Observation of Changes in Bacterial Cell Morphology Using Tapping Mode Atomic Force Microscopy

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Atomic force microscopy (AFM) was used in tapping mode to probe morphological changes in surface-confined cells resulting from adhesion-modifying chemicals. Bacteria (Burkholderia cepacia G4 and Pseudomonas stutzeri KC) were exposed to Tween 20, heparin, disodium tetraborate, sodium pyrophosphate, low ionic strength water, lysozyme/ethylenediaminetetraacetic acid (EDTA), and 3-(4-morpholinol)-propanesulfonic acid sodium salt (MOPS) buffer (as a control), and the surface topography of the cells was examined after exposure to each chemical. Cells were attached to glass slides for AFM imaging by a new method of cross-linking carboxyl groups on the bacterial surfaces with amine groups that had been coupled to glass slides. Topographic images, phase images, traces of surface topography, and analyses of surface roughness were performed on all samples. We are not aware of any studies besides the present one in which phase imaging has been used on bacteria. Height traces illustrated the effect of different chemical treatments on the cells by showing whether the topography of the cell was altered by the different treatments. The surface roughness was quantified in terms of the root-mean-square (RMS) average of the height deviations. All of the treatment chemicals except disodium tetraborate caused higher RMS values to be measured for G4 and KC. Disodium tetraborate flattened the cells and, therefore, resulted in slightly lower or equal RMS values as the control (MOPS buffer). RMS values were correlated with the qualitative shapes of the cells. Lysisocyte/EDTA, sodium pyrophosphate, and disodium tetraborate produced the most damage to cellular morphology, as observed by topographic images and surface traces, and decreased cellular viability. These data show that AFM operated in tapping mode can provide a useful method for investigating the consequences of bacterial exposure to surface-modifying chemicals.

Introduction

Chemicals have been used to alter the adhesion of bacteria to surfaces,1—3 yet most of these chemicals have not been examined for their effect on cellular morphology. The atomic force microscope (AFM) is an ideal tool for determining changes in cellular morphology. AFM imaging can be performed in contact or tapping mode. In contact mode, the tip is dragged across the sample surface and maps of surface topography can be constructed by monitoring tip deflection. Biopolymers,4 biofilms,5 yeast cells,6,7 proteins,8 and bacteria9,10—12 have all been imaged using contact mode AFM. In tapping mode, the tip makes intermittent contact with the sample as the tip is oscillated near its resonance frequency. Two advantages of tapping mode are that the sample is less likely to be damaged by the tip and that lateral forces are greatly reduced. The accuracy and reproducibility of tapping mode height profiles for many kinds of samples have been verified by comparison of AFM images with TEM or other types of electron microscopy for gold nanoparticles, polysaccharides, and humic substances.14—16 Despite these advantages, the only study in which tapping mode AFM was used to image bacteria is that of Grantham and Dow7 although other types of cells have been examined in tapping mode.18 It is possible to probe bacterial surfaces in air or in liquid. Preparation of bacteria for air imaging is easier, and air imaging is able to reveal the overall morphology of cell surfaces. Imaging in liquid is more difficult because the cells often fail to adhere strongly enough to the substrate and can be displaced easily.15,19,20 Scanning probe measurements of many biological samples have success-
fully been performed in air, but only in contact and not tapping mode. For example, Butt et al.10 imaged Halo-
bacterium halobium in air and were able to resolve features as small as 10 nm, although they did not explicitly identify
these features. Gunning et al.11 examined Pseudomonas putida biofilms in air to determine their morphology. Changes in cell surfaces resulting from a single treatment have been reported. For example, Kasas et al.11 studied
the morphology of Escherichia coli as small as 10 nm, although they did not explicitly identify
bacterium halobium have been reported. For example, Kasas et al.11 studied
Changes in cell surfaces resulting from a single treatment
putida biofilms in air to determine their morphology.

**Table 1. Summary of Chemical Treatment Conditions**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Buffer Conc</th>
<th>Final Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>disodium tetraborate</td>
<td>0.01 M MOPS</td>
<td>1 mM</td>
</tr>
<tr>
<td>heparin</td>
<td>0.01 M MOPS</td>
<td>1 g/L</td>
</tr>
<tr>
<td>low IS water</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>lysozyme/EDTA</td>
<td>0.01 M MOPS</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>(lysozyme)</td>
<td></td>
<td>(EDTA)</td>
</tr>
<tr>
<td>MOPS buffer</td>
<td>0.07 M mophosphate buffer</td>
<td>0.1 mg/mL</td>
</tr>
<tr>
<td>proteinase K</td>
<td></td>
<td>0.1 M</td>
</tr>
<tr>
<td>sodium pyrophosphate</td>
<td>0.01 M MOPS</td>
<td>1 mM</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.07 M mophosphate buffer</td>
<td>0.1% v/v</td>
</tr>
</tbody>
</table>

G4 cultures were grown in M9 buffer containing 20 mM lactate and a mineral salt solution,26,27 while KC was grown in modified medium D containing 3.0 g/L sodium acetate amended with trace nutrients22 (TN2 solution). The media for KC was prepared as described previously22 except that the concentration of trace nutrients was increased by an order-of-magnitude and sterife-
filtrated instead of autoclaved so that a precipitate would not form.

**Chemical Treatments for AFM Studies.** Chemicals were chosen for their varying effects on bacteria. MOPS buffer is a control and did not adversely affect cell morphology. Low ionic strength (IS) water has been used without causing damage to cells in bacterial adhesion studies.28 Lysozyme/EDTA and disodium tetraborate each denature lipopolysaccharides. Heparin and sodium pyrophosphate are large polyanion chains that can adsorb irreversibly to cells and make the cell surface charge appear more negative.9 Proteinase K is an enzyme that causes
cells to release proteins by cleaving peptide bonds.

After growing to mid-log phase in their growth media, cells were diluted to ~10^7 cells/mL, as determined by acridine orange counting,29 and resuspended in 0.1 M 3-(4-morpholinopropyl)-sulfonate sodium salt (MOPS; pH = 8.6) buffer. To remove the growth media from the suspension and resuspend the bacteria in the desired chemical solution (Table 1), cells were captured on a 0.2-µm syringe filter (Acrodisc). A few milliliters of Milli-Q water was passed through the filter to remove any components of the media or extracellular material that may have been present. The filter was reversed and attached to a new syringe containing the desired suspension media, and filter contents were back-
washed into that suspension (10 mL) and placed in 25-mL scintillation vials. This procedure is useful for working with low concentrations of bacteria, where centrifuging and resuspending the cells might be difficult if a visible pellet does not form. After
the contents of the vials were mixed on a shaker table for 45 min, the cells were resuspended into 0.1 M MOPS by the same filtration procedure to remove the treatment chemical (so that only irreversible effects on the cells were studied), and the cells were bonded to glass slides as described below. A viability assay was performed on cells exposed to these chemical treatments. Cells were stained with a green fluorescent nucleic acid stain (SYTO 9) for detecting live cells and a red fluorescent nucleic acid stain (propidium iodide) for detecting dead cells as part of a viability kit20 (LIVE/DEAD BacLight; Molecular Probes). Stained cells were mounted on black membrane filters (Poretics) and enumerated under blue light using a microscope.

**Experimental Section**

**Chemicals.** EDC [1-(3-dimethylaminopropyl-3-ethylcarbo-
diimide) was obtained from Aldrich. Sulfo-NHS (hydroxysulfo-
succinimide) was obtained from Pierce. The biological growth
buffer, MOPS [3-(4-morpholinopropyl)sulfonate sodium salt] was purchased from Calbiochem. Chemicals were used as received
from the manufacturer. Some chemical solutions (as indicated in Table 1) were prepared in a phosphate buffer, which consisted of (per L of Milli-Q water (Millipore Corp.)): 0.51 g of KH_2PO_4, 0.52 g of K_2HPO_4, 0.147 g of CaCl_2.7H_2O.

**Cultures.** Two bacterial strains that have been extensively investigated for use in bioremediation,22-24 Burkholderia cepacia


G4 and Pseudomonas stutzeri KC, were used in most experiments. G4, provided by D. F. Dwyer (Department of Civil Engineering, The University of Minnesota), is able to cometabolically degrade TCE in an appropriate growth environment.24 KC, provided by C. S. Criddle (Department of Civil and Environmental Engineering, Stanford University) can reductively dehalogenate carbon tetrachloride under appropriate conditions.24 Pseudo-
monas fluorescens P17 was used in preliminary studies of sample preparation techniques. Its growth conditions and properties have been described previously.25

**Covalent Bonding of Bacteria to Slides.** The slides were rigorously cleaned,31 rinsed with ultrapure water (Milli-Q), and stored at room temperature in a beaker of ultrapure water. Slides were treated with amine solutions so that the base of the amine could be coupled to the amino group, using a modified procedure that Grabar et al.14 developed to study the self-assembly of gold
Table 2. Effects of Chemical Treatments on Bacteria

<table>
<thead>
<tr>
<th>chemical</th>
<th>chemical properties</th>
<th>% viability (G4)</th>
<th>% viability (KC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>disodium tetraborate</td>
<td>denatures polysaccharides</td>
<td>50</td>
<td>31</td>
</tr>
<tr>
<td>heparin</td>
<td>polysaccharide that attaches to positively charged amino groups on cell</td>
<td>&gt;95, N</td>
<td>&gt;95</td>
</tr>
<tr>
<td>low IS water</td>
<td>increases electrostatic repulsive barrier around cell, may lyse some cells</td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>N-acetyl-l-glucosamine/EDTA</td>
<td>hydrolyses polysaccharides</td>
<td>&lt;1, N</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MOPS (Control)</td>
<td></td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>proteinase K</td>
<td>cleaves peptide bonds</td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>sodium pyrophosphate</td>
<td>large polyanion chain which sorbs to cell, increasing overall negative charge of cell</td>
<td>39, N</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tween 20 in phosphate buffer</td>
<td>reduces hydrophobicity, may provide steric barrier to attachment</td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>EDC/NHS</td>
<td></td>
<td>91</td>
<td>85</td>
</tr>
</tbody>
</table>

(N) = no cells were found using AFM.

Cells, slides. Slides were soaked for 10 min in a 10% silane solution (either 3-aminopropyltrimethoxysilane or 3-aminopropyltrimethoxysilane, United Chemical Technologies) in distilled quality methanol (EM Science) and then rinsed with copious amounts of methanol (at least 50 mL per slide) followed by Milli-Q water to remove excess silane.

Bacteria were coupled to amino groups of the aminosilane compounds with a protein–protein cross-linking reaction. Carboxyl groups on the bacterial surfaces react with a pH 5.5 solution of EDC (31,32) (1-(3-dimethylaminopropyl-3-ethylcarbodiimide) to form an unstable intermediate, an O-acylisourea. A pH 7.5 solution of sulfo-NHS (hydroxysulfosuccinimide) reacts with this intermediate and then undergoes nucleophilic substitution by the amine group of the amosilane compound. The combination of bacteria in MOPS (buffer only, no carbon source or nutrients), EDC, and NHS was added to cleaned, silanized slides in Petri dishes and allowed to shake (125 rpm) overnight. It was necessary to allow the reaction to proceed for 6–8 h in order for the reaction to work at near-neutral pH. If a lower pH was used, the bonding reaction would be quicker, but we did not want to risk damaging the cells. Samples were kept hydrated while shaking and then air-dried by gently blowing air across the glass slide. Slides were imaged immediately after being removed from the liquid media, but since the imaging process can take several hours, the slides may have air-dried for an additional period of several hours. Previous studies suggest that AFM imaging under ambient conditions allows organic macromolecules, such as polysaccharides to retain their water of hydration. 15,19 The bacterial viability kit was also used on EDC/NHS treated cells to ensure that this technique was not damaging to the bacteria.

Other Preparation Methods to Attach Bacteria to Slides. Pseudomonas flourescens P 17 was deposited onto 0.2-μm filters (Poretics). The filter was placed on a vacuum filtration flask, the cells, suspended in Milli-Q water, were added to the top of the filter, and a vacuum was applied for ~10 s. P 17 was also attached through electrostatic interactions (physical adsortion) by placing in contact with a glass slide or piece of silica that had been coated with poly-L-lysine hydrobromide, a positively charged compound. 31 After cleaning the slide with methanol and Milli-Q water, a drop of 0.01% (wt/vol) poly-L-lysine hydrobromide solution was added. When dry, the slide was rinsed with Milli-Q water and dipped into the bacterial solution (with and without a formaldehyde/glutaraldehyde fixation).

AFM experiments. Samples were analyzed using an AFM (BioScope, Digital Instruments, DI) mounted on an inverted light microscope (Zeiss), in tapping mode using TESP tips specially designed for tapping mode (DI). Tips were 125 μm long and had typical resonant frequencies between 294 and 375 kHz. Another AFM (Multimode III, DI) was used in some of the preliminary experiments with the same type of tips and operated in the same manner. “Light” tapping was used, which involved maintaining a high amplitude set point relative to the free amplitude of the cantilever. Typically, we began by scanning a 15 μm x 15 μm area that would contain several bacterial cells. Gradually, the image size was reduced to isolate individual cells. Bacteria were scanned in both directions several times before capturing an image, to help ensure that tip artifacts, such as hysteresis, were not altering the images. At least two and up to six high-quality images were captured for each type of treatment, and one image was selected from these. Tips were replaced frequently, or when there was any indication of artifacts present in the images. After images were collected, an offline section analysis was performed on each image in order to gain information on sample topography. When a line was drawn across the image, the topography of the sample as a function of distance was displayed. These height traces were performed to provide more information on how each treatment affected the surface topography. Roughness analyses were also performed on some samples, conducted according to the manufacturer’s software program. The root-mean-square (RMS) average of the surface roughness value was calculated as the standard deviation of all the height values within the given area.

Phase images were captured simultaneously with height or topographic images. In phase imaging, the phase shift of the oscillating cantilever is measured as a function of tip position on the surface. Height images reveal surface topography and are more accurate in detailing the height of features on the surface. Phase images have proved to yield better resolution of surface features, 32 but since the different factors that cause phase shift cannot be separated, interpretation of phase images can be much more difficult. Phase imaging is useful as a complement to topographic imaging for providing information on sample heterogeneity.

Results

Chemical bonding of bacteria to silanized glass slides permitted AFM imaging of bacterial cells to be performed in air without moving cells during the scanning process for most chemically modified bacteria. Cells attached to poly-L-lysine coated slides or silica could not be imaged because the cells did not adhere strongly enough to the substrate. Bacteria could be deposited on a filter, but extracellular polysaccharides obscured the images of the cells. In addition, filtered cells appeared flattened as a result of the applied vacuum needed to pull the cells onto the filter.

Most bacteria remained viable following chemical treatments. However, disodium tetraborate, heparin/EDTA, and sodium pyrophosphate each reduced cell viability to <50% of the nontreated controls, and in several cases to <1% (Table 2). The cross-linking compounds (EDC/NHS) that were used to attach bacteria to the slide

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(33) Baker, G. Oklahoma State University College of Osteopathic Medicine. Personal communication.
did not appreciably affect bacterial viability, with viabilities of 85% and 91% for G4 and KC, respectively.

**Surface Topography after Chemical Treatment.**

Cells exposed only to MOPS buffer displayed reproducible surface traces and appeared undamaged, as shown in Figure 1 for KC. Several cells were examined, and although some variations exist in the exact size and height of surface features, the surface traces are reproducible. On the basis of the good reproducibility of the images as shown by the height profiles in Figure 1C, only a single image is shown for subsequent chemical treatments.

Cells in MOPS buffer are undamaged and serve as controls. When cells of KC were exposed to different chemical treatments, the cell morphology was affected (Figure 2). Low IS water (Figure 2B) caused the surface of KC to be more irregular than the surface exposed only to MOPS buffer, but the overall height and shape of the cells were maintained in low IS water. Disodium tetraborate (Figure 2C), sodium pyrophosphate (Figure 2D), and the combination of lysozyme/EDTA (Figure 2E) each severely damaged KC at the concentrations tested. Proteinase K (Figure 2F) did not damage KC.

For G4, there was little cellular topographic damage observed for most of the chemical treatments where attachment to the slide was successful (Tween, low IS water, proteinase K, Figure 3; also MOPS which is not shown). For disodium tetraborate, an image of a damaged cell was obtained (Figure 3C). For the cases where G4 was treated with heparin, sodium pyrophosphate, and lysozyme/EDTA, no cells could be found for AFM imaging.

Acridine orange (AO) stains were performed on treated cells to distinguish between chemical treatments that harmed the cells and treatments that interfered with the attachment of the cell to the slide. At least some G4 cells in heparin and sodium pyrophosphate were apparently undamaged, as confirmed by AO stains and the viability assay (Figure 4, Table 2), although they could not be imaged with AFM. However, G4 could not be imaged with AFM or AO staining after exposure to lysozyme/EDTA. The cells found after AO staining were obviously damaged and/or coagulated (Figure 4D). For sodium pyrophosphate, the viability was low (39%) and so it is not known if the cells shown in Figure 4C were viable at the time of slide preparation as AO staining does not distinguish between live and dead cells.

The chemicals that had the most noticeable effects on KC were lysozyme/EDTA and disodium tetraborate, each of which caused a substantial reduction in the surface height and irregularity in the cell surfaces (Figure 5A). KC exposed to Tween 20 shows a similar distribution in height profile as KC in MOPS; however, KC exposed to Tween 20 is taller by more than 100 nm. Since the linear dimension of Tween 20 is < 3 nm, it is not likely that this difference in height was due to adsorption of the surfactant.

For G4, disodium tetraborate caused a flattening in the surface trace (Figure 5B). Low IS water, Tween 20, MOPS, and proteinase K each resulted in similarly shaped height traces for G4.

All of the treatments except disodium tetraborate resulted in higher RMS values for both KC and G4 (Table...
3), indicating surfaces were made more rough or irregular by the chemicals. The lower RMS values for disodium tetraborate are probably indicative of flattening of the cells' surfaces, which was also apparent in the topographic images.

**Phase Images.** The phase signal is useful in providing information on sample heterogeneity. The phase signal is a measure of energy dissipation, but depending on the operating regime, different factors can exert more of an influence on the phase signal. In light tapping (as was used here), the phase signal is most likely related to adhesion and long-range tip–sample interactions, while in hard tapping, the signal is dominated by elastic properties. Our control phase image of KC in MOPS (Figure 6A) showed surface features on the scale of 50–200 nm. After exposure to disodium tetraborate, the overall height of KC was substantially decreased (Figure 5), but the surface structures were still present in the phase image (Figure 6B). Also, there is more contrast in the phase image, indicating more sample heterogeneity. Heparin and Tween 20 each had unusual effects on the cell surface chemistry. After exposure to heparin (Figure 6C), the phase image of KC showed no contrast, indicating a uniform surface. The cell exposed to Tween 20 (Figure 6D) was heterogeneous in composition and showed the presence of a corona region around the cell of a different composition than either the cell or the glass slide.

**Discussion**

**Cell Preparation.** If cells are not firmly attached to surfaces, they may move and disappear from the viewing area after AFM imaging has begun. At a low

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(37) Stemmer, A.; Engel, A. Ultramicroscopy 1990, 34, 129.
attachment rate, there may not be enough cells to make it likely that a typical scan size of 10–15 μm will include a cell. Kasas et al.38 found that for cells that were known to grow in monolayers on glass in the presence of culture media, 3 of 20 disappeared after the first scan because they did not adhere strongly enough.

Figure 3. Topography of G4 cells exposed to (A) Tween 20, (B) low ionic strength water, (C) disodium tetraborate, and (D) proteinase K. No images could be obtained for G4 in lysozyme/EDTA, heparin, and sodium pyrophosphate.

Figure 4. Acridine orange stains of G4 exposed to (A) low IS water, (B) heparin, (C) sodium pyrophosphate, and (D) lysozyme/EDTA. Images were captured using a digital camera (Pixera) connected to a fluorescent microscope under blue light at 1000×.

A variety of methods have been used to prepare cells for AFM imaging, including trapping living cells in filters, capturing growing cells in agar, allowing cells to colonize a surface such as copper or mica, or TEM because the latter did not retain its water of hydration. Also, AFM studies that have been done on natural organic matter with that of a polymer, poly(acrylic acid), that could not be imaged accurately by either AFM or TEM because the latter did not retain its water of hydration. Also, AFM studies that have been done on bacterial surfaces.

In general, the bacterial cells we observed were not smooth but contained spheroidal structures ranging from 20 to 200 nm. Regardless of whether cells were deposited on a filter (with and without the application of vacuum for drying) or immobilized using the EDC/NHS reaction, these structures were always observed and have spots appeared to correspond to the size of the pores in the filter (the pores are clearly visible in the upper portion of Figure 7D), which led us to presume that the cells were sinking into holes in the filter. This limitation would have to be overcome before cells could be successfully imaged on filters.

In several studies biological materials have been prepared (bacterial surface proteins, archaebacteria, red and white blood cells, plant cells, eubacteria) by allowing the material to evaporate and form a film on glass slides or coverslips. For our bacteria, allowing cells to air-dry on a slide did not adhere the cells strongly enough to the glass, and so AFM imaging was not possible.

Other methods for preparing cells have relied on electrostatic interactions or covalent bonds to hold cells to the slides, as we have done in this study. Nermut succeeded at attaching bacteria to (positively charged) poly-L-lysine-coated surfaces. He also used a technique for coating binding of cell surface proteins to gullaraldehyde coated supports or to supports coated with a specific ligand on a silanized glass slide. This “cell monolayer technique” has been used to prepare bacteria for ultrastructural studies using scanning electron microscopy. A very effective two-step reaction for cross-linking proteins using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) was later developed with the advantage that this technique can be used in multicomponent systems when reagents such as EDO are present.

It is difficult to avoid contamination of the tip by sample material when the sample is a bacterial cell. Many other researchers have experienced adsorption of biological sample material onto the tip, with the result being poorer quality images or measurements of high surface forces. Grantham and Dove observed AFM tips under an optical microscope after performing imaging on Shewanella putrefaciens in air. The bacteria were seen to adsorb to the cantilever and thus obscured the image quality that was obtainable with the AFM. In our study, the image quality was closely monitored, and if broadening in the image appeared, the tip was immediately replaced.

It is possible that performing AFM work in air will produce artifacts due to the dehydration of the bacteria. However, many studies have shown that hygroscopic samples, such as plant cell walls, polysaccharides, and natural organic matter retain enough of their hydration water to be imaged with the AFM reproducibly and accurately for several hours. Wilkinson et al. compared AFM and TEM imaging of polysaccharides and natural organic matter with that of a polymer, poly(acrylic acid), that could not be imaged accurately by either AFM or TEM because the latter did not retain its water of hydration. Also, AFM studies that have been done on bacterial surfaces in liquids produce contour images that are very similar to the tapping mode (air) images that we obtain and show the same types of profiles and structures on the bacterial surfaces.

Morphology. In general, the bacterial cells we observed that occasionally the cells seemed highly compressed and sunk into holes in the filter (Figure 7D). For all the bacteria we studied with this preparation technique, dark spots in the topographic images were observed, which indicated a very low surface height. The size of these dark spots appeared to correspond to the size of the pores in the filter (the pores are clearly visible in the upper portion of Figure 7D), which led us to presume that the cells were sinking into holes in the filter. This limitation would have to be overcome before cells could be successfully imaged on filters.

Another artifact of depositing bacteria on a filter was that occasionally the cells were deposited on a filter (without and without the application of vacuum for drying) or immobilized using the EDC/NHS reaction, these structures were always observed and have

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also been observed on bacteria imaged in liquids.\textsuperscript{19} Therefore, these features are not likely to be artifacts of either the drying or immobilization procedure.

The surface roughness of bacterial cells has been previously observed using AFM\textsuperscript{7,19} but was not quantified in terms of RMS values as done here. The spheroidal structures visible on bacterial surfaces were speculated to be proteins because of their size, 40 nm.\textsuperscript{7} The rough but regular surface structures that are observed in Figure 6 are therefore probably large surface proteins, but may also be lipopolysaccharides or EPS. We do not think that the spherical structures represent precipitate from the drying process because bacteria suspended only in low IS water show similar features. These structures are also unlikely to be artifacts of either tip shape or immobilization procedure because they have been observed on many bacteria, in liquid and air images, and for cells prepared in a variety of manners.\textsuperscript{7,11,19} Chemical treatments known to affect surface proteins, such as proteinase K, did cause a flattening of the cells' surfaces observable in the section analyses, although the RMS values were still high. Lysozyme/EDTA can cause cells to release their periplasmic proteins.\textsuperscript{45} However, the cells imaged were so damaged (Figure 1E) that it was not possible to determine if proteins were specifically targeted by the treatment chemicals. The surface of KC was greatly altered by the addition of these two chemicals, and G4 cells exposed to lysozyme/EDTA were damaged so greatly that imaging was not possible.

Lipopolysaccharides (LPS) are an important component of the outer surface of a bacterium since 3.4% of the whole cell's dry weight is LPS, and the outer wall is predominantly LPS.\textsuperscript{45} The combination of lysozyme/EDTA hydrolyzes polysaccharides, which causes bacteria to lyse.\textsuperscript{45} This is consistent with the damage seen for KC and G4 exposed to this combination. Disodium tetraborate is a chemical that denatures polysaccharides, and both G4 and KC were damaged by the addition of this chemical.

The effects of heparin and Tween 20 on the bacteria are interesting: while neither chemical damaged KC, there was an unusual effect on the morphology of KC. Each of these chemicals increased the height of the cells' surfaces. This could lead to a steric repulsion between the bacteria and surfaces that may prove useful in preventing the adhesion of bacteria to various surfaces.

**Surface Characteristics Observed with Phase Imaging.** Phase images of samples are less commonly reported in the literature than height (topographic) images because the different components that give rise to phase shifts of the cantilever are more complex. It is difficult to separate the effects of mechanical, chemical, and topographic variations in the sample that each give rise to changes in the phase response. So far, most studies employing phase images have focused on polymer systems.\textsuperscript{1,34,46} In one colloidal study, Omoike et al.\textsuperscript{47} used a combination of height and phase images to obtain


information on the morphology and viscoelastic properties of aluminum oxide particles from simulated wastewater. We are not aware of any studies besides the present one in which phase imaging was used on bacteria.

Caution must be applied in drawing conclusions from phase images since the degree that the tip penetrates into the sample will also affect the resulting phase shift. In soft samples, the tip can penetrate further and the duration of tip-sample contact is longer. To overcome this limitation, it has been recommended to use moderate degrees of tapping to obtain the most useful phase images for distinguishing between relative degrees of surface stiffness.\textsuperscript{46} In our study, we used “light tapping”, defined as a high set point amplitude relative to the free drive amplitude of the cantilever (the amplitude the tip would have if there was no interaction between the sample and tip), because we did not want to damage the bacteria. With light tapping, there is a concern that the probe response will be strongly influenced by the surface contamination layer on the sample and tip.\textsuperscript{46} However, if the tip was being drawn to the sample through attractive capillary forces, which is an indication that tapping is too light,\textsuperscript{35} then we would see only attraction between the bacteria and the tip, or even cell damage. Preliminary results from our laboratory indicate that in some cases there is attraction and in some cases there is repulsion in force measurements on these cells, depending on the chemical treatment the cells were exposed to (unpublished data). Also, there was no evidence of cell damage such as holes in the cell, which we would probably have observed if the tip was drawn in by capillary forces and forced into contact with the surface. The dilemma of how hard to tap could be overcome by imaging cell surfaces in liquids, where surface contamination from evaporated materials is less problematic.

Despite our limited ability to interpret complicated phase images in a quantitative manner, phase images are useful because they provide a measure of sample heterogeneity. Upon exposure to disodium tetraborate (Figure 6B), KC became more heterogeneous than the control cell exposed to MOPS (Figure 6A). This can be attributed to cell damage caused by disodium tetraborate, since it denatures polysaccharides on the cell surfaces. When KC was exposed to Tween 20 (Figure 6D), the phase image showed the presence of some material surrounding the cell that was of a different composition than either the cell itself or the glass slide. This may represent adsorbed surfactant. When KC was exposed to heparin (Figure 6C), the phase image did not show any contrast. This may be because heparin sorbed to the cell and coated it, giving it

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Examples of image artifacts and problems encountered with some preparation techniques. (A) G4 cells deposited on a 0.2-\textmu m filter. The image of the cells is obscured, presumably by the presence of extracellular polysaccharides (EPS). (This image was taken with a Multimode III AFM (DI).) (B) Example of imaging artifact that occurs when the tip picks up debris from the sample. (C) Enlargement of lower right corner of previous image to demonstrate that the triangular shape seen over the whole background is due to tip contamination. (D) Representative image of bacteria deposited on a filter [Pseudomonas fluorescens P17 on a 0.2-\textmu m filter (Poretics)].}
\end{figure}
a uniform surface composition. Phase images provide information that is complementary to the information obtained from topographic images and at this time can only be interpreted broadly as an indication of sample heterogeneity, rather than differences in some specific property, such as elasticity or adhesion.

**Conclusions**

Covalently bonding carboxyl groups of bacterial cells to aminosilane groups on glass slides is an effective method of bonding bacteria for AFM imaging in air. Bacteria that were exposed to various chemicals known to affect adhesion were examined. A combination of height, line, and phase images, as well as surface traces and roughness analyses, provided information on how each chemical treatment affected the morphology and structure of bacterial cells. Low ionic strength water, Tween 20, and heparin, which have been used to decrease adhesion of bacteria to soil and other surfaces, could be used without causing damage to the bacteria. Proteinase K may be used to promote adhesion of bacteria to some surfaces without damaging the cells. This preparation technique can be used to obtain AFM images of bacteria for a wide variety of applications.

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