ± 14.88 nm. However, this indentation depth increased significantly after incubation of germinants for 2 or 4 h. Higher indentation depth values were observed for spores treated with L-alanine compared to inosine. For the germinant-treated spores, the highest indentation depths were observed for spores treated with both L-alanine and inosine for 2 h (223.97 ± 82.14 nm) or with incubation of the cells in L-alanine for 4 h (225.37 ± 98.65 nm). Lower concentrations of L-alanine and/or inosine (25 and 2.5 mM, respectively) showed similar indentation depth values, suggesting that there is no appreciable effect of germinant concentration on surface elasticity and indentation depth, once an optimal germination condition has been achieved
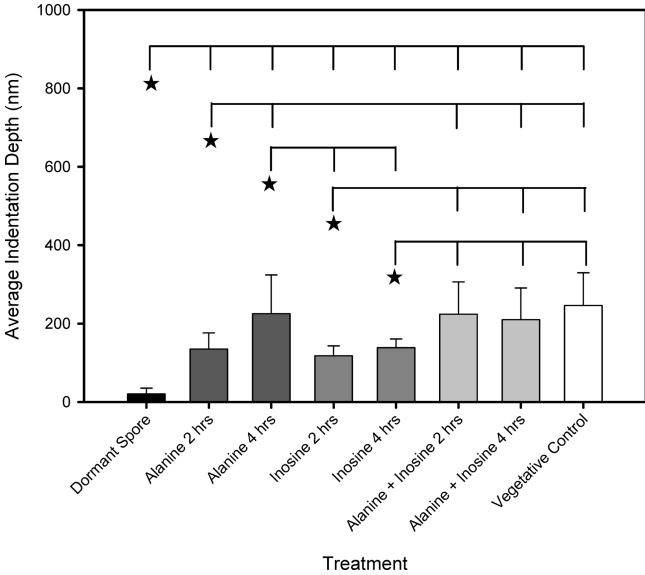

Figure 4. Average indentation depths of *B. anthracis* Sterne spores probed with a silicon AFM tip as a function of treatment. Spores germinated with 50 mM L-alanine and/or 5 mM inosine for 2 or 4 h. Error bars show the standard deviation of 50 measurements obtained for each condition. The top lines connect statistically distinct points ($P < 0.05$) calculated by Kruskal−Wallis one-way ANOVA on ranks The star symbol indicates the condition that is being compared to other treatments.

(Table 1). The incubation of *B. anthracis* spores in TSB supplemented with yeast extract resulted in the highest indentation depths (246.23 ± 84.31 nm). Our results suggest that the surface of dormant (untreated) *B. anthracis* spore is harder and therefore higher loading forces are required to indent the cell. Once germination is initiated, there is a degradation of the spore coat that allows the spore to outgrow and become a vegetative cell.[5] Vegetative *B. anthracis* need to have a much thinner cell wall with more flexibility to allow the release of waste products as well as toxins during infection and invasion of macrophages.[5,35] Because of the more flexible and thinner surface, the AFM probe can indent the vegetative cell more than the sporulated cell. Analogous changes in the elasticity have been observed for *S. cerevisiae* at the location where the bud emerges during cell division.[22] When the cell undergoes cellular division, a chitin ring of polysaccharide of (β1−4)-linked *N*-acetylglucosamine forms to strengthen the bud scar, marking the division site.[22] The proteins that form this chitin ring are highly glycosylated peptides that are 50−95 wt % carbohydrate, which makes chitin fibers insoluble, causing stiffening of the bud scar.[36] Because of the rigidity of the chitin ring, the

(35) Leuschner, R. G. K.; Ferdinando, D. P.; Lillford, P. J. *Colloids Surf., B* **2003**, *19*, 31−41.

(36) Alsteens, D.; Dupres, V.; Mc Evoy, K.; Wildling, L.; Gruber, H. J.; Dufrene, Y. F. *Nanotechnology* **2008**, *19* (38), 384005/1−384005/9.

portion of the cell that had the bud scar had higher Young's modulus values and lower indentation depths made by the AFM probe compared to the rest of the cell surface. In another study, the spring constant of the *S. cerevisiae* cell body and the bud scar were determined from the slope of the linear portion of the force−distance curve, and the bud scar displayed higher cell spring constant due to the increased chitin content of the area.[37]

Conclusions

The rigidity of the spore coat of dormant *B. anthracis* cells is severely compromised by exposure to germinants or nutrients, such as TSB and yeast extract. During germination, the spores start losing their coat, making the cell's surface more flexible, lowering the surface elasticity, and allowing the AFM probe to indent the surface more during the force cycle. Vegetative *B. anthracis* showed the weakest cell surface with elasticity values that were 15 times lower than sporulated cells. This decrease in elasticity accompanied by a decrease in the thickness of the spore coat, which we previously observed,[3] would explain why vegetative cells are sensitive to

(37) Pelling, A. E.; Sehati, S.; Gralla, E. B.; Valentine, J. S.; Gimzewski, J. K. *Science* **2004**, *305* (5687), 1147−50.

sporicidal compounds. Based on our findings, the study of elasticity of spores may be a valuable tool to predict how to weaken spores, thus making them more susceptible to spore inactivation processes based on mechanical forces, such as the high-pressure, high-temperature inactivation method practiced in the food industry. Indeed, on the basis of our results, we anticipate that the decrease in the elasticity of the spores caused by germinators such as L-alanine, inosine, dodecylamine, etc., either alone or in mixtures, would make spore inactivation possible at lower pressures compared to that in the absence of such germinators. It may also be possible to reduce the small population of "difficult to kill" or "superdormant" spores that often survive the current high-pressure inactivation methods by the use of germinants in the high-pressure spore inactivation process.