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AFM imaging of bacteria in liquid media immobilized on gelatin coated mica surfaces

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Abstract

Immobilization of particulates, especially biomolecules and cells, onto surfaces is critical for imaging with the atomic force microscope (AFM). In this paper, gelatin coated mica surfaces are shown to be suitable for immobilizing and imaging both gram positive, *Staphylococcus aureus,* and gram negative, *Escherichia coli,* bacteria in both air and liquid environments. Gelatin coated surfaces are shown to be superior to poly-L-lysine coated surfaces that are commonly used for the immobilization of cells. This cell immobilization technique is being developed primarily for live cell imaging of *Rhodopseudomonas palustris.* The genome of *R. palustris* has been sequenced and the organism is the target of intensive studies aimed at understanding genome function. Images of *R. palustris* grown both aerobically and anaerobically in liquid media are presented. Images in liquid media show the bacteria is rod shaped and smooth while images in air show marked irregularity and folding of the surface. Significant differences in the vertical dimension are also apparent with the height of the bacteria in liquid being substantially greater than images taken in air. In air immobilized bacterial flagella are clearly seen while in liquid this structure is not visible. Additionally, significant morphological differences are observed that depend on the method of bacterial growth.

Keywords: Atomic force microscopy; Gelatin; MacMode; Bacteria; *Rhodopseudomonas palustris*; In situ microscopy; Cell immobilization

1. Introduction

Characterizing molecular interactions in the context of a live cell represents a significant challenge for next phase genomics studies. As genomics and proteomics information is collected, the questions turn towards how and where gene products interact to form metabolic and regulatory pathways. Imaging tools that can localize and/or validate molecular interactions, within the framework of the cell, will facilitate functional assignment. Complicating such studies is the

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dimensional scale of biological systems. Typical bacterial cells are on the order of a micron in diameter while eukaryotic cells can extend more than 100 μm. Molecular complexes range from approximately one nanometer to tens of nanometers in size. Therefore, imaging capabilities that span greater than five orders of magnitude are needed. Further, imaging under conditions that are compatible with the natural environment of living systems will be necessary for determining function and dynamics.

Many of these imaging requirements can be met by scanning probe microscopes. Unlike the conventional high-resolution electron microscope that must operate in vacuum, the AFM [1] uses a position sensitive probe that allows imaging in any environment. This includes liquid mediums where both composition and temperature can mimic the natural environment. Further, the AFM is capable of sub-nanometer resolution and cantilever sensitivity can be used to measure other phenomena including molecular binding [2–8], and cell surface interactions [9]. Structural characterizations of individual proteins, nucleic acids and biological complexes using atomic force microscopy (AFM) are abundant [10–12]. Further, imaging of bacteria [13–15] and cultured mammalian cells [16–18] has been demonstrated.

Applying AFM, or other types of scanning probe microscopes, to the examination of living biological cells requires a robust technique for cell immobilization. Effective immobilization techniques must position the cells such that they are stable to tip forces in liquid environments that favor viability, and ideally, they must be applicable to a range of cell types. Here, strategies involving poly-L-lysine or gelatin for immobilizing bacterial cells to mica surfaces are presented. Bacteria representative of gram positive, *Staphylococcus aureus*, and gram negative, *Escherichia coli* are examined. Additionally, images of the gram negative bacteria *R. palustris* are also presented. *R. palustris* is a photoheterotrophic bacterium that can degrade a variety of aromatic compounds and is the focus of ongoing genomic studies. http://bahama.jgi-psf.org/prod/bin/microbes/rpal/home.rpal.cgi. The clinical and environmental importance of bacterial cells, combined with their relatively small genomes, makes these organisms ideal targets for functional analysis by imaging techniques.

2. Materials and methods

2.1. Cell cultures

E. coli and *S. aureus* were grown at 37°C on standard Luria broth (LB) agar plates. *Rhodopseudomonas palustris* CGA009 was cultured anaerobically in PM, a defined mineral medium [19]. Aerobic cultures were grown in PM supplemented with 0.2% yeast extract and 0.5% casamino acids. The carbon source was succinate at a final concentration of 10 mM. Cultures were grown anaerobically in light at 30°C or aerobically at 30°C with shaking. Growth was monitored spectrophotometrically at a wavelength of 600 nm.

2.2. Preparation of mica surfaces for AFM

A 20 mm hole punch (Ralmikes TOOL-A-RAMA, South Plainfield NJ) was used to punch circular mica disks out of a flat mica sheet. The disks were then cleaved several times to obtain thin individual disks that were freshly cleaved on both sides. For coating with poly-L-lysine, the disks was incubated from 1 h to overnight in 0.01% aqueous (nanopure deionized water). Poly-L-lysine of different molecular weights were obtained from Sigma Aldrich, Saint Louis, MO and included Sigma #P6516, MW 4K–15 K, Sigma #P8920 MW 150–300 K, Sigma #P1524 (MW $\geqslant$300 K). After incubation in the solutions of poly-L-lysine, the disks were removed and allowed to dry overnight by standing on edge on a paper towel. Gelatin solutions were prepared by dissolving 0.5 g of gelatin and 10 mg of chromium ammonium sulfate in 100 ml nanopure deionized water at 60°C. Different gelatin stocks were examined including Sigma #G6144, Polysciences Inc., Warrington, PA #9000-70-8, and J. T. Baker Chemical Co., Phillipsburg, NJ #2124. After cooling to 40°C, thin freshly cleaved mica disks were vertically dipped into the solution and allowed to air dry overnight by standing on edge on a paper towel.

2.3. Sample preparation

Samples of *S. aureus* and *E. coli* were prepared by scraping a small quantity of the bacteria off of a culture plate with a sterile loop and transferring into a micro-centrifuge tube containing 150 μl of distilled water. After mixing, a 20 μl aliquot was pipetted onto a poly-L-lysine or gelatin treated mica disk and spread with the pipette tip to a diameter of roughly 5–7 mm. The sample was allowed to stand for 10 min, rinsed in deionized water, and either allowed to dry for imaging in air or covered with distilled water or media for liquid imaging. Samples of *R. palustris* grown in liquid culture media to logarithmic phase were prepared for AFM imaging by simply placing a 20 μl droplet onto a mica disk and following the procedure outlined above.

2.4. Atomic force microscopy

AFM images were recorded using a PicoSPM scanning probe microscope (Molecular Imaging Inc., Tempe, AZ) with a 100 μm scanning head. The instrument was operated in MacMode, which is a non-contact mode of operation. MacMode operates by placing a solenoid beneath the sample and establishing an AC field to excite a magnetically treated cantilever into oscillation at its resonance frequency. Proximity of the cantilever tip to the surface is identified by reduction of the oscillation amplitude. This method is especially valuable for imaging in liquids. Images were taken using a silicon cantilever with a spring constant of 0.6 N/m at a scan speed of 0.8–1.2 Hz at 256 pixels per line scan. All of the images presented in this paper were first-order flattened.

3. Results and discussion

Establishing reliable sample mounting techniques is essential for scanning probe based imaging studies. For imaging bacteria, it is desirable to avoid drying and to maintain biologically relevant conditions. For these studies, bacterial immobilization was evaluated using freshly cleaved mica surfaces that were coated with either poly-L-lysine or gelatin solutions. Cultures obtained from both liquid minimal media (*R. palustris* grown to logarithmic phase) and scraped from culture plates and suspended in deionized water (*S. aureus*, *E. coli* and *R. palustris*) were evaluated. Initial studies qualitatively tested bacterial immobilization by spreading a 20 μl drop of bacterial suspension onto different substrate surfaces. The samples were spread to a circular diameter of roughly 5–7 mm. After standing for 10 min, the samples were rinsed vigorously in a stream of deionized water and dried by a focused jet of nitrogen. If the bacteria adhered to the surface, an opaque film was clearly visible.

The results obtained for mica surfaces that were coated with different commercial preparations of either poly-L-lysine or gelatin are presented in Fig. 1. This test was used to either eliminate or qualify surfaces for further evaluation by AFM-based analyses. It is clear from Fig. 1 that rinsing in water substantially removed bacteria from some of the surfaces, while on other surfaces varying degrees of immobilization were observed. The poly-L-lysine polymers, obtained from Sigma

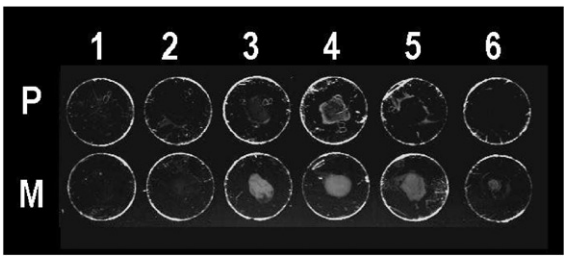

Fig. 1. Evaluation of methods used to immobilize bacteria on mica surfaces. Shown are 2 cm freshly cleaved mica disks that have been coated with either poly-L-lysine or gelatin as described in the methods. Top row P (Plate): A bacterial culture was grown on a plate, scraped into 150 ml deionized water, spotted (20 μl) onto each treated mica surface, spread to a diameter of 5–7 mm, incubated for 10 min, rinsed in a stream of distilled water for 30 s, and dried. Bottom row M (Media): 20 μl of a bacterial sample grown in minimal media was applied to each disk and processed as described above. If bacteria are immobilized on a disk an opaque spot is seen and the degree of opaqueness can be used to judge the degree of immobilization. Column 1: poly-L-lysine, Sigma #P6516 (MW 4-15 K), Column 2: poly-L-lysine, Sigma #P8920 (MW 150-300 K), Column 3: poly-L-lysine, Sigma #P1524 (MW $\geqslant$300 K), Column 4: Gelatin, Sigma #G6144, Column 5: Gelatin, Polysciences #9000-70-8, Column 6: Gelatin, Baker #2124.

Aldrich, P6516 (MW 4–15 Kd) and P8920 (MW 150–300 Kd) poorly adsorbed bacteria. Sigma P8920 is the recommended polymer for immobilizing mammalian cells on surfaces [20] but appears unsuitable for holding bacteria on surfaces. The 300 Kd poly-L-lysine polymer (P1524) showed improved affinity for immobilizing bacteria from minimal media cultures but only weak affinity when immobilizing bacteria that were removed from culture plates and subsequently suspended in deionized water.

Surfaces coated with Sigma gelatin (G6144) gave the best results of the three gelatin surfaces that were tested. As seen in Fig. 1, cells from the minimal media culture are immobilized to a greater extent than are cells scraped from plates and suspended in deionized water. The poorest results were recorded with gelatin obtained from J. T. Baker (2124) where little to no bacterial immobilization was evident. With gelatin from Polysciences (9000-70-8), relatively moderate immobilization was obtained with bacteria cultured in media while poor immobilization was observed for bacteria derived from culture plates.

In general, complex media (those that contain multiple carbon sources) tended to interfere with bacterial immobilization onto all surfaces (data not shown). This may be due to competition for surface binding sites due to undetermined media components, such as yeast extract. The presence of these, or similar, media components in bacterial samples derived from cultures plates may explain the relatively lower immobilization efficiency when compared to similar samples derived from minimal media. Additionally, the bacterial surface structure may be different when either grown under or treated under different conditions [21,22].

In Fig. 2, AFM images of both gram positive and gram negative bacteria immobilized on Sigma G6144 gelatin and imaged in liquid media are shown. The images were recorded on a PicoSPM scanning probe microscope operating in the non-contact MacMode configuration, where cantilever resonance is achieved by magnetically exciting the cantilever. A dampening of the oscillation amplitude identifies proximity to the sample. MacMode has been found to be superior to acoustic excitation of the cantilever for imaging in liquids [14] presumably because only the cantilever, and not the cantilever assembly, is excited. Therefore, there is less acoustic disturbance of the liquid.

In Fig. 2A and B, the images of *S. aureus* and *E. coli* were taken from bacterial cultures harvested from plates and imaged in minimal media while the image of *R. palustris* was made from a culture grown in liquid minimal media. Although bacteria isolated from plates gave universally poor results in the qualitative tests discussed above (Fig. 1), bacterial images comparable to those derived from liquid media were obtained by AFM. In all cases, the bacteria maintain a hydrated appearance with no evidence of collapse of the cell wall. The images of both *S. aureus* (Fig. 2A) and *E. coli* (Fig. 2B) show relatively dispersed bacteria when compared to the image of *R. palustris* (Fig. 2C). The tightly packed bacteria in this latter image may be due to differences in the concentration of the spotted cultures. Nevertheless, the repeated imaging of an isolated bacterium serves as a real test of the immobilization technique. Packing of bacteria leads to an increased contact area and may contribute to greater collective stability. In all cases examined attachment was robust enough to allow individual bacterium to be repeatedly imaged.

In higher resolution images under liquid, *R. palustris* appears to have a smooth outer surface with no apparent ultrastructural features (Fig. 3). Similar results were obtained for cultures grown either aerobically or anaerobically (data not shown). In contrast, images in air are dramatically different. As seen in Fig. 4, bacteria imaged in air have a flattened scalloped shape with raised edges. These features are likely due to dehydration effects and were observed for cultures grown either aerobically or anaerobically. Additionally, bacterial flagella are seen when imaged in air. These structures are especially evident in the amplitude image. The absence of flagella in the under liquid images indicate that the flagella may not be rigidly immobilized. Forces exerted by the scanning probe tip may be repositioning this structure. Alternatively, the resolution in the under liquid images may be lower due to interactions between the tip and bacterial surface structures [14,22].

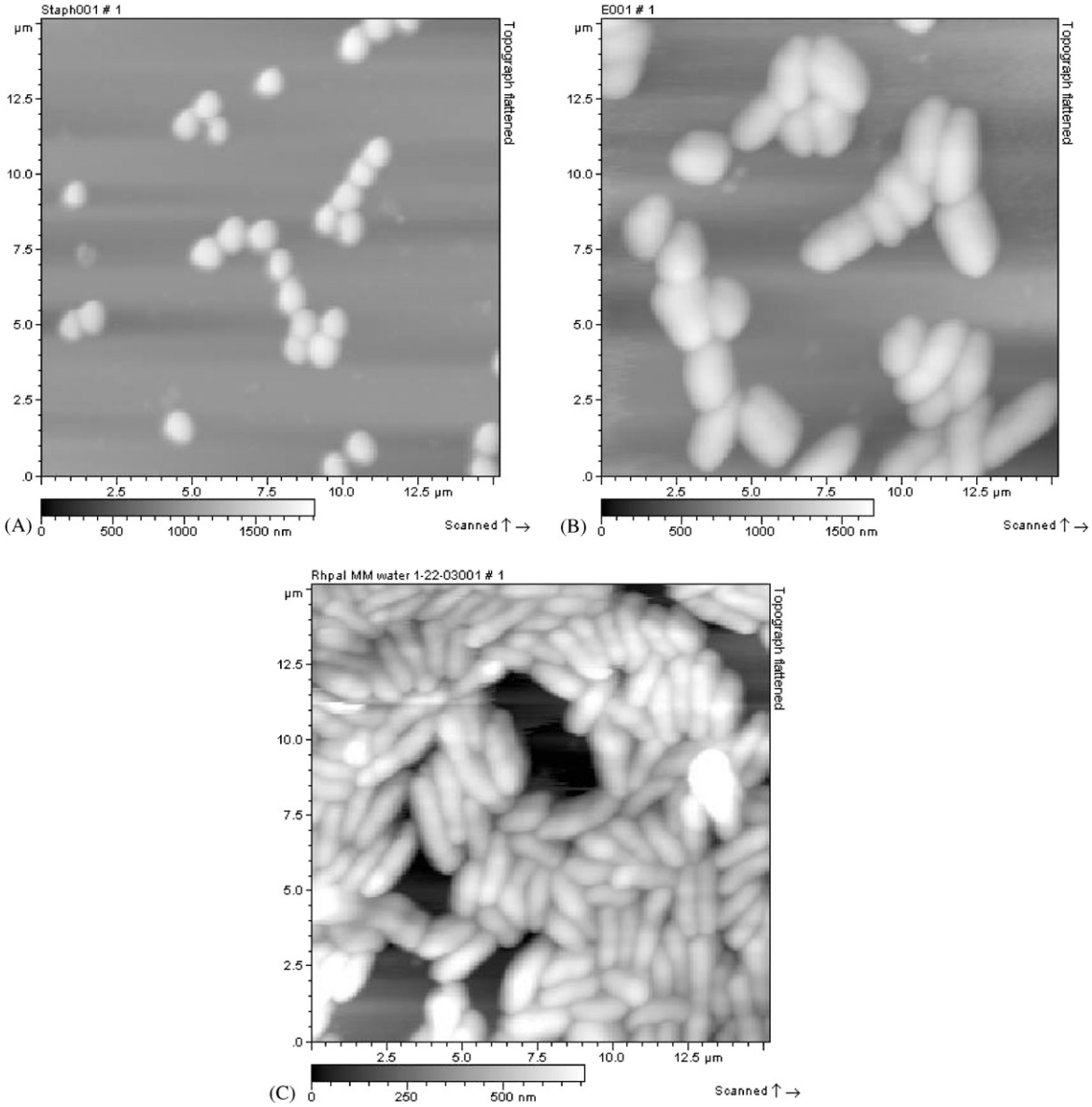

Fig. 2. Bacteria immobilized on a surface and imaged in liquid media. Gram positive *S. aureus* (A) and gram negative *E. coli* (B) bacteria were grown on culture plates and suspended in minimal media while *R. palustris* (C) was grown in minimal media. The bacterial samples were immobilized on Sigma gelatin (G6144) coated mica surfaces and imaged in minimal media with a PicoSPM operating in MacMode at minimal force using silicon cantilevers with spring constant of 0.6 N/m. Images were taken at the same magnification (15 μm^2), at a scan rate of 0.8–1.0 Hz, and were first-order flattened. In all of the images, the bacteria appear to be hydrated and the outer surface appears smooth and without apparent ultrastructure.

In contrast to the smooth bacterial surfaces seen in liquid media grown bacteria, the bacteria prepared from culture plates and imaged in air show increased surface ultrastructure and a distinctly different overall morphology. Fig. 5 shows *R. palustris* imaged under these conditions. In this image, the bacterium appears to maintain a rigid shape with height measurements comparable

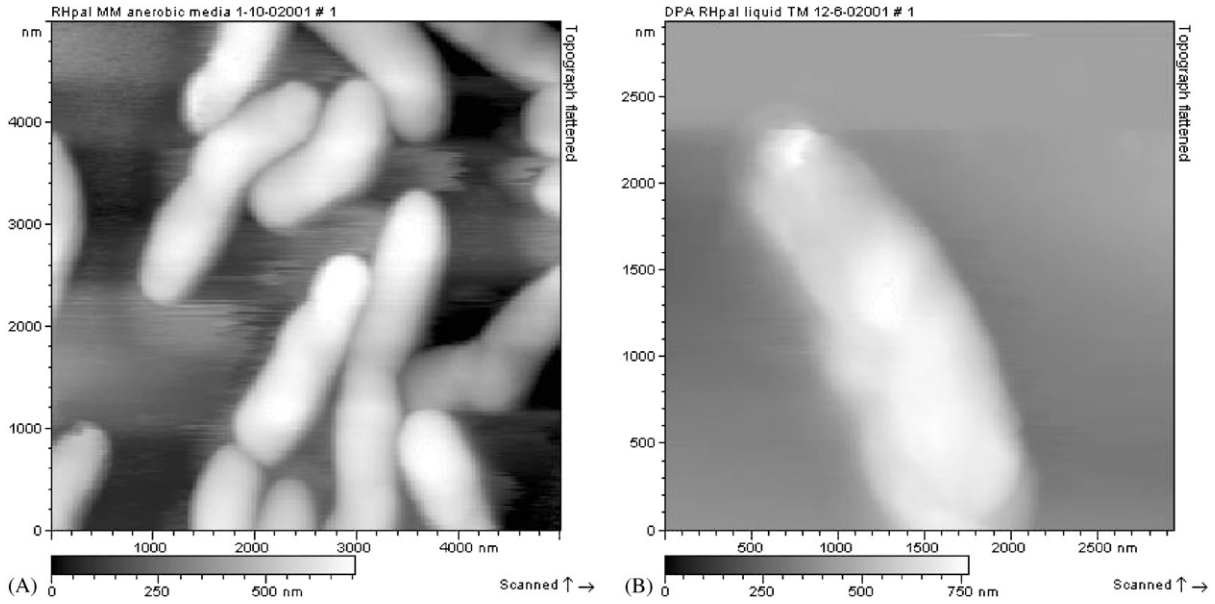

Fig. 3. Higher resolution images of *R. palustris* taken with the same imaging parameters used in Fig. 2. Note that at higher magnification there is still no evidence of changes in surface ultrastructure from the smooth appearance that was observed in Fig. 2. Also, the image shown in 3B was imaged several times without any apparent loss in resolution or any apparent destruction of the bacterium. Image magnification is shown.

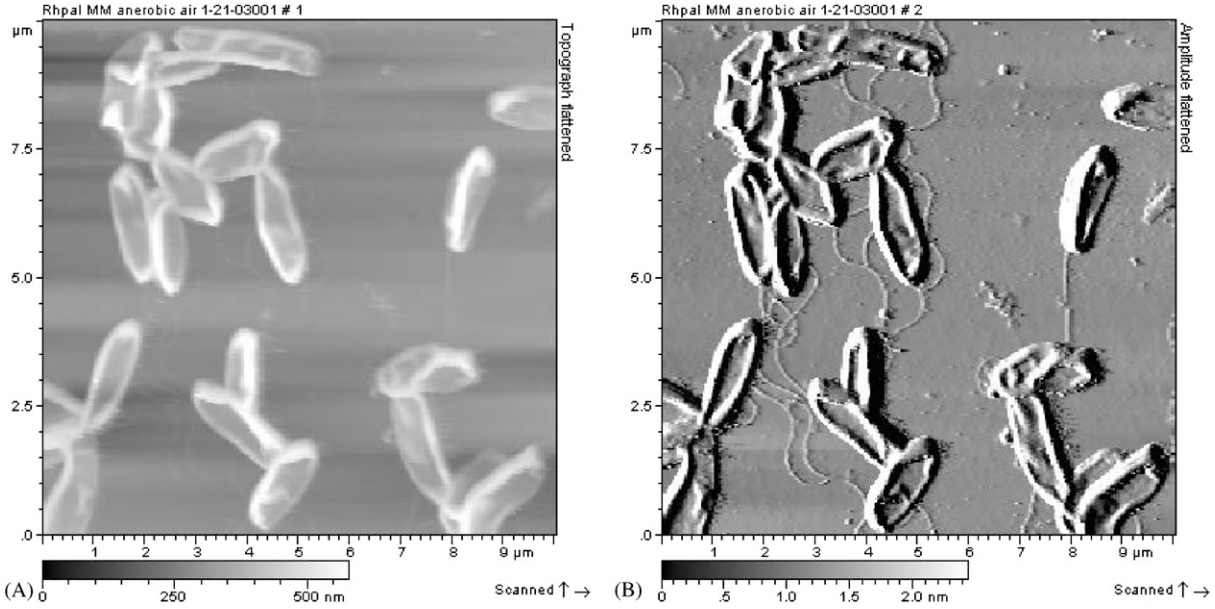

Fig. 4. AFM images taken in air of *R. palustris* that were grown in minimal media, and applied to Sigma (G6144) gelatin coated mica surfaces as described in the methods. These images show a marked collapse of the cell structure. Although the image presented in the figure was made from a culture grown anaerobically, no differences were found when imaging aerobically grown cultures. Note that flagella are present in cultures imaged in air while no evidence of flagella is observed when bacteria are imaged in liquid. The parameters used for imaging in air were the same as those used for imaging in liquid with the exception that the scan rate was increased to 1.28 Hz.

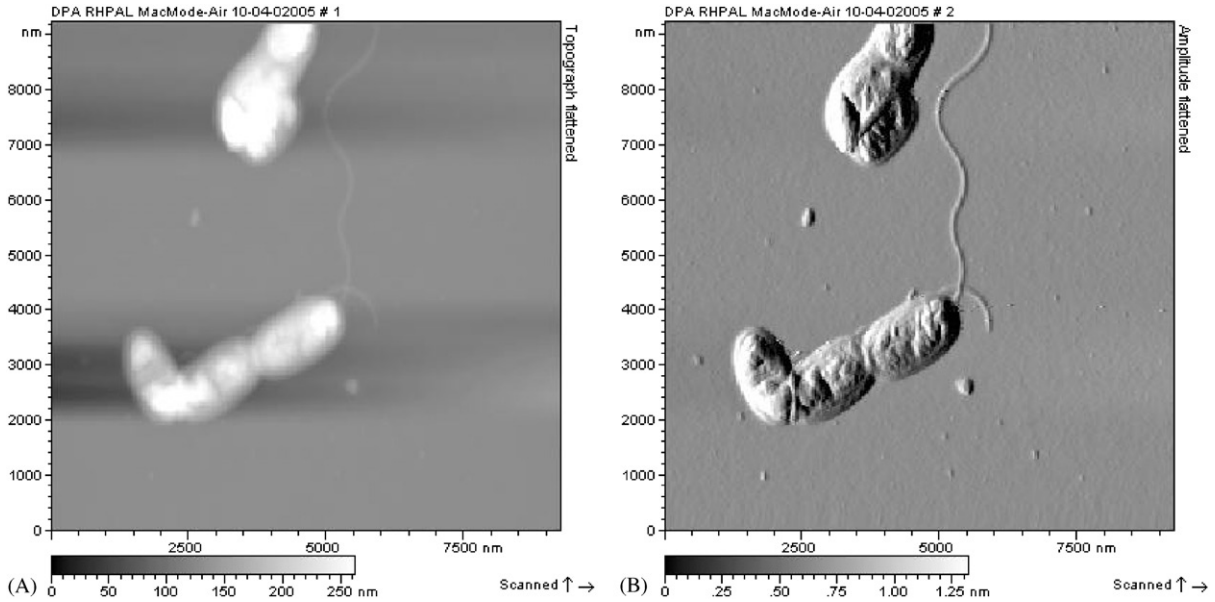

Fig. 5. An AFM image of *R. palustris* prepared by scraping a quantity of the bacteria from a culture plate, suspending in water, and placing on a Sigma gelatin (G6144) coated mica surface as described in the methods. A slightly wrinkled appearance of the bacterial surface is observed which is possibly due to dehydration or the presence of budding structures. The overall morphology and surface details are significantly different from the bacteria presented in Fig. 4. Flagella are clearly present in the amplitude image.

to those obtained from bacteria imaged under solution. This is unlike the flattened appearance of these bacteria when grown in liquid minimal media and imaged in air (Fig. 4). The origin of these structural differences may be due to changes in the rigidity or hydration state of the cell wall. However, this is currently speculation and will require further study.

4. Conclusion

A reliable technique for immobilizing bacterial cells has been established. The technique employs gelatin coated mica surfaces and has been demonstrated to be superior to poly-L-lysine coated surfaces. The immobilization strategy appears general and has been effective in immobilizing *S. aureus,* a gram positive bacteria, and *E. coli* and *R. palustris*, which are gram negative bacteria. Significantly, the technique is compatible with AFM imaging under solution or in air, allowing for repeated imaging of individual bacteria. Initial examinations of *R. palustris* have demonstrated significant morphological differences that depend on the bacterial growth conditions and imaging environment. Bacteria imaged in liquid appeared to have smooth surfaces while images in air showed different structures, including flagella, and apparently greater resolution of cell surface structures.

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