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Atomic Force Microscopy Examination of the Topography of a Hydrated Bacterial Biofilm on a Copper Surface

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Abstract. A bacterium, designated CCI#8, that was isolated from a corroded copper coupon colonized both polished and unpolished copper surfaces under batch culture conditions. Atomic Force Microscopy (AFM) images revealed that the biofilm was heterogeneous in nature, both in depth and in cell distribution. Bacterial cells were shown to be associated with pits on the surface of the unpolished copper coupons. These observations support previous studies that CCI#8 is associated with the pitting corrosion of copper.

Copper tubing is generally guaranteed to function for 20 years; however, in the past 5 years there has been an increase in the number of failures of copper tubing in industrial and municipal water systems. The failures have involved the formation of pits that have perforated the wall of potable water pipes within a few months. This type of corrosion has occurred in water lines in hospitals in such diverse places as Hellersen, Germany; Dundee, Scotland; and Riyadhl, Saudi Arabia. In every instance large numbers of bacteria were found within the pit and the associated corrosion deposits [5, 6, 15].

Although, by their presence, bacterial biofilms have been implicated, the mechanism of the corrosion process has not been elucidated. Demonstration and characterization of this type of surface phenomenon have been difficult owing to a lack of analytical techniques capable of examining both the surface and the associated biofilm under hydrated conditions. A number of aspects of biofilms, about which we know very little, are considered to be important in corrosion processes on surfaces. For example, one of the most elusive features to describe has been the topography of the biofilm at the bulk/liquid interface. Information of this nature is necessary in order to predict mass transport phenomena the rates of which are considered to be important in controlling the rates of reactions at the biofilm/metal interface [2].

It has been postulated that the activities of biofilm bacteria depend on the maintenance of the structural integrity of the biofilm [13]. The physical relationships between different bacterial cells and the surfaces with which they associate are most commonly maintained by extracellular polymeric material produced by the bacteria [10]. These polymers form a matrix that influences the diffusion of molecules within the biofilm. Thus, the chemical conditions within the biofilm and at the surface are often quite different from those in the overlying bulk fluid [3]. Recently the binding of metal ions by these polymers has been implicated in the corrosion of metals [11, 14].

The heterogeneous nature of a biofilm is also considered to play a role in the corrosion of a metal surface; a number of mechanisms for this effect have been proposed, such as the formation of differential aeration or copper concentration cells on the surface of the metal [16]. By examining a hydrated biofilm by AFM, we hope to be able to view the spatial distribution of cells within a biofilm with a clarity and realism that until now have not been possible. Information gained will enable us to relate cell distribution to the reactions postulated to be responsible for the pitting corrosion of copper.

Materials and Methods

Atomic force microscopy. Unlike traditional light microscopes, which use lenses to magnify the image of an object, scanned probe microscopes use a variety of probes to examine the surface of objects at very close range [18]. An atomic force microscope (AFM) uses X, Y, and Z piezotranslation to position a sample in contact with a microfabricated silicon nitride (Si_3N_4) cantilever. The specimen is scanned in a raster pattern, its parallel tracks separated by as little as a fraction of a nanometer. The repulsion

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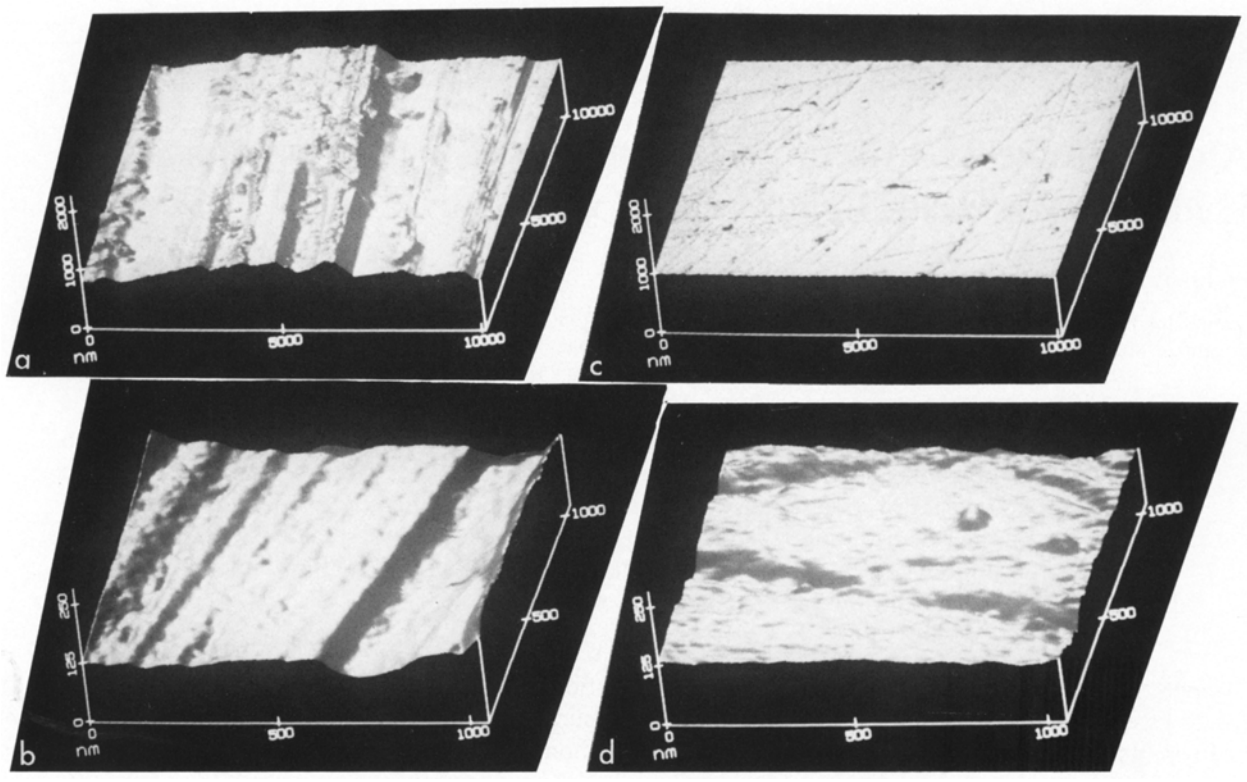

Fig. 1. AFM contour images of copper coupons imaged in the presence of air. (a and b) Unpolished coupons; note the presence of milling lines on the surface of the copper coupons. (c and d) Polished coupons.

generated by the overlap of the electron cloud at the AFM tip with the electron clouds associated with the surface atoms causes the tip to deflect. This deflection is measured by means of a laser beam that is reflected off the cantilever to a photodiode. The photodiode output, fed into a digital feedback loop, acts to maintain a steady tip displacement by varying voltage to a z piezoelectric control. The variations in the voltage on the z piezoelectric control mimic the sample topography and serve as the basis for the image [17, 18]. In these studies all samples were imaged by use of a Nanoscope II AFM (Digital Instruments, Santa Barbara, California). Silicon nitride cantilevers, 120 μm long (Digital Instruments) with a spring constant (k) of approximately 0.6 N m^{-1} were used on all of the samples. The applied force while imaging was approximately 10^{-7}–10^{-8} N. All bacterial samples were transferred to the AFM under defined culture medium (DCM) and remained under DCM during insertion into the sealed fluid cell of the AFM and during subsequent imaging.

Organism and culture media. The bacterium used in this study (CCI#8) was isolated from a corroded copper coupon. The culture medium (DCM) consisted of 0.1 g $MgSO_4$, 0.2 g K_2HPO_4, 0.2 g $NaNO_3$, 0.2 g sodium succinate, 0.5 g sodium citrate, 5 g sucrose, 10 mg vitamin B_6, 10 mg vitamin B_1, 10 mg thiamine-HCl, 10 mg asparagine, 10 mg L-lysine, 10 mg L-cysteine, 10 mg L-ornithine, 10 mg L-glutamic acid, 2.4 mg $CoCl_2 \cdot 6H_2O$, 2.5 mg $CuSO_4 \cdot 5H_2O$, 2.4 mg $Ca(NO_3)_2 \cdot 4H_2O$, 2.8 mg $FeSO_4 \cdot 7H_2O$, 1.7 mg $MnSO_4 \cdot 2H_2O$, and 2.9 mg $ZnSO_4 \cdot 7H_2O$, dissolved in 1 L of ultrapure water. The pH was adjusted to 7.0, and the medium was sterilized by membrane filtration (0.45 μm pore size, Millipore Corp.).

Imaging of CCI#8 on the surface of copper coupons. Sheet dead soft copper (0.004 inch thick) (Thin Metal Sales Inc. Anaheim, California) was used as a surface for bacterial colonization. Both unpolished and polished copper surfaces were used. The copper was polished with a polishing cloth and 0.05-μm alumina particles (Buehler, Illinois). Coupons (14 mm × 14 mm) were cut from the sheet copper, a hole was punched in the corner of the coupons, and they were suspended in a glass flask by a Teflon thread. The coupons were sterilized by autoclaving and were aseptically transferred to flasks containing culture medium, immediately prior to the inoculation of the flasks with a suspension of the bacterium CCI#8. The bacterial suspension used as an inoculum was obtained by growing a batch culture of CCI#8 in a flask containing DCM and copper coupons, on a rotary shaker (100 rpm) at 25°C. After 21 days the medium in the flask was discarded, and the cells adhering to the copper coupons and to the flask were resuspended in 5 ml of fresh medium. By preparing an inoculum in this manner, we hoped to select cells that adhered to copper coupons.

Once inoculated, flasks were incubated for 7 days before the copper coupons were removed and transferred under DCM to the flow stage of the AFM and kept under DCM during examination by AFM. Replicate coupons were removed from the culture flasks and placed into sterile Petri dishes, where they were gently agitated in a solution of DCM minus the carbon sources (DCM salts)

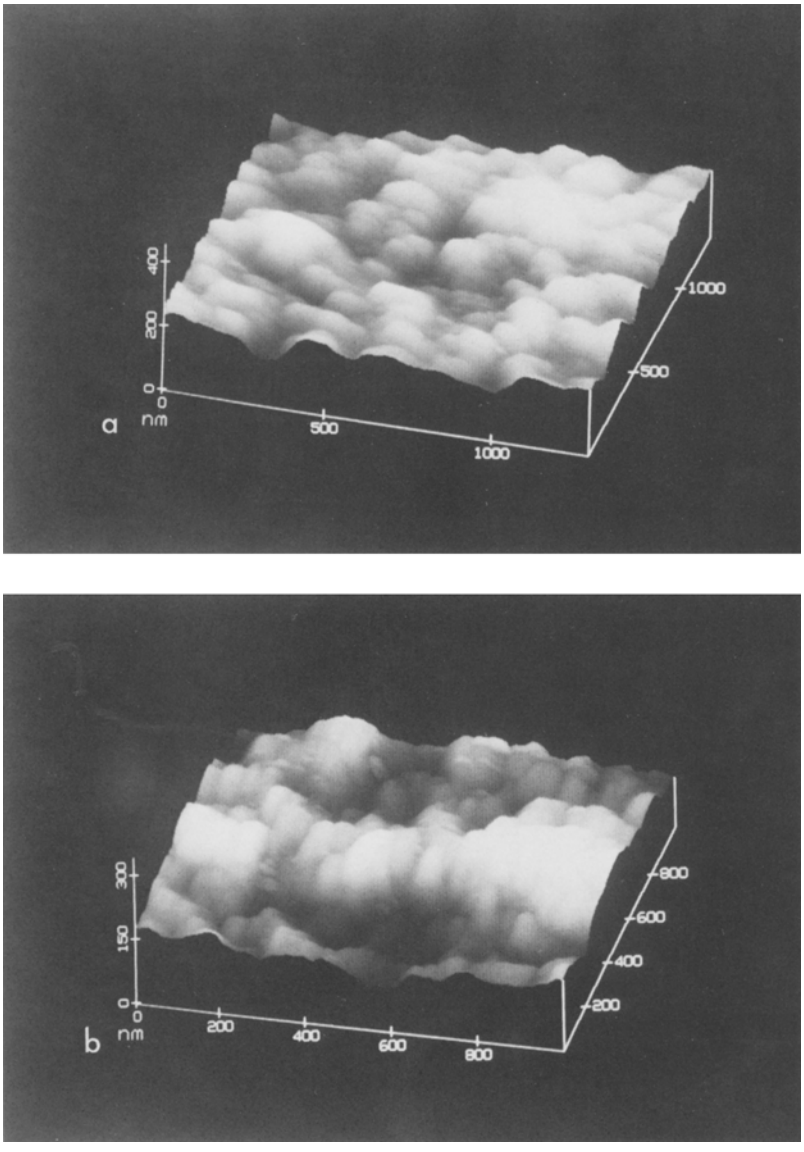

Fig. 2. AFM contour image of the surface of an (a) unpolished and (b) polished copper coupon after exposure to defined culture medium (DCM) for 7 days. Samples imaged under DCM.

and stained with a 0.01% solution of acridine orange. The coupons were rinsed (DCM salts), air dried, and examined by epifluorescent microscopy. Autoclaved copper coupons that had been exposed only to sterile culture medium were also examined by AFM and epifluorescent microscopy.

A comparison of the colonization of unpolished and polished copper coupons by CCI#8. To six replicate flasks (250 ml) containing DCM (100 ml) and both a polished and unpolished copper coupon was added a suspension of CCI#8. After 2 and 7 days of incubation coupons were removed from three flasks, rinsed, stained with acridine orange, and examined by epifluorescent microscopy as previously described. Cell densities were determined by averaging cell numbers at 10 random locations over the surface of the coupon with a 10 × 10 μm grid. Two flasks containing medium and polished and unpolished coupons were included and maintained as sterile controls.

Results

Unpolished and polished copper coupons were imaged prior to exposure to sterile culture medium (Fig. 1a–d). Milling lines formed during the manufacture of the copper coupons are visible on the surface of the unpolished coupon (Fig. 1a), the variation in surface contour over a 1-μm^2 area is in the order of 200 nm (Fig. 1b). Polishing of the coupons resulted in a decrease in the variation of

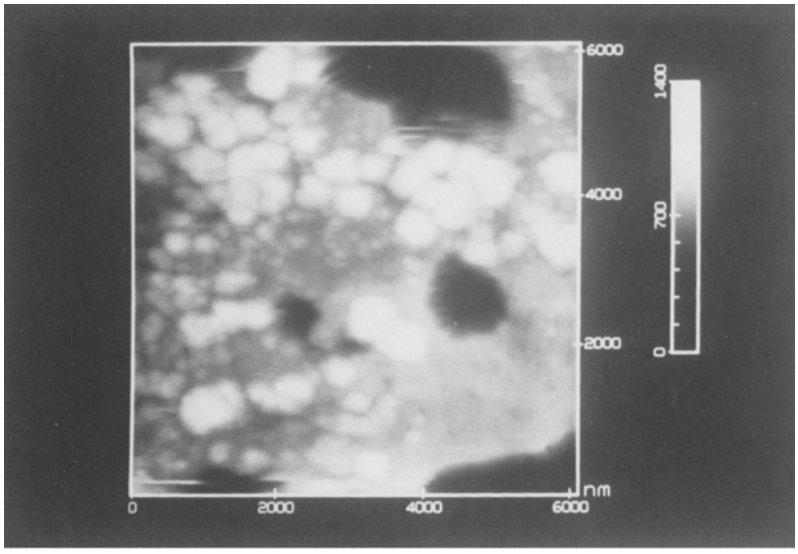

Fig. 3. AFM image of a biofilm of the bacterium CCI#8 on the surface of an unpolished copper coupon. Note the presence of pits on the surface of the copper coupon. The scale bar on the side of the image indicates the z axis height, the lowest points on the image are black (0 nm), and the highest points are white (1400 nm). Sample was imaged under DCM.

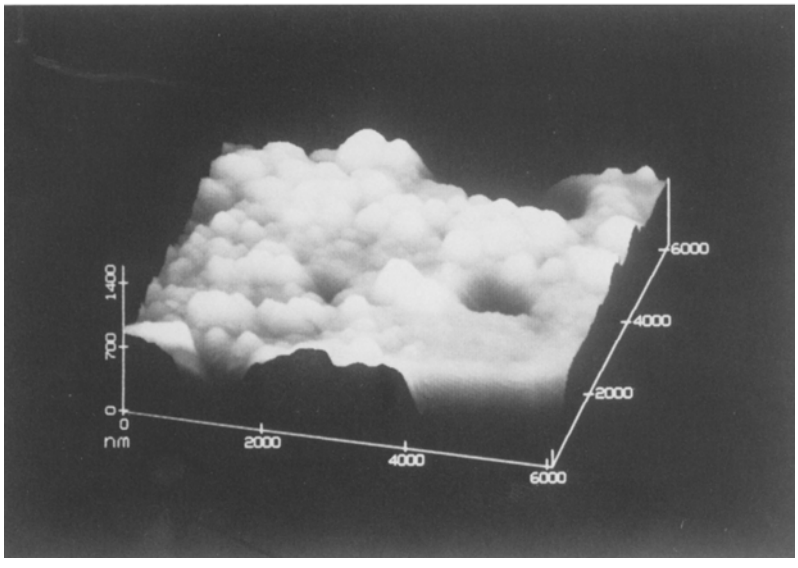

Fig. 4. AFM contour image of Fig. 3.

surface contour to <50 nm over a 1-μm^2 area (Fig. 1c and d).

Exposure of the copper coupons to sterile culture media for 7 days (Fig. 2a and b) resulted in an apparent increase in surface contour for both the unpolished and polished copper coupons. This increase is considered to be owing to the deposition of an organic conditioning film on the copper surface. It is this "conditioned" surface with which the bacteria initially interact.

The bacterium CCI#8 adhered to the surface of unpolished copper coupons and formed a biofilm that was several cells thick in places (Figs. 3 and 4). The biofilm appeared to be distributed heterogeneously over the surface of the coupon with considerable variation in both surface contour and in the arrangement of bacterial cells within the biofilm. The AFM images suggest that the portion of the biofilm in contact with the bulk fluid may be significantly greater than the area of the underlying substrate.

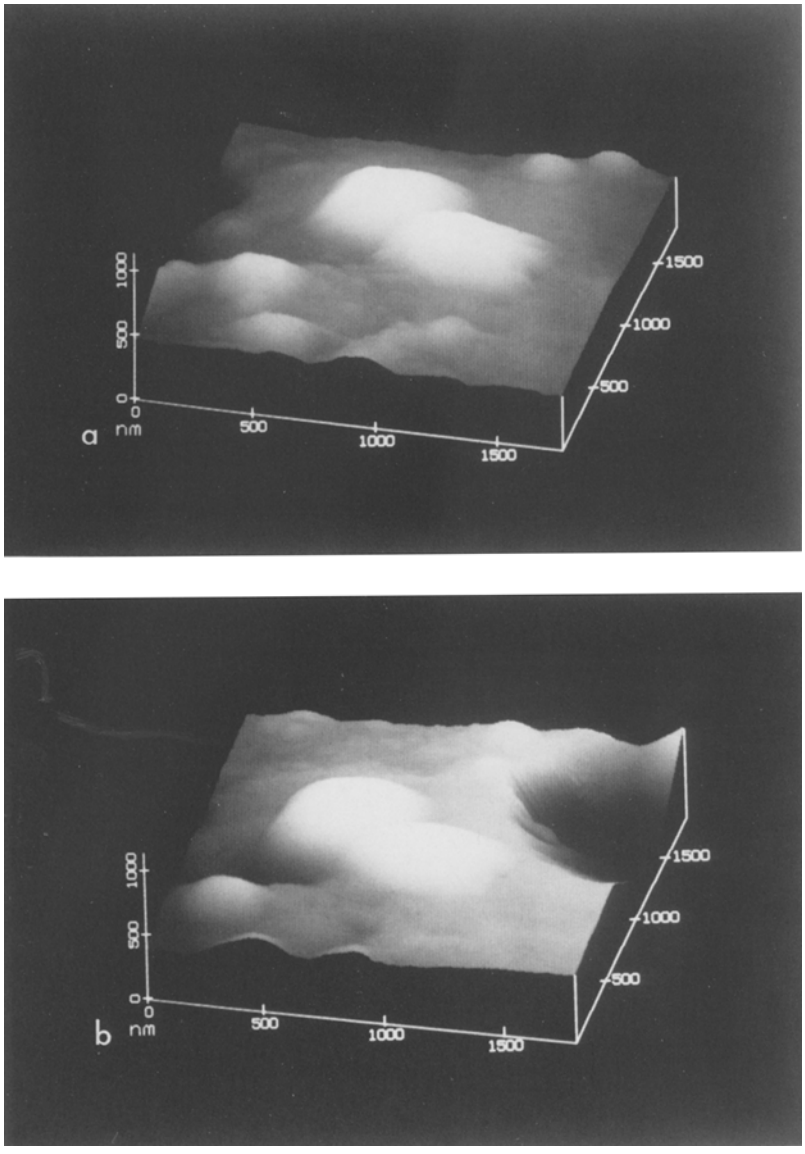

Fig. 5. (a, b) AFM contour images of a portion of Fig. 3, showing the spatial relationship between bacteria on a surface and a pit on the surface of the copper coupon.

Images of higher magnification (Fig. 5a and b) revealed the presence of bacterial cells that appear to have just recently divided, on the surface of the copper coupons; the newly divided cells were approximately 0.7 μm long, 0.3 μm wide, and 0.5 μm thick and were covered by extracellular material. In some instances, the bacterial polymers appear to extend down into pits on the copper surface.

AFM images of the polished coupons after exposure to the bacterium CCI#8 revealed that the bacterium adhered to the surface of the polished copper coupons; however, no pits were detected on the surface of the copper coupons (Figs. 6 and 7). The bacterium had the following dimensions: length, 1.1 μm; width, 0.66 μm; and height, 0.75 μm.

Examination of replicate polished and unpolished coupons by epifluorescent microscopy, following acridine orange staining (Fig. 8a and b), revealed the presence of bacteria on the surface of the copper coupons. The length of acridine orange-stained cells was on the order of 1.1 $\pm$ 0.2 μm (n = 30). In the case of the unpolished coupons the bacte-

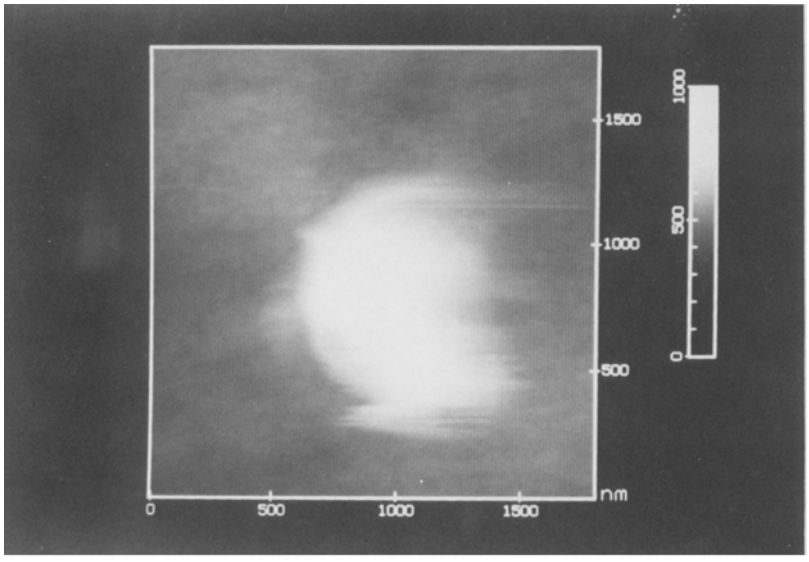

Fig. 6. AFM image of a bacterial cell (CCI#8) on the surface of a polished copper coupon. Sample was imaged under DCM.

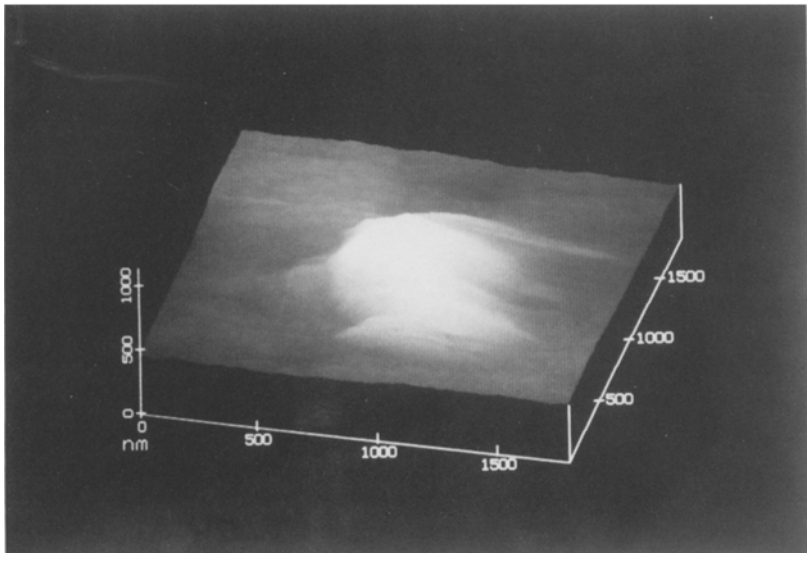

Fig. 7. AFM contour image of Fig. 6.

ria in some instances were in lines on the surface (Fig. 8b), suggesting that perhaps they were associated with the milling lines seen in the AFM images of the copper coupons (Fig. 1).

In a separate experiment it was determined that after a 2-day incubation period bacterial cell numbers on polished coupons ($4.2 \pm 0.8 \times 10^6$ cells/cm^2) were significantly greater (99.9% confidence interval, Student t-test) than bacterial cell numbers on the unpolished copper coupons ($2.6 \pm 0.3 \times 10^6$ cells/cm^2). Cell numbers on both surfaces increased, from 2 to 7 days, to such a degree that colony formation made it impossible to obtain an accurate count. This indicated that the bacteria not only were capable of adhering to the copper surface, but were able to multiply on the surface of the coupons.

Discussion

We previously reported [1, 7, 8] that the bacterium CCI#8 promoted the deterioration of a Cu thin film

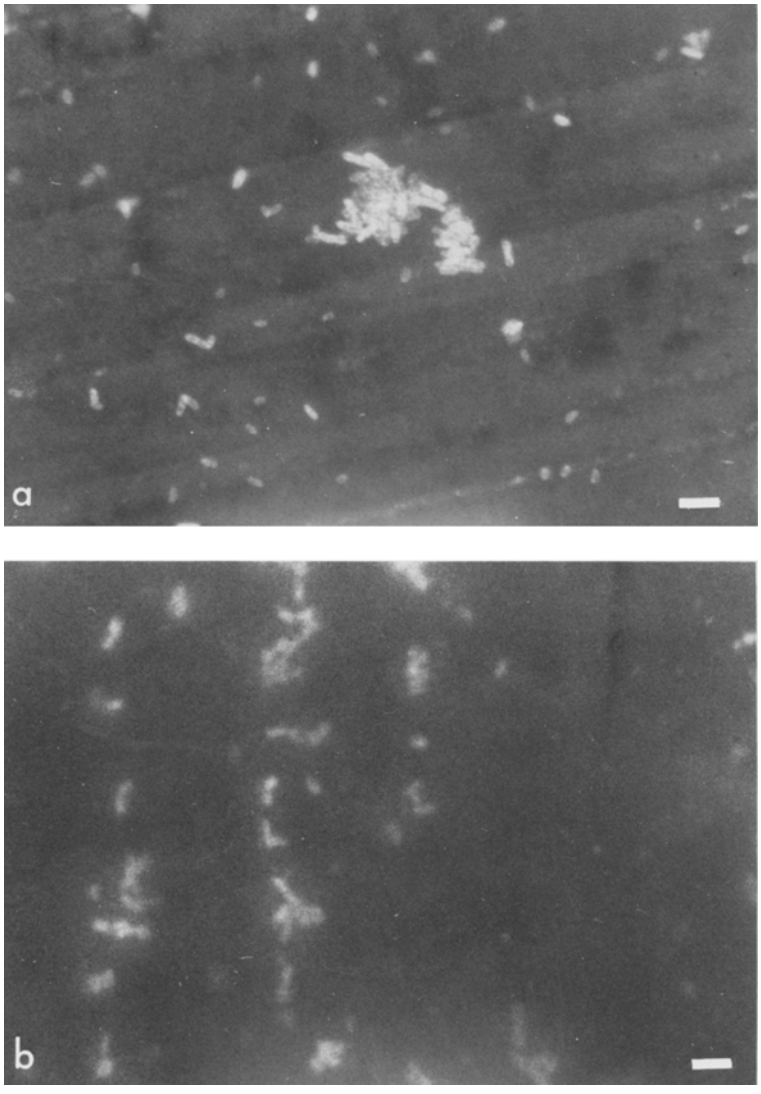

Fig. 8. Acridine orange epifluorescence photographs of the bacterium CCI#8 on the surface of (a) polished and (b) unpolished copper coupons after exposure to DCM for 7 days. Bar = 2 μm.

under both batch and continuous culture conditions. It was, therefore, of great interest that this bacterium was found to be associated with pits on the copper surface. Although we cannot be certain that these depressions are the result of microbial colonization and activity, these images have given us a new perspective of the surface heterogeneity associated with a biofilm. As previously mentioned, this heterogeneity has been postulated to be important in the corrosion process, because adsorbed growing cells constitute anomalies on a metal surface that may result in the formation of differential aeration cells. The areas under respiring colonies become anodic to the surrounding uncolonized areas. Little et al. [16] suggest that the micro-roughness of the substratum is an important factor in the corrosion process as the corrosion currents are thought to flow between the peaks and valleys on the metal surface. This phenomenon may explain why we saw pits associated with bacteria colonizing only unpolished copper coupons. It is of interest, however, that the pits we imaged on the copper surface were beside rather than beneath the bacterial cells, with associations between the pits and the bacteria maintained by the presence of exopolymeric substances (EPS).

Extracellular polysaccharides that appear to be associated with cells of CCI#8 on the surface may play an important role in the formation of pits on the copper surface. The widespread existence of EPS stems from their participation in the adhesion of bio-

film microorganisms to surfaces [4]. It has been proposed that the complexation of copper ions by the polysaccharides reduces the free ion concentration at the metal surface and promotes further ionization of metallic copper in order to establish equilibrium conditions [9, 11, 12]. Hence, once corrosion has begun, it is maintained by the fact that the polymers depolarize the anode by binding the free copper ions.

In conclusion, AFM is a relatively simple way to visualize the topography of attachment surfaces and biofilm surfaces. The great advantage of AFM over the traditional techniques such as scanning or transmission electron microscopy is that the sample does not require dehydration prior to viewing. This is important, because a dehydrated biofilm renders little information about the native state or morphology of the surface of a hydrated biofilm. Even the recently developed environmental scanning electron microscope, which permits visualization of samples in a partially hydrated state, provides little information on the surface contour of a biofilm or on the arrangement of extracellular materials, which are typically transparent in a light or electron microscope. The information obtained has given us a better understanding of the relationship between bacteria, the EPS they produce, and the copper surface; such information is useful in appraising the current models of copper corrosion.

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