THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

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Interactions of Antimicrobial Peptide Chrysophsin-3 with *Bacillus anthracis* in Sporulated, Germinated, and Vegetative States

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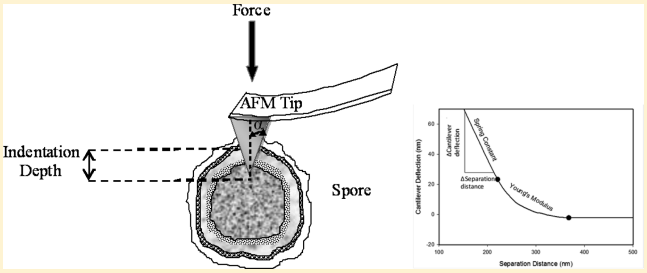
ABSTRACT: *Bacillus anthracis* spores contain on their surface multilayered protein coats that provide barrier properties, mechanical strength, and elasticity that aid in protecting the sporulated state and preventing germination, outgrowth, and transition into the virulent vegetative bacterial state. In this work, the antimicrobial peptide (AMP) chrysophsin-3 was tested against *B. anthracis* in each of the three distinct metabolic states (sporulated, germinated, and vegetative) for its bacteria-killing activity and its ability to modify the surface nanomechanical properties. Our results provide the first demonstration that chrysophsin-3 killed *B. anthracis* even in its sporulated state while more killing was observed for germinated and vegetative states. The elasticity of vegetative *B. anthracis* increased from 12 ± 6 to 84 ± 17 MPa after exposure to 0.22 mM chrysophsin-3. An increase in cellular spring constant was also observed for chrysophsin-3-treated vegetative *B. anthracis*. Atomic force microscopy images suggested that the changes in mechanical properties of vegetative *B. anthracis* after chrysophsin-3 treatment are due to loss of water content and cellular material from the cell, possibly caused by the disruption of the cell membrane by the AMP. In contrast, sporulated and germinated *B. anthracis* retained their innate mechanical properties. Our data indicate that chrysophsin-3 can penetrate the spore coat of *B. anthracis* spores and kill them without causing any significant mechanical changes on the spore surface. These results reveal a yet unrecognized role for chrysophsin-3 in the killing of *B. anthracis* spores without the need for complete germination or release of spore coats.



INTRODUCTION

Over the last 120 years, significant progress has been made in understanding the biochemical changes that occur during germination and killing of *Bacillus anthracis* spores.[1] However, little attention has been paid to understanding the mechanical changes that occur during these processes and how these changes may provide physical insight into the mechanism of action of potential antisporal compounds.

B. anthracis, the microorganism that causes anthrax infections, is one of the most resistant life forms known because of its ability to form spores.[2] In their sporulated state, the genetic material of *B. anthracis* is protected and encased in a thick protein coat composed of the exosporium, an outer spore membrane that surrounds the cortex, the germ cell wall, and an inner spore membrane that surrounds the spore core where all genetic information is kept (Figure 1a).[3] Together, these peptidoglycan and proteinaceous coat layers surrounding the spore core form a protective ∼130 nm thick[4] barrier that cannot be penetrated by chemical and environmental insults.

Although the mechanical properties of the spore coat have not been investigated, it is thought that the strength and elasticity of the spore surface are important in maintaining a state of relative dehydration of the spore core, where any influx of water and swelling of the spore is resisted.[5]

Dormant spores can resume their metabolic state during the process of germination. Amino acids and purine nucleosides, such as L-alanine and inosine, trigger germination by binding to receptors on the inner membrane of the spore.[3,6] During germination, monovalent cations, Zn^{2+}, Ca^{2+}, and dipicolinic acid (DPA) are released from the spore core,[7,8] while water molecules start entering the spore, rehydrating its core and cortex. Increases in hydration levels of the spore result in the degradation and thinning of the cortex and in the swelling of the spore core (Figure 1b). As the spore swells, the coats that once were a barrier against antisporal agents start to rupture (Figure 1c).

Degradation and changes in elasticity of the outer spore membrane and cortex result in the release of an emerging virulent vegetative cell from the spore coat remnants.[9] Vegetative *B. anthracis* are devoid of the spore cortex, the outer spore coat, and the outer spore membrane (Figure 1d). Only a cell wall surrounds the cytoplasm of vegetative *B. anthracis*, which makes vegetative *B. anthracis* susceptible to common disinfectants. Vegetative *B. anthracis* are in the

Received: January 15, 2013
Revised: April 26, 2013
Published: April 30, 2013

dx.doi.org/10.1021/jp400489u | *J. Phys. Chem. B* 2013, 117, 6364−6372

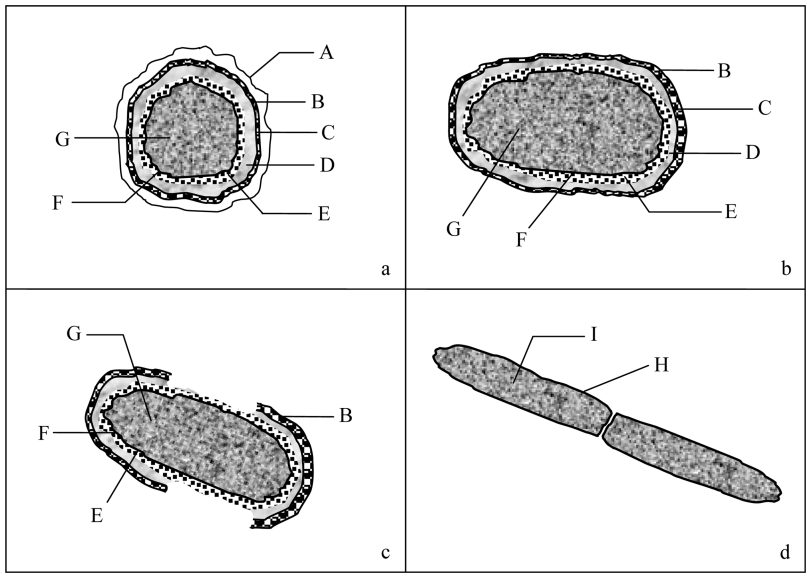


Figure 1. Schematic representation of *B. anthracis* spore germination at (a) 0 h, (b) ~30 min of germinant contact, (c) 1−2 h, and (d) 3−7 h. A, exosporium; B, spore coat; C, outer spore membrane; D, spore cortex; E, germ cell wall; F, inner spore membrane; G, spore core; H, cell wall; I, cell cytoplasm.

virulent state because in this state the bacteria produce and release toxins.[10]

Current treatments of anthrax infections are mostly effective on vegetative *B. anthracis*; therefore, alternative therapies against *B. anthracis* in the sporulated state are needed. Antimicrobial peptides (AMPs) are short polypeptides that are associated with the innate immune system of host organisms and are widely distributed in the animal and plant kingdoms.[11] Chrysophsin-1, -2, and -3 are a family of peptides that have been isolated from the gills of the red sea bream (*Chrysophrys major*).[12] Chrysophsin-3 is an amphipathic, cationic α-helical peptide that is rich in histidine residues with a 20-amino-acid sequence (FIGLLISAGKAIHDLIRRRH) and a molecular mass of 2287 Da.[12] The peptide has an unusual RRRH motif that makes the molecule positively charged, causing secondary amphipathicity and a change in hydrophobicity between the N and C termini (Figure 2).[13]

Chrysophsin-3 was previously shown to kill vegetative *Bacillus subtilis* at concentrations lower than 40 μM.[12] Although the bactericidal activity of chrysophsin-3 is encouraging, no prior study has investigated the effects of this peptide on bacteria in their sporulated state. It was previously assumed that AMPs would not be able to penetrate the spore coat, and thus they would be ineffective against spores. Furthermore, there is not a clear understanding of how the AMP interacts with the spore coat, vegetative bacteria, or germinating spores.

In this study, we investigated *B. anthracis*−chrysophsin-3 interactions when the bacteria were in different metabolic states: sporulated, germinated, and vegetative. Because the strength and elasticity of the spore surface seem to play important roles during dormancy and spore germination, we used atomic force microscopy (AFM) to quantify the changes in elastic modulus and spore spring constant during germination and killing of spores. Changes in mechanical properties of spores during germination and killing contribute to a mechanistic understanding of the mode of action of AMPs against bacterial spores.

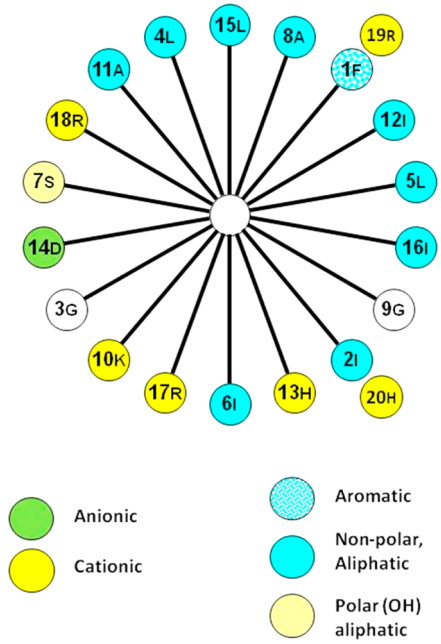


Figure 2. Helical wheel diagram demonstrating the amphipathic α-helical conformation of chrysophsin-3. The solid blue color indicates amino acids that are hydrophobic.

MATERIALS AND METHODS

Antimicrobial Peptide (AMP). The AMP chrysophsin-3 was synthetically produced with the sequence FIGLLISAGK-AIHDLIRRRH (Bachem Americas, Inc., Torrance, CA). The peptide has a relative molecular mass of 2287 Da and is more than 80% pure, measured by reverse phase high-performance liquid chromatography (HPLC). A 4.4 mM stock solution of the peptide was prepared in 50 mM Tris-HCl and stored at 4 °C. The peptide is known to have an α-helical secondary structure.

Spore Preparation. *B. anthracis* Sterne 34F2 (pXO1$^+$ pXO2$^-$; referred to heareafter as *B. anthracis* 34F2) were

dx.doi.org/10.1021/jp400489u | J. Phys. Chem. B 2013, 117, 6364−6372

kindly provided by the Edgewood Chemical Biological Center (Edgewood, MD). *B. anthracis* 34F2 were grown in sporulation media as described previously.[14] Sporulation media, consisting of 8 g of nutrient broth, 4 g of yeast extract, 0.001 g of $MnCl_2 \cdot 4H_2O$, 5 g of peptone, and 15 g of agar in 1 L of ultrapure water (Milli-Q water, Millipore Corp., Bedford, MA), were sterilized by autoclaving for 60 min and poured into Petri dishes.

Fresh cultures of *B. anthracis* 34F2 were obtained by adding 100 μL of spores from a glycerol stock solution to a flask with 25 mL of sterile nutrient broth. The flask was placed in a water bath at 37 °C and was agitated at 200 rpm for 20 h. This culture was used to inoculate plates of sporulation media using aliquots of a 50 μL solution. The plates were incubated at 37 °C for 4 days to allow vegetative *B. anthracis* to form spores. Harvesting of the spores was done in autoclaved ultrapure water, and spores were collected by centrifugation at 5000 rpm for 20 min. The spores were washed until all vegetative and partially sporulated *B. anthracis* were removed and only spores remained. The state of sporulation was verified through phase contrast microscopy, and 99% of the spores examined under the microscope were phase bright. After spores were prepared, they were heat activated for 30 min at 65 °C.

Germination Treatments of Spores. To trigger germination of spores, *B. anthracis* at a concentration of 10^8 cells/mL was exposed to 50 mM L-alanine (Sigma-Aldrich, St. Louis, MO) and/or 5 mM inosine (Sigma-Aldrich) in 50 mM Tris-HCl buffer (pH 8.0) and was incubated for 2 h at 37 °C. To achieve outgrowth of spores into vegetative *B. anthracis*, a 1 mL sample of dormant spore solution was suspended in tryptic soy broth supplemented with 1% yeast extract and grown for 6 h at 37 °C with a rotation of 18 rpm. Germination was assessed through phase contrast microscopy. During phase contrast microscopy, sporulated *B. anthracis* are characterized by having bright bodies and bright halos surrounding the spore. In contrast, spores that are undergoing the process of germination and vegetative *B. anthracis* appear as black or dark cells. Untreated spores incubated in Tris-HCl buffer for 2 h were used as a control.

Exposure to the AMP Chrysophsin-3. Sporulated (untreated), germinated, and vegetative *B. anthracis* were exposed to 0.22 mM chrysophsin-3 for 1 h at 37 °C. After a 1 h exposure to chrysophsin-3, bacteria were washed by centrifugation at 5000 rpm for 5 min and were resuspended in ultrapure water to stop AMP treatment.

Testing Whether Chrysophsin-3 Can Cause Any Germination of *B. anthracis* Spores. Chrysophsin-3 was tested for its ability to germinate spores through optical density measurements and phase contrast microscopy. Spore germination was monitored spectrophotometrically where the loss of light diffraction followed by the addition of germinants is reflected by a decrease in optical density (OD) at 600 nm.[6] To evaluate chrysophsin-3 as a germinant of spores, heat-activated *B. anthracis* were resuspended in a 0.22 mM chrysophsin-3 solution in 50 mM Tris-HCl. The spore solution was mixed at 18 rpm at 37 °C, and the optical density was monitored for 2 h. As a positive control, germination of *B. anthracis* after exposure to 50 mM L-alanine and 5 mM inosine was monitored for changes in optical density. *B. anthracis* exposed to 50 mM Tris-HCl served as a negative control. Phase contrast images were obtained after 2 h of exposure to chrysophsin-3, 50 mM L-alanine and 5 mM inosine, and 50 mM Tris-HCl to correlate optical density measurements with microscopy data.

Viability of *B. anthracis* after Chrysophsin-3 Treatment. To determine the effects of chrysophsin-3 on *B. anthracis* viability in sporulated, germinated, and vegetative states, the AMP-treated bacterial solution (in each state) was serially diluted, and 100 μL was inoculated onto tryptic soy agar (TSA) plates and incubated at 37 °C for 18 h. The number of colony-forming units (cfu) on the agar plates was counted, and the experiment was done in triplicate. Control experiments on TSA plates were performed also for *B. anthracis* in sporulated, germinated, and vegetative states but without exposure to the peptide.

Characterization of *B. anthracis* with Atomic Force Microscopy. Changes in cellular spring constants and elastic moduli of the spores after treatment with germinants and/or chrysophsin-3 were calculated from AFM experimental measurements.

Sporulated, germinated, and vegetative *B. anthracis* were prepared by depositing droplets of treated *B. anthracis* 34F2 (5 μL), in the appropriate state, onto freshly cleaved mica and drying for 18 h. AFM experiments were carried out with a Dimension 3100 with a Nanoscope IIIa controller (Veeco Metrology, Santa Barbara, CA) operated in intermittent contact mode in air at 30−35% relative humidity. A single rectangular cantilever with a conical silicon tip having a specified spring constant of 14 N/m and a resonant frequency of 315 kHz was used for all AFM measurements (Mikromasch, San Jose, CA). Once a cell was located, it was centered so force measurements were not made near the edges. Forces were measured on five cells for each treatment, and ten force cycles were recorded per cell. All force cycles recorded per cell were taken at the same location to verify that no deformation of the surface was taking place. AFM images were taken before and after force measurements to ensure that no damage to the cell occurred during force measurements (scan rate of 1.0 Hz). The AFM tip was kept in contact with the sample for 1 ms before retracting. The velocity of the tip was 2 μm/s with a loading rate of 3×10^{-5} N/s. Data in ASCII format were exported to a spreadsheet and converted from deflection to force as previously described.[14]

Spring Constant of *B. anthracis* 34F2. Cantilever sensitivity was used together with the cantilever spring constant (k_c) to convert raw AFM data into deflection (nm) versus separation distance (nm) curves (Figure 3). When bacteria were probed with the AFM tip, the curve observed during approach represented the indentation of the tip into the spore coat, before the region of constant compliance was reached (linear response; Figure 3). Once there was no further indentation of the cell surface by the AFM tip, the linear cantilever deflection−separation distance relation observed during the force cycle was due to the *B. anthracis*' turgor pressure.

The slope of the constant compliance region, which characterizes the rigidity of the whole spore, was then used to calculate the effective cell spring constant k_s by

$$k_s = \frac{k_c s}{1 - s} \tag{1}$$

where s is the slope of the constant compliance region (Figure 3).[15]

A total of 10 curves were analyzed per cell, and the overall mean and standard deviation (SD) of the sample were calculated on the basis of the k_s values of five *B. anthracis*

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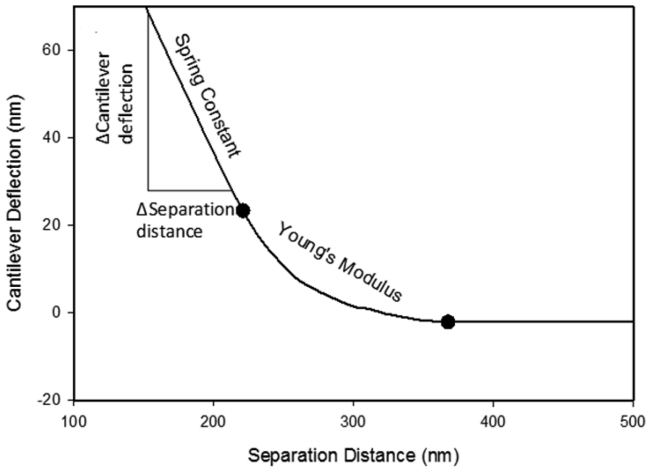

Figure 3. Representative AFM approach curve from a *B. anthracis* 34F2 cell. The linear region of the curve in the upper left corner represents the region of constant compliance that was used to calculate the spring constant of the cell. The nonlinear region between the two black dots denotes the indentation of the AFM tip into the cell and was used to calculate the Young's modulus of *B. anthracis*.

cells. Hence, data were collected on a total of 50 force curves per test condition.

Elastic Modulus of *B. anthracis* 34F2. The deflection data obtained during force measurements were converted to loading force versus indentation data following procedures described previously.[14] The elastic modulus was obtained by applying the Hertz model of continuum mechanics of contact to the nonlinear portion of the loading force−indentation depth data (Figure 3). The geometry of the AFM tip was taken into account and was related to the AFM data by

$$F_{\text{cone}} = \frac{2}{\pi} \tan \alpha \frac{E}{1-\upsilon^2}\delta^2 \quad (2)$$

where α is the half-opening angle of the conical tip used (taken as 40° as specified by the manufacturer), E the modulus of the cell, ν the Poisson ratio of the spore (taken as 0.5),[16] and δ the indentation depth of the cell.

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

Statistical Analysis. Elasticity, spring constant, and viability data were analyzed using the Kruskal−Wallis one-way analysis of variance (ANOVA) on ranks for repeated measurements. The null hypothesis tested was that there were no differences in the distribution of values between different groups. Tukey's test was used for comparisons between untreated spores and those treated with chrysophsin-3. A difference was considered significant if $P < 0.05$.

RESULTS

We characterized viability, cellular spring constant, and elastic modulus of *B. anthracis* exposed to chrysophsin-3 in three metabolic states: sporulated, germinated, and vegetative.

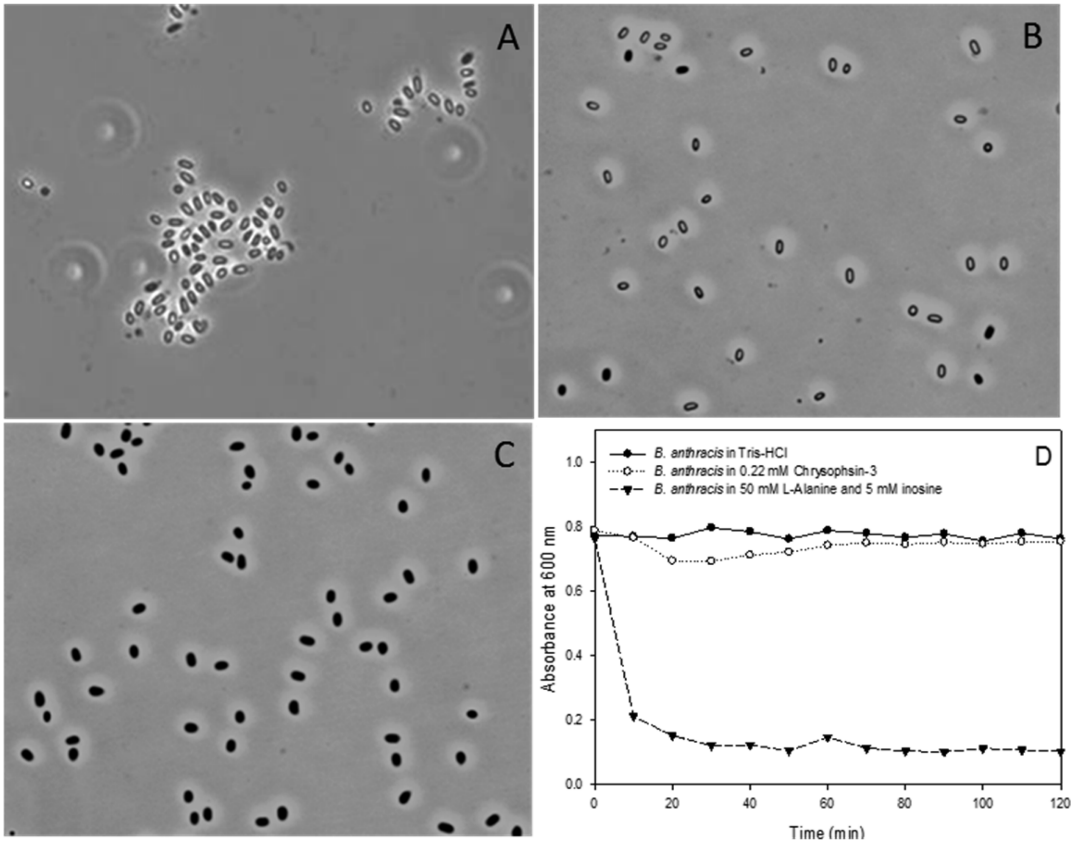

Figure 4. Phase contrast microscopy images and optical density measurements of *B. anthracis* 34F2 spores in their dormant state exposed to (A) 50 mM Tris-HCl buffer, (B) 0.22 mM chrysophsin-3, and (C) 50 mM L-alanine and 5 mM inosine. Exposure was for 2 h at 37 °C where the optical density of the spore solution was measured every 10 min at 600 nm (D). Phase bright cells indicate spores in their sporulated state while dark spores indicate germinated spores. Magnification 1000×.

dx.doi.org/10.1021/jp400489u | J. Phys. Chem. B 2013, 117, 6364−6372

Can Chrysophsin-3 Cause Germination of *B. anthracis*? We evaluated the ability of chrysophsin-3 to germinate *B. anthracis* 34F2 spores. There were no significant differences between the appearance of untreated spores (Figure 4A) and chrysophsin-3-treated spores (Figure 4B). *B. anthracis* 34F2 in 0.22 mM chrysophsin-3 remained sporulated because more than 95% of the cells remained phase bright when observed under phase contrast microscopy (Figure 4B). In contrast, 100% of spores germinated after 2 h of exposure to 50 mM L-alanine and 5 mM inosine because they turned phase dark when observed under the microscope (Figure 4C). These results were supported by optical density studies. While untreated spores and chrysophsin-3-treated spores remained unchanged with the same optical density of ~0.7−0.8 at 600 nm, the optical density of spores exposed to the germinants L-alanine and inosine decreased significantly, reaching an absorbance of 0.1 at 600 nm. There was a 75% OD loss within 10 min of exposure to the germinants, suggesting that *B. anthracis* germinate rapidly in 50 mM L-alanine and 5 mM inosine. No significant loss in optical density was observed for the chrysophsin-3 spores during the 2 h of exposure, indicating that 0.22 mM chrysophsin-3 does not germinate *B. anthracis* spores.

Viability of Sporulated, Germinated, and Vegetative *B. anthracis* after Exposure to Chrysophsin-3. The ability of chrysophsin-3 to kill *B. anthracis* depended on the bacterial state. As anticipated, chrysophsin-3 had the greatest antibacterial effect against vegetative *B. anthracis* 34F2, resulting in killing of >99.9% of vegetative *B. anthracis* after 1 h of peptide treatment, which corresponds to a log-kill of ~5.9 (Table 1). In contrast, the totally unexpected finding was that even in their sporulated state, exposure of *B. anthracis* 34F2 to 0.22 mM chrysophsin-3 resulted in a significant decrease in the number of surviving spores (Table 1). Without any germinant, the peptide killed ~79% of the spores in their dormant state. To the best of our knowledge, this is the first observation of any spore killing by antimicrobial peptides.

Exposing *B. anthracis* 34F2 to the germinants 50 mM L-alanine and 5 mM inosine for 2 h before exposure to chrysophsin-3 resulted in ~84% and ~82% killing of spores, respectively. When both germinants were applied together before exposure to the AMP, >99% of the germinating spores were killed, corresponding to a log-kill of ~2 (number of surviving spores was reduced by two-log).

Table 1. Antisporal Efficacy of Chrysophsin-3 against *B. anthracis*

treatment[a]	number of surviving spores (cfu/mL)	% killed[b]	log-kill[c]
B. anthracis, no peptide or germinant	7.4×10^6		
B. anthracis + chrysophsin	1.6×10^6	78.8	0.67
B. anthracis + inosine	7.2×10^6		
B. anthracis + inosine + chrysophsin	1.4×10^6	81.6	0.74
B. anthracis + L-alanine	8.2×10^6		
B. anthracis + L-alanine + chrysophsin	1.2×10^6	84.3	0.81
B. anthracis + L-alanine + inosine	2.6×10^5		
B. anthracis + L-alanine + inosine + chrysophsin	5.4×10^4	99.3	2.14
vegetative *B. anthracis*	4.5×10^6		
vegetative *B. anthracis* + chrysophsin	10	99.9	5.86

[a]Chrysophsin-3 concentration = 0.22 mM; L-alanine concentration = 50 mM; inosine concentration = 5 mM. [b]Against control. [c]log-kill = log(number of original spores/number of surviving spores).

Effects of Chrysophsin-3 on the Spring Constant of Sporulated, Germinated, and Vegetative *B. anthracis* 34F2. Comparison of chrysophsin-3-treated *B. anthracis* 34F2 with untreated *B. anthracis* 34F2 revealed that the cellular spring constants were not statistically different from one other, regardless of whether AMP treatment was applied, except for vegetative *B. anthracis* 34F2 (Figure 5).

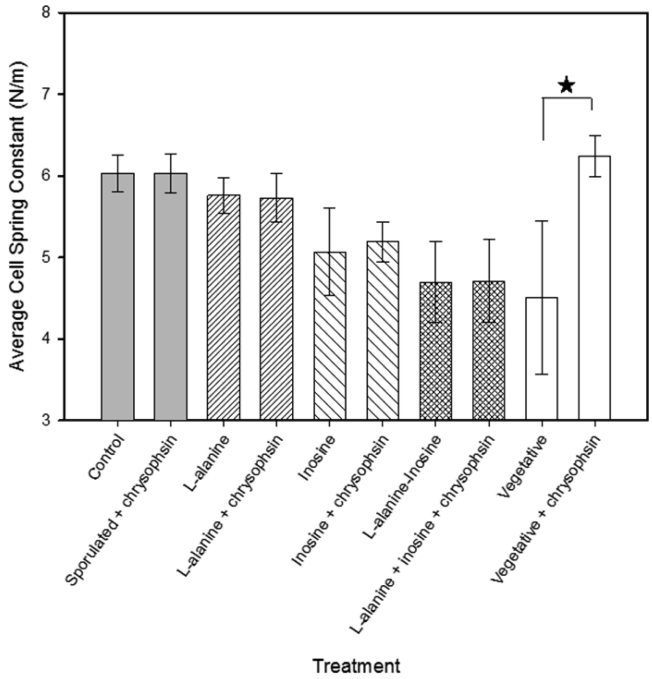

Figure 5. Average spring constant of *B. anthracis* 34F2 as a function of treatment. Spores were germinated with 50 mM L-alanine and/or 5 mM inosine for 2 h at 37 °C. After treatment with germinants, the spores were exposed to 0.22 mM chrysophsin-3 for 1 h at 37 °C. Bars depict the average value of 50 force profiles per condition and standard deviation. The top line and star connect statistically distinct points ($P < 0.05$) calculated by one-way ANOVA on ranks.

The spring constant of untreated *B. anthracis* 34F2 in the sporulated state remained the same before and after exposure to 0.22 mM chrysophsin-3 for 1 h (6.03 ± 0.23 and 6.03 ± 0.24 N/m, respectively). Germination of *B. anthracis* 34F2 with 50 mM L-alanine resulted in a slight decrease in the cellular spring constant when compared to that of the sporulated cell, but this change was not statistically significant. Exposure of *B. anthracis* in the germinated state (attained using L-alanine) to chrysophsin-3 did not cause any changes in the spring constant, and the values remained similar to that of the untreated, L-alanine-germinated spore (5.76 ± 0.22 and 5.73 ± 0.29 N/m, respectively). Use of 5 mM inosine as the germinant resulted in a spring constant of 5.07 ± 0.54 N/m, which remained nearly the same after exposure to the AMP (5.19 ± 0.25 N/m).

dx.doi.org/10.1021/jp400489u | J. Phys. Chem. B 2013, 117, 6364−6372

Treatment of *B. anthracis* 34F2 with both germinants together resulted in a cellular spring constant of 4.70 ± 0.49 N/m, and the value remained the same after additional exposure to the peptide (4.71 ± 0.51 N/m). Thus, for all germinations considered, the spring constant of *B. anthracis* 34F2 in the germinated state remained the same after exposure to 0.22 mM chrysophsin-3.

Only for the bacteria in the vegetative state did the exposure to the AMP have a significant effect on the spring constant. The lowest spring constant values were observed for *B. anthracis* in the vegetative state. The spring constant of vegetative *B. anthracis* 34F2 was 4.51 ± 0.93 N/m (Figure 5). After exposure to the AMP, the spring constant of the vegetative cell increased significantly to 6.24 ± 0.25 N/m.

Elastic Modulus of Sporulated, Germinated, and Vegetative *B. anthracis* 34F2 after Exposure to Chrysophsin-3. Elastic moduli of *B. anthracis* were affected by the sporulation state but were not affected by the AMP, except for vegetative bacteria (Figure 6). Chrysophsin-3 did not have an effect on the elastic moduli of the spore surfaces in their dormant form or after germination had been initiated with L-alanine and/or inosine (Figure 6).

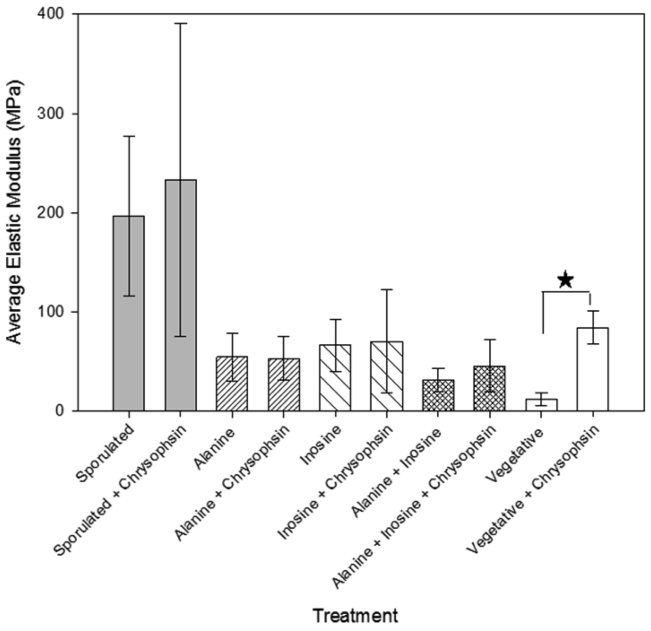

Figure 6. Average elastic modulus of *B. anthracis* spores as a function of treatment. Spores were first treated with 50 mM L-alanine and/or 5 mM inosine for 2 h at 37 °C. After treatment with germinants, 0.22 mM chrysophsin-3 was introduced, and spores were exposed to the AMP for an additional 1 h at 37 °C. Bars depict the average value of 50 spores per condition and standard deviation. The top line and star 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 highest modulus was obtained with the untreated dormant spores (197 ± 74 MPa; Figure 6) and *B. anthracis* spores treated with 0.22 mM chrysophsin-3 (233 ± 158 MPa; Figure 6). The elastic modulus decreased significantly for the germinated state, independent of the choice of the germinant. L-Alanine- or inosine-treated *B. anthracis* 34F2 had an elasticity of 53 ± 22 and 70 ± 52 MPa, respectively, whereas treating *B. anthracis* 34F2 with both germinants together resulted in an elastic modulus of 45 ± 26 MPa. The exposure of the germinated spores to chrysophsin-3 did not cause any statistically different change in the elastic moduli for any of the three kinds of germinated spores (Figure 6). *B. anthracis* 34F2 in the vegetative state had the lowest modulus (12 ± 6 MPa), but this value increased significantly after exposure to 0.22 mM chrysophsin-3 (84 ± 17 MPa; $P < 0.001$). A significant difference in the indentation depth was also observed for vegetative *B. anthracis* 34F2, decreasing from 246 ± 83 to 108 ± 14 nm after exposure to the AMP ($P < 0.001$). No significant changes in indentation depths were observed for the bacteria in the sporulated or germinated states before and after being exposed to chrysophsin-3.

Morphological Changes of *B. anthracis* 34F2 after Chrysophsin-3 Treatment. Chrysophsin-3 caused significant changes in the elastic modulus, indentation depth, and spring constants of vegetative *B. anthracis*. Therefore, the morphology of vegetative *B. anthracis* 34F2 cells was characterized using AFM (Figure 7).

Untreated vegetative *B. anthracis* 34F2 had an elongated shape, with lengths up to 7 μm, and were found in chains of three or four cells (Figure 7A). Treatment of vegetative *B. anthracis* 34F2 with chrysophsin-3 resulted in lysis of bacteria (Figure 7B). Images showed cellular debris, which appears to be intracellular material being lost from the cell.

DISCUSSION

Sporulated *B. anthracis* Exposed to Chrysophsin-3. Our experimental data show that chrysophsin-3 does not cause any germination of *B. anthracis* spores but at the same time can kill nearly 75% of the spores. Some prior studies have shown that AMPs can kill bacterial spores after inducing germination, but this is the first observation in the literature, to the best of our knowledge, that a peptide can kill the bacteria in the sporulated state in the complete absence of germination. For example, two larger AMPs, E4I subtilin and nisin, have been shown to kill spores, but contact with the AMPs also promoted germination before killing took place.[17,18] Nisin, a 34-amino-acid peptide isolated from *Lactococcus lactis,* triggers germination, which is required for the peptide to cause disruption of membrane integrity.[17] Subtilin, a cationic peptide that is ribosomally synthesized by *B. subtilis*, promoted germination and caused lysis of vegetative cells.[18] Research suggests that in the case of subtilin and nisin, germination needs to take place in order to minimize the obstruction of AMP's passage into the cell by the spore coat.[18]

We studied the mechanical properties of spores to determine whether chrysophsin-3's action on the spores in the absence of any germination could be related to mechanical strength and/or elasticity. The spring constant and the elastic modulus of sporulated *B. anthracis* remained the same before and after exposure to the peptide (Figures 5 and 6), suggesting that killing is not related to changes in mechanical properties of the spores.

We observed a high standard deviation for the sporulated *B. anthracis* with or without AMP treatment. This rather high variation in modulus within one treatment is not uncommon in AFM studies because the AFM probe makes measurements at multiple locations within the spore surface that may not be completely homogeneous. High-resolution images of *B. anthracis* spores suggest that ridges and rodlets are found throughout the surface of the spores, giving them a rough appearance.[19] To overcome this problem of probing a

dx.doi.org/10.1021/jp400489u | J. Phys. Chem. B 2013, 117, 6364–6372

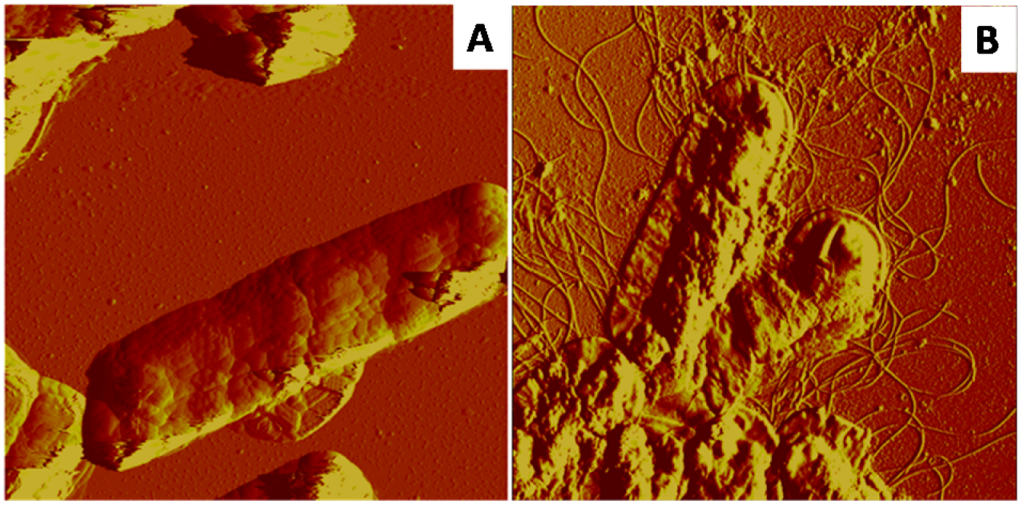

Figure 7. AFM amplitude images of *B. anthracis* Sterne before (A) and after (B) exposure to 0.22 mM chrysophsin-3 for 1 h at 37 °C. Imaging was done in tapping mode in air. Scan size: 10 μm × 10 μm.

somewhat heterogeneous surface, we took multiple AFM measurements at various locations.

The surface of spores is composed of protein layers that are densely packed and difficult for large molecules that may be harmful to the cell to penetrate. However, previous research has suggested that the outermost surface layer in *B. anthracis* spores (the exosporium) is a semipermeable barrier because 20 Å pores can be found throughout this layer.[20] Prior research also suggested that molecules in the range of 2−8 kDa can pass through the spore coat.[21] Chrysophsin-3 has a molecular mass of 2.3 kDa and therefore may be able to pass through the spore coat and exosporium without disrupting the mechanical properties of the spore surface. Although the mechanism is still not fully understood, once delivered through the spore coat, the peptide may act to compromise the cell wall in the same manner as in bacteria in the vegetative state.

Germinated *B. anthracis* Exposed to Chrysophsin-3. *B. anthracis* spores that were undergoing germination were much easier to kill by the AMP, with ∼99% of germinating spores being killed after exposure to 0.22 mM chyrsophsin-3 (Table 1). Germinating spores have less resistance to antisporal compounds, because the spore cortex and spore coats are hydrolyzing and degrading during germination to allow the entry of water molecules into the core.[8] In our previous study, we observed that the roughness of the spore surface changes during germination of the spore, which may result in the formation of pores on the surface that would allow the passage of larger molecules into the spore.[22] Because larger pores may be forming during germination, a larger amount of the AMP may get inside the spore, resulting in an increased killing effect. Killing of germinating spores by other AMPs has also been observed.[17,23−25] The AMP licochalcone caused the release of calcium from different strains of *Bacillus* spores and the inhibition of spore growth.[25] Nisin also inhibited the growth of *B. anthracis* spores at low concentrations; however, germination was a necessary step for nisin to stop spore growth.[17]

The spring constant and elastic modulus values of germinated *B. anthracis* remained the same after exposure to chrysophsin-3. However, we observed that there was a decrease in the elastic modulus of germinated spores when compared to that of sporulated *B. anthracis*. The decrease in elastic modulus occurs as a result of the degradation of spore coats that takes place during germination and not the exposure of spores to the AMP.

The mechanisms of action of chrysophsin-3 against sporulated or germinated *B. anthracis* are not well understood. On the basis of our research, it is plausible that the elastic modulus and spring constant of the spore may be changing but at levels that cannot be detected by AFM because the radius of the AFM tip is large when compared to the size of the small pores that may be present on the surface of *B. anthracis*. Studies done in our laboratory have suggested that at low concentrations chrysophsin-3 causes pores to form on the surface of a supported lipid bilayer, resulting in partial loss of lipid mass from the membrane.[26] Pore formation and mass loss from the cellular membrane destabilize the bacterial surface and result in the loss of cellular material. AMPs such as magainin, alamethicin, and retrocyclins form pores on lipid membranes that have a diameter of <10 nm.[11,24] Retrocyclin is a synthetic defensin peptide with a molecular mass of ∼25 kDa that has been shown to be effective in the killing of vegetative *B. anthracis*.[24] Defensins, such as retrocyclins, form pores with diameters estimated at 25 Å.[27] Because the molecular mass of retrocyclins is ∼12 times greater than that of chrysophsin-3 (∼2.3 kDa), the pores formed by chrysophsin-3 may be of a much smaller diameter. The small diameter pores that may be formed by the peptide would have been impossible to detect by the AFM tip during the elasticity measurement because the radius of curvature of the AFM tip used was >10 nm (∼8 times larger than the diameter of retrocyclin-formed pores).

There have been a number of AFM studies that have reported the formation of pores by AMPs.[28] However, these studies investigated only the effects of AMPs on supported lipid bilayers instead of whole bacterial cells, or the diameter of the pores were >10 nm. Our results show that the elastic modulus of *B. anthracis* spores would be similar for chrysophsin-3-treated and untreated spores because the AFM tip would be too large to detect small pores formed by the AMP.

Vegetative *B. anthracis* Exposed to Chrysophsin-3. Chrysophsin-3 had the most sporicidal effect against vegetative *B. anthracis* 34F2. The complete release of spore coats after outgrowth results in a vegetative bacterium with a susceptible, thin cell membrane. Chrysophsin-3 may act on vegetative *B. anthracis* the same way other similar AMPs act on vegetative

dx.doi.org/10.1021/jp400489u | *J. Phys. Chem. B* 2013, 117, 6364−6372

bacteria. Chrysophsin-3 forms pores that destabilize the cell membrane, causing vital cellular content to be lost from the cell. Recent studies have found that chrysophsin-1 (another isoform of chrysophsin) causes the release of calcein (a fluorescent calcium indicator) from large unilamellar vesicles that were preloaded with the dye.[13] This loss of calcein may be caused by pores being formed on the membrane, leading to the loss of intracellular material. AFM images confirmed this idea because chrysophsin-3 caused the lysis of vegetative *B. anthracis* (Figure 7). Lysis of *B. anthracis* was characterized by intracellular material being lost from the cell and apparent dehydration. Similar lysing events have been observed through AFM studies when *Escherichia coli* is lysed by the surfactant sodium deoxycholate.[29]

Chrysophsin-3 seemed to affect the stiffness of only vegetative *B. anthracis* and not that of the spores or *B. anthracis* undergoing the process of germination. Similar to our study, Fernandes et al. observed that antimicrobial chitooligosaccharides (COS), chitin byproducts derived from crustaceans and insects, are more effective against vegetative *Bacillus cereus* than against sporulated cells.[30] The slope of the constant compliance region of AFM curves taken on vegetative *B. cereus* changed significantly and became steeper after treatment with the antimicrobial compound, which indicates an increase in cellular stiffness. However, COS treatment of *B. cereus* spores did not affect the AFM force profile of the spore, suggesting that spore stiffness remained unchanged after exposure to COS.[30] The penetration of chrysophsin-3 into vegetative *B. anthracis* may cause the loss of water content from the cell, which would result in an increase of the stiffness or spring constant of the cell.

Similar to the effect on spring constant values, the 0.22 mM concentration of chrysophsin-3 may have been high enough to cause changes in the elastic modulus of vegetative *B. anthracis* but not sufficient to cause any changes in the elastic moduli of sporulated and germinated cells. The cell surface of vegetative *B. anthracis* is weaker and more susceptible to common disinfectants because the ∼130 nm coat of proteins that protects the genetic material in spores is no longer present once the spore has germinated and outgrown into a fully virulent bacterium.[4] The cell membrane of *B. anthracis* 34F2 in its vegetative state is composed only of a peptidoglycan layer (murein sacculus) that protects the cells from destructive osmotic changes.[24] This peptidoglycan layer contains multiple 41 Å diameter pores that are capable of allowing small molecules, such as retrocyclins, to pass.[24] Therefore, it is unlikely for vegetative *B. anthracis* to impede the passage of even smaller molecules, such as chrysophsin-3, into the membrane. The passage of chrysophsin-3 into the membrane of vegetative *B. anthracis* may result in destabilization of the membrane due to the formation of multiple pores that will cause the loss of cellular contents from *B. anthracis*. Lysis of vegetative bacteria and loss of water content would result in dehydration and hardening of the cell.

While these results show promising antisporal action for the eradication of vegetative *B. anthracis*, a more in-depth study on the kinetics of the interaction of chrysophsin-3 and other similar AMPs with vegetative *B. anthracis* needs to take place to fully understand the mechanism of action of these AMPs. Furthermore, other techniques need to be studied to address the leakage of the cytoplasmic content from *B. anthracis* that would explain the morphological changes observed for the bacteria in their vegetative state.

CONCLUSIONS

This is the first study to demonstrate that antimicrobial peptides can kill *B. anthracis* in its sporulated state without causing any germination, even though the extent of spore killing is only about 75%. The absence of any structural and mechanical changes in the spores on exposure to the peptide chrysophsin-3 suggests that the spore coat may have some limited permeability to the peptide allowing it to cause spore killing. This opens the possibility of designing other small peptide molecules that could directly kill the spores, providing a new and simple approach to the eradication of bacterial spore threats.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof. Nancy Burnham and Ms. Stephanie Marcott for helpful discussions regarding this project.

This work was supported in part by the National Science Foundation (CBET 0827229 and CBET 0922901) and Defense Threat Reduction Agency Project BA07PRO102.

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