Microbiology

Effect of Water Washing Purification on the Surface Morphology of Bacillus cereus Spores

Jessica M. Goss1, Eric J. McCullough2, Cristina Stanciu1, Vamsi K. Yadavalli2, Christopher J. Ehrhardt1*

1Department of Forensic Science, Virginia Commonwealth University, Richmond, VA 23284, USA. 2Department of Chemical and Life Science Engineering, Virginia Commonwealth University, Richmond, VA 23284, USA

The architecture of spore surfaces plays an important role in the pathogenesis and natural ecology of many Bacillus organisms. However, changes to the laboratory preparation method for Bacillus spores may be a source of phenotypic variability in cell surfaces that can affect our understanding of in situ morphology and make cross-study comparisons more difficult. In this work, we examined how variation in the number of water washing steps during spore purification influenced the three-dimensional morphology of Bacillus cereus spore surfaces. Two strains of B. cereus, str. 14579 and T-strain, were cultured in liquid medium and purified using 0, 1, 3, or 5 water washing steps. Results showed that the nanoscale morphology of the spore surface is affected by the number of water washes such that early wash step samples had higher levels of roughness, quantified with a Root Mean Square (RMS) algorithm, which decreased with successive washes. The presence of large (>200 nm) surficial step samples also changed the nanomechanical signatures of the outermost surface but did not affect the properties of the underlying cell. This suggests that variation in purification method can affect the nanoscale morphology and mechanics and should be considered when analyzing micro- to nano-scale surface properties of Bacillus cereus spores. Journal of Nature and Science, 1(6):e116, 2015

Bacillus cereus | spore | atomic force microscopy | nanomechanical

Phenotypic profiling of organisms within the Bacillus ACT group (anthracis, cereus, thuringiensis) can be a valuable tool for investigating the natural ecology and pathogenic properties of virulent species/strains. Specific morphological features on Bacillus spore surfaces correlate well with taxonomic divisions and can differentiate closely related species (1, 2), an important goal for public health and biodefense agencies. Surface phenotypes may also indicate key aspects of the production method for Bacillus spores relevant to a microbial forensics investigation (3). Although many of the surface characteristics reported in previous studies (Table 1) are linked to intrinsic properties of the cell, spore surfaces are also dynamic features that can be affected by culturing environment and/or preparation method in the laboratory (2, 4, 5). Since the culturing protocol for spores often differs between studies and across laboratories, this may create artificial variability in surface textures that is unrelated to the underlying biology of the cell. This may complicate interpretation of surface phenotypes and make cross-study comparisons more difficult.

One particular aspect of spore preparation that varies across studies is the purification method. After culturing Bacillus in the appropriate sporulation medium, cells are typically harvested and subjected to procedures designed to remove cell debris, vegetative cells, and residual growth medium from the intact spores (6, 7). There are many established purification methods and one of the most common is washing the spores several times in ultrapure water (e.g., Table 1 and references therein). The number of successive water wash steps can range anywhere from one to more than ten. Because the overall ‘purity’ of the spore culture (i.e., the proportion of vegetative cells and/or cell debris in solution or physically associated with cell surface) changes with each wash step (6, 8), the number of water wash steps has the potential to influence surface textures of individual spore cells. While previous work has indicated a correlation between the extent of purification and the nanoscale texture of individual Bacillus spores (9), this has not been explicitly tested across multiple strains and a relevant range of wash steps.

Therefore, the goal of this study is to investigate how the surface morphology of Bacillus cereus spore surfaces is affected by the number of water washing steps used to clean the spore culture after harvesting. We use atomic force microscopy (AFM) as a tool to characterize the nano- and microscale topography of spores that have undergone different processing histories after laboratory culturing. AFM is particularly well suited for analyzing spore surfaces because it allows for high precision imaging and real-time measurement of cells without the need for destructive sample preparation steps (10, 11). Additionally, in comparison to other types of high resolution microscopic techniques (e.g., electron microscopy), morphological imaging with the AFM can be performed in conjunction with nanomechanical and biochemical analysis of the cell surface (12, 13). In this study, the nanomechanical properties of single spore cells were characterized before and after washing to further elucidate physical changes that occur with water washing purification of Bacillus cereus spores.

Experimental

Spore culture and preparation

Two different strains of Bacillus cereus were used for all experiments: Bacillus cereus T-strain (BcT) which was donated by the FBI Laboratory’s Counterterrorism and Forensic Science Research Unit (Quantico, VA) and Bacillus cereus 14579 (ATCC#14579, Manassas, VA). B. cereus was chosen because it is biochemically and structurally similar to Bacillus anthracis (14, 15) and can be manipulated at Biosafety Level 1. All cultures of Bacillus cereus were maintained at 30°C on Trypticase Soy Agar (TSA) (30 g Trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ), 15 g agar (American BioAnalytical, Natick, MA)).

Broth starter cultures were made by picking single colonies of Bacillus cereus from TSA medium and inoculating into 125 mL of Trypticase Soy Broth (TSB). Starter cultures were incubated for ~16-18 hours at 30°C and 225 rpm. To induce sporulation, either 1 mL or 20 mL of starter culture (BcT14579 and BcT, respectively) were added to 250 mL of sporulation medium. Preliminary experiments were initially conducted with Bacillus cereus spores prepared in four different medium recipes, ‘G’ Medium (16), ‘GPep’ (G Medium supplemented with 8 g L-1 of meat Peptone), GTryp (G Medium supplemented with 8 g L-1 Tryptone), and ‘GBHI’ (G Medium supplemented with 8 g L-1 Brain Heart Infusions). AFM analysis of various individual spores from each medium showed that variation in the complex nutrient source (peptone, tryptone, brain-heart, etc.) had no discernible effect on the surface morphology of the spore (data not shown). Therefore, only GTryp medium formulation was used for this study.

Sporulation cultures were incubated at 30°C and 300 rpm in an orbital shaker. The cultures were monitored throughout the incubation period and were harvested when the proportion of spores in the cell population reached ≥ 90 %. Cells were typically harvested between 24 and 48 hours depending on the strain.

Conflict of interest: No conflicts declared.

*Corresponding Author. Department of Forensic Science, Virginia Commonwealth University, 1015 Harris Hall South, Richmond, VA 23284, USA. (804)828-8420. Email: cehrhardt@vcu.edu

© 2015 by the Journal of Nature and Science (JNSCI).
After culturing, spores were harvested after 0, 1, 3, and 5 wash steps. Each wash step consisted of centrifugation of spore biomass at 3,000 x g for 15 minutes at 4 °C, decanting the supernatant and replacing it with 50 mL of cold (4 °C), ultrapure water (18 MΩ). For the zero wash step samples, a 10 mL aliquot was taken directly from the culture broth and stored at 4 °C until analysis.

Atomic Force Microscopy imaging and nanomechanics

The surface morphologies of the spores were imaged using an Asylum Research MFP-3D Atomic Force Microscope (AFM) (Asylum Research, Santa Barbara, CA) in non-contact mode. This is particularly suited for softer and more fragile cell surfaces and can reveal more surface detail (17). For the AFM study, glass slides were used as the imaging substrate. 10 µL of the spore suspension were deposited onto a glass slide cleaned with 50 mL of nanopure water, 50 mL of 100% ethanol, and dried at room temperature in a biological safety cabinet prior to imaging. Spores were imaged in air using TR-800PSA silicon nitride tips (Olympus, Japan) with a nominal force constant of 0.57 N/m and resonance frequency of 73 kHz. Height, amplitude, and phase images were collected simultaneously. Three-dimensional height and amplitude images were predominantly used for analysis and presentation. For the wash step experiments, efforts were made to image spores that were positioned on the slide with the same orientation (i.e., long axis parallel to slide) as well as individual spores that were not physically associated with other cells. Because of the variability across spore cultures in these two parameters, the number of spores imaged from each sample group varied from 8-25 cells. In order to distinguish spore samples that had been processed through a different number of wash steps, the spore surfaces were quantitatively compared on the basis of their surface topography.

AFM-based nanoindentation experiments were carried out using PPP-ZEIRH cantilevers (k~20 N/m) (Nanosensors, Neuchatel, Switzerland). Spring constants of cantilevers were measured prior to each experiment using the thermal fluctuation method (18). Stiffer cantilevers were used owing to the well-known high rigidity of bacterial spores (19). A strategy of elasticity mapping was used for these experiments. Regions containing cells were identified and indent curves were obtained by collecting a series of sequential indent curves in an m×n grid. Each indent curve was obtained at the same loading rate (300 nN/s) by indenting the cantilever to the samples until a constant load force (150 nN) was reached. All the procedures including analysis of the indent curves on the basis of the Hertz model, (20, 21) generating elasticity maps as well as the overlays of height maps and elasticity maps were carried out with Igor Pro 6.32 software. Elasticity maps were obtained by collecting 50×50 indent curves over a defined area (~3×3 µm) and estimating the Young’s modulus values.

Analysis and flattening of AFM images

The most often used parameters used to describe a surface are the average roughness (R_a) and root mean square roughness (R_m), providing a measure of the deviation of the height values across a surface. Typically, for flat samples, estimation of the roughness from the x, y and Z data is relatively straightforward, using the surface function $Z = f(x, y)$ to calculate the roughness. However, the curvature of the spore surfaces presents a complex parameter in this calculation (22). When a sample has a large curvature (for example, highly curved architectures that are spherical or fibrillar in nature), it tends to distort roughness measurements whereby small feature present on the top of the spores are masked or hidden by the large range of values (vast minimum and maximum values). This results in inaccurate RMS values and representation of the surface. To analyze the nanoscale roughness of the spore surface, we developed a methodology wherein each image was analyzed by estimating reference lines and planes (23, 24). To the best of our knowledge their application to, and effect on, micro- and nanoscale images collected by AFM for the purpose of surface roughness analysis have not been well established. Here, we describe the important considerations to be accounted for when determining the surface roughness of a curved surface. In most cases, it is not a trivial matter of using inbuilt software or simply removing the background, but an active choice of parameters to obtain an accurate representation of the surface. We describe isolating the relevant area of the surface, determining a best fit reference plane, and utilizing it for calculating the $R_{RMS}$. Subtraction of the underlying curvature due to the spherical shape of the spores then allows small surface features to be observed and accounted for in estimating the true morphology of the surface (23, 25). The techniques described will have an impact in accurately quantifying variations in surface properties and roughness at the nanoscale for highly curved cellular systems. These analyses were performed in Igor Pro 6 (WaveMetrics, Oswego, OR). Data was exported into PAST software program (26) for univariate statistical analyses. Significant differences between $R_{RMS}$ values at each wash step were assessed with a Student’s t-statistic.

Results and Discussion

The roughness of cells is an important metric that can be correlated to the purification and washing of Bacillus spores as well as other properties such as cell-cell and cell-substrate adhesion (22) or the germination state of bacteria (27). The AFM is a valuable tool in this regard owing to the ability to directly investigate the cell surface morphology in both dry and aqueous (physiological) environments (28). A brief summary of previously reported work on AFM-based characterizations of different bacterial spore systems is shown in Table 1. However, the variation of surface roughness of Bacillus spores as a function of the processing conditions has not been systematically investigated. Here, we demonstrate a flattening algorithm for highly curved spore surfaces first and then use that to obtain accurate values of $R_{RMS}$ values of two strains of B.cereus: T-strain (BcT) and 14579 (Bc14579). Next, we observe changes due to water washing. Finally, we determine the mechanical properties of the spores before and after washing and present 3D maps of the surfaces.

| Table 1. High Resolution Surface Studies of Bacillus spores. |
|-----------------|-----------------|-----------------|
| **Surface analysis** | **Organism** | **Reference** |
| Morphology and imaging | B. thuringiensis | (35) |
| B. anthracis | (37) |
| B. cereus | (2) |
| B. subtilis | (1) |
| B. cereus | (38) |
| B. atrophaeus | (5) |
| B. thuringiensis | (29) |
| B. subtilis | (13) |

Flattening of images for analysis

In order to obtain accurate quantitative metrics of spores, it is first necessary to develop suitable algorithms to analyze the surfaces. Spore surfaces are typically curved with a height difference between the highest and lowest points on the order of several micrometers, necessitating the use of flattening algorithms. In the absence of accurate flattening, a “roughness” value that is near the difference between the highest and lowest point would be obtained. However, the roughness of the cell surface if it were flattened out, would actually be different. This is because the extrema are not
making it easy to distinguish specific features on the surface. Here, we used this technique to analyze all the spores imaged under different conditions.

The surface topology is also clearer, not necessarily due to the large curvature of the cell but could arise from objects on the cell surface such as adhered debris or simply the nature of the membrane including surface receptors. In our procedure, shown in Figure 1, the spore top was isolated from the background substrate and the edges of the spore that could contain AFM-tip imaging artifacts. Flattening is performed using a least squares polynomial plane fitting, widely used for calculating reference planes (24). The order of the plane is determined by the underlying nature of the sample image and deduced mathematically by selecting the plane equation that best fits the surface. The procedure to find the best fit plane of a curved surface has been shown previously (23), but is generally applicable. The equation for the fitting plane is of the form of a three variable polynomial, \( Z(x,y) = A_0 + A_1x + A_2y + A_3x^2 + A_4xy + A_5y^2 \). Where \( n \) is the degree of the polynomial and \( A_r \) are the coefficients that need to be determined. The fit of the polynomial is checked by calculating the coefficient of determination, \( R^2 \). To fit a polynomial plane to the raw data, a series of equations, one for each height point, is solved simultaneously to determine the coefficients (23, 24). Once the underlying least squares plane (or line) equation is determined, the surface roughness can be correctly calculated. The plane is subtracted from the original data to produce a flattened representation of the data, and \( R_{A,M,S} \) is calculated by:

\[
R_{A,M,S} = \sqrt{\frac{1}{nm} \sum_{i=1}^{m} \sum_{j=1}^{n} (Z_{ij} - \bar{Z})^2}
\]

For each point, is solved simultaneously to determine the coefficients (23, 24). Once the underlying least squares plane (or line) equation is determined, the surface roughness can be correctly calculated. The plane is subtracted from the original data to produce a flattened representation of the data, and \( R_{A,M,S} \) is calculated by:

\[
R_{A,M,S} = \sqrt{\frac{1}{nm} \sum_{i=1}^{m} \sum_{j=1}^{n} (Z_{ij} - \bar{Z})^2}
\]

where \( m \) is the number of points collected in the \( x \) direction, \( n \) is the number of data points collected in the \( y \) direction and \( Z \) is the height value for each point. A second order plane was fit to the surface using the least squares method, and then subtracted from the original height data to obtain a flattened spore surface for analysis (Figure 1b). The effect on the \( R_{A,M,S} \) value is clear, as the original section image has an \( R_{A,M,S} \) of 49.6 nm while the flattened image 6.1 nm, a difference of about one order of magnitude. The surface topology is also clearer, making it easy to distinguish specific features on the surface. Here, we used this technique to analyze all the spores imaged under different conditions.

**Morphology of Bacillus cereus spore surfaces**

In this study, the surface morphology of *Bacillus cereus* spores were compared at four different points in the purification procedure corresponding to zero washes, one wash, three washes, and five washes. In general, unwashed *Bc*T and *Bc*14579 spore cells sampled directly from the culturing medium (zero wash step, ‘W0’) were irregular in shape and had several features ranging from ~20 nm to ~200 nm in size distributed over the surface (Figures 2a, 3a). Such spore samples also had poorly defined edges which prevented systematic measurements of the overall dimensions of the spore. In contrast, spore preparations washed the maximum number of times (W5) had fewer features and appeared ellipsoidal in shape with well-defined boundaries (Figure 2d, 3d). Laterally traversing ridges characteristic of *Bacillus spores* (2, 29) were observed on many of the spores washed five times and occasionally on spores after the third wash step (Figure 2c, 3c). Importantly, ridges were not apparent on any of the W0 spores. Spores from the first or the third wash step generally showed a morphology that was intermediate to spores sampled from W0 or W5. The abundance of 20-200 nm surface features noticeably decreased after the first wash step (Figure 2a and 3a compared to subsequent wash step images). Similarly, well-defined cell boundaries and ridge structures were only observed on spores in W1, W3, and W5 samples. These results were consistent across both *Bc*T and *Bc*14579.

To quantify the effect of the number of wash steps on the spore morphology, \( R_{A,M,S} \) measurements were made on the spore surfaces sampled from each wash steps following the flattening protocol discussed above. Overall, both *Bc*14579 and *Bc*T spores showed the highest average \( R_{A,M,S} \) values in W0 samples (17.4 nm and 13.1 nm, respectively). Average values then decreased incrementally after each successive wash step (Table 2). For *Bc*14579 spores, \( R_{A,M,S} \) variance remained high (~20 nm) through the first three washes then decreased to 5.6 nm in spores after the fifth wash. \( R_{A,M,S} \) Variance in *Bc*T spores was also high in zero and one wash samples (22.0 nm, 19.0 nm) but decreased to 12.4 nm and 10.8 nm in the third and fifth washes, respectively.
Figure 2. Three dimensional height AFM images of BcT spores grown in Tryptone supplemented G medium at different washing steps. (a) Spore without any washing; (b) Spore after one wash in water; (c) Spore after three washes in water; (d) Spore after five washes in water. All images 2.5 x 2.5 µm and presented to the same z-scale.

Figure 3. Three dimensional height images of Bc14579 spores across five wash steps. (a) unwashed spores, (b) one wash, (c) three washes, (d) five washes. The scale bars on each images = 0.5 µm. All spores are presented to the same z-scale.

Table 2. Statistical analysis of RMS surface values for each wash step (RMS values are in nm, variance values are in nm²).

<table>
<thead>
<tr>
<th></th>
<th>0 Washes (n=10)</th>
<th>1 Wash (n=10)</th>
<th>3 Washes (n=6)</th>
<th>5 Washes (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bc14579</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>8.8</td>
<td>7.6</td>
<td>9.2</td>
<td>9.2</td>
</tr>
<tr>
<td>Max</td>
<td>24.7</td>
<td>26.1</td>
<td>25.0</td>
<td>17.1</td>
</tr>
<tr>
<td>Mean</td>
<td>17.4</td>
<td>15.3</td>
<td>13.7</td>
<td>12.6</td>
</tr>
<tr>
<td>Variance</td>
<td>21.8</td>
<td>34.8</td>
<td>21.9</td>
<td>5.6</td>
</tr>
<tr>
<td>BcT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>4.3</td>
<td>5.8</td>
<td>6.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Max</td>
<td>23.0</td>
<td>26.3</td>
<td>22.4</td>
<td>13.3</td>
</tr>
<tr>
<td>Mean</td>
<td>13.1</td>
<td>12.2</td>
<td>11.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Variance</td>
<td>22.0</td>
<td>19.0</td>
<td>12.4</td>
<td>10.8</td>
</tr>
</tbody>
</table>
Comparisons of $R_{RMS}$ values taken from each wash step using the Student’s t-test indicated that the differences between W0 and W5 were statistically significant ($p<0.001$). Significant differences in average $R_{RMS}$ were also observed when images of all spores from early washes (W0 and W1) were combined into one group and compared to a second group containing spores images from later washes (W3 and W5). Similarly, average $R_{RMS}$ from W0 samples was significantly different from pooled data from W3 and W5 ($p<0.001$). The overall trend of decreasing RMS across successive wash steps indicates changes to the nanoscale morphology during purification. However, surface roughness of individual spores did exhibit heterogeneity throughout the wash steps as indicated by high levels of $R_{RMS}$ variance. For example, variance in Bc14579 spores was above 20 nm$^2$ through the first three wash steps and decreased to 5.6 nm$^2$ only after the fifth wash. Variance in BcT spores was also high in the first two wash steps (W0 - 22.0 nm$^2$, W1 - 19.0 nm$^2$) but decreased to 12.4 nm$^2$ and 10.8 nm$^2$ in the third and fifth washes, respectively. This suggests that some surface features are retained after the first wash step for both Bc14579 and BcT spores but are mostly removed by the fifth wash.

Although the specific chemical properties of the nanoscale surface features observed with AFM are unknown, their size and decreasing abundance through successive water washes is consistent with biological debris and fragments that are generated from cell lysis during culturing. This can occur when spores are released from the mother cell or during breakdown of dead vegetative cells or spores (8, 30). The persistence of cell debris in batch spore preparations and their subsequent removal through purification steps is well established (6, 31). However, these results suggest that this process significantly affects the nanoscale morphology of the spore surface through the third wash step.

The results also indicate that the abundance of small surface features (< 200 nm) and the associated $R_{RMS}$ values may be useful indicators for the number of wash steps used during spore preparation. Because there is significant overlap in the observed surface textures and distribution of $R_{RMS}$ values across each wash step, it is difficult to extract distinct ranges for each wash step. However, obvious visual differences and statistically significant variation in roughness was observed between spore preparations from early wash steps (W0, 1) and later steps (W3, 5) indicate a clear relationship between cell surface texture and the purification procedure on harvested spore cultures. Overall, this shows that variation across the first three wash steps can be an important source of phenotypic variability for Bacillus cereus spore surfaces at the micrometer to nanometer scale.

Nanomechanics of Bacillus cereus spore surfaces

In addition to the variation in morphology, measuring the mechanical properties of the spores is another important parameter to characterize various cellular systems (32, 33). There have been previous reports on measuring mechanical properties of bacteria using AFM based nanoindentation (12, 34). As shown above, morphology of the spore surface is observed to change on account of the cellular debris that is removed in sequential washing steps. We hypothesize that the soft external debris is likely not to affect the underlying nature of the spore itself. The unique properties of the AFM allow us to determine this progression via nanoindentation coupled with a technique of “elasticity mapping” to determine the mechanical nature of the entire spore at the nanoscale. Representative samples of spores at W0, W3, and W5 were collected and their nanomechanical distribution was obtained using elasticity mapping. The indent curves fit well to a Hertzian mechanical model, allowing us to obtain spatially resolved Young’s modulus. In each case, the mechanical elasticity map could be overlaid on the topography of the cell to show the variation of properties across the entire spore surface (Figure 4).

Figure 4 shows how the mechanical properties vary across the 3D surface of Bacillus spores. Here, we show measurements of elasticity over 2500 points over a smaller area of the cell surface in the form of a 50x50 array. The images bear some analysis as on first glance it appears as if the mechanical properties change with the wash step. However, a closer analysis reveals that each spore is surrounded by debris in the form of a thin layer. This was also seen by the imaging discussed earlier. As the AFM cantilever collects mechanical information, the debris in the unwashed samples presents as higher modulus areas owing to the probing of the
underlying substrate. Indeed, if we remove the debris and focus on the spore alone, it is seen that the modulus distributions are very similar across wash steps which confirms our hypothesis that the spore itself is unaffected by these washing protocols. The sizes of the cells are also in agreement with the imaging results presented above. Taken together, our nanoindentation data further confirm the morphology observations from the spatially observed progression of this process. It must be noted here that Bacillus spores (as indeed several cellular systems) are highly curved in nature. Therefore, an accurate determination of the exact elasticity values across the entire cell, especially at the edges is challenging. In our results therefore, there may be some errors at the edges of the cells. This may be due to two reasons: the curvature/edge effects of nanoindentation and the collapsed outer coat of sample during air drying. However, the flat center areas of the spores provide reliable elasticity values to confirm our observations. Future efforts can therefore study the endospore mechanical properties in liquid. In addition, imaging and indenting biological samples in liquid, as a unique ability of AFM compared to other conventional techniques, provides us a powerful toolkit to evaluate the spore morphology as a function of the environment and processing conditions.

**Conclusions**

The goal of this study was to determine if variation in Bacillus spore purification methods produced quantifiable morphological signatures on the cell surface. Using AFM as a sensitive, single cell technique, it is possible to investigate the nano and microscopic topographies of these bacterial spores quickly and non-destructively. Overall, the roughness and nanomechanical profiles suggest that the spore surface is affected by the purification procedure through the first five wash steps. Considering the wide range of washing methods reported for Bacillus spores, our results suggest that the purification protocol may need to be tested and/or optimized for surface studies to ensure that taxonomically- or environmentally-relevant morphological properties are being characterized. The study can be the foundation for future efforts that examine the quantitative changes to surface morphology across either wash steps or other purification procedures relevant to Bacillus spore production.

