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D. T. Goddard

*Springfields Works, dtg3@bnfl.co.uk*

A. Steele

*University of Portsmouth*

I. B. Beech

*University of Portsmouth*

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## TOWARDS *IN SITU* ATOMIC FORCE MICROSCOPY IMAGING OF BIOFILM GROWTH ON STAINLESS STEEL

D.T. Goddard\*, A. Steele<sup>1</sup> and I.B. Beech<sup>1</sup>

Company Research Laboratory, BNFL, Springfields Works, Salwick, Preston, Lancashire PR4 0XJ, U.K.

<sup>1</sup>Department of Chemistry, Physics and Radiography, University of Portsmouth, Portsmouth PO1 2DT, U.K.

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### Abstract

Atomic force microscopy (AFM) has been used to visualise the formation of bacterial biofilms on polished surfaces of 316 stainless steel. Imaging under ambient conditions revealed both the bacterial cells and the matrix of exopolymeric substances (EPS). These images exhibited good resolution with cell surface features as small as 30 nm distinguishable. *In situ* imaging was also carried out, and although the resolution was considerably reduced, images revealing the process of bacteria division have been obtained.

**Key Words:** Atomic force microscopy, biofilm, exopolymeric substances, tapping mode.

### Introduction

In recent years, the use of atomic force microscopy (AFM) techniques in the study of biological materials has expanded rapidly. Although most attention has focused on the smallest structures, such as DNA (Hansma *et al.*, 1992), there have also been a number of studies of larger samples such as bacterial cells (Farina *et al.*, 1993; Kasas *et al.*, 1994) and eukaryotic cells (Hörber *et al.*, 1992; Henderson, 1994; Le Grimmellec *et al.*, 1994). On the scale of a single bacterium, AFM can be used alongside more conventional techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM). However, unlike these techniques, samples require little or no preparation prior to AFM imaging. Furthermore, AFM images contain information in three dimensions, valuable when attempting to accurately visualise complex heterogeneous structures such as biofilms.

Biofilms are ubiquitous in nature (Ellwood *et al.*, 1982) forming on almost any surface. In industry, the presence of biofilms is often considered a problem, and has been acknowledged as contributing to the accelerated deterioration of unprotected metal surfaces (Tatnall, 1981), a process known as microbially influenced corrosion (MIC). This phenomenon has been studied extensively using electrochemical techniques (Mansfeld and Little, 1991), however, the detailed nature of the bio-corrosion mechanism remains to be fully understood.

The complex structures formed by biofilms depend on the physical, chemical and biological nature of the environment. A simplified model consists of bacterial cells encased in a matrix of exopolymeric substances (EPS) (Trulear and Characklis, 1982). The use of microscopy techniques allows the direct observation of the structure and interaction of biofilms with their substrata. Of the techniques currently available, optical methods lack the resolution to study individual bacterial cells in detail, whilst SEM and TEM require potentially damaging sample preparation in the form of fixation and dehydration. AFM has the potential to overcome both of these drawbacks.

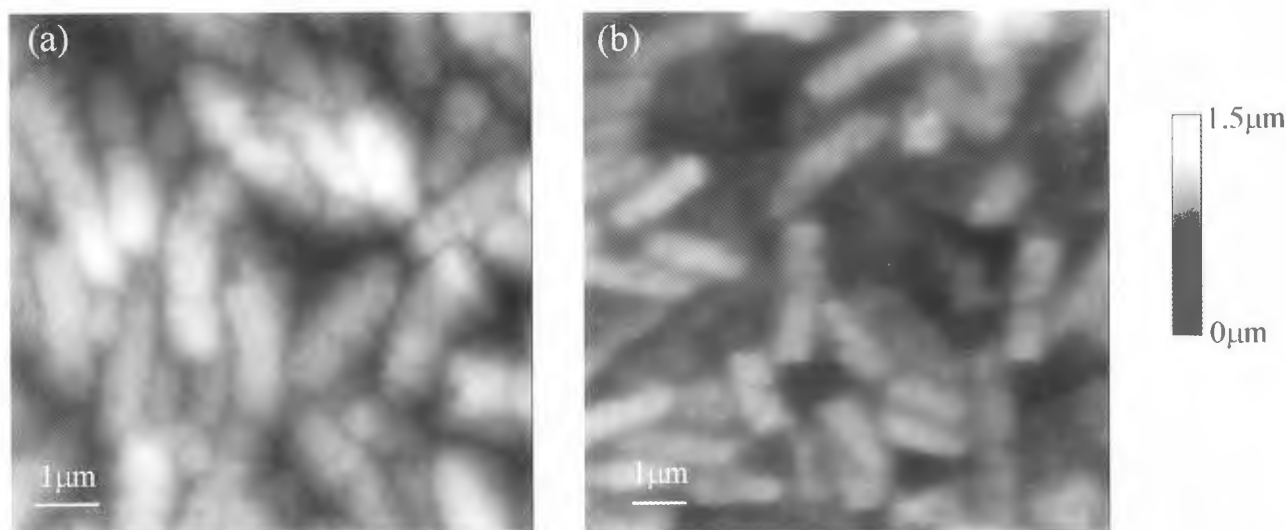
\*Address for correspondence:

D.T. Goddard, address as above.

Telephone number: (44) 1772 763939

FAX number: (44) 1772 762470

E-mail: dtg3@bnfl.co.uk



**Figure 1.** AFM images of an air-dried pipeline consortium biofilm on 316 stainless steel obtained using (a) contact mode and (b) tapping mode.

To date only a few studies of biofilms have been carried out using AFM (Bremer *et al.*, 1992; Steele *et al.*, 1995a,b). Steele *et al.* (1995b) have shown that air dried samples provide the best resolution images and have used these to monitor the pitting of 316 stainless steel. Bremer *et al.* (1992) have reported *in situ* imaging of biofilms grown on copper. However, little is known of the potentially damaging effects of tip-surface interactions or how dehydration affects these delicate structures. In this paper, we report on the AFM operating and preparative conditions which give most information about biofilm structures and discuss prospects for future imaging capabilities.

### Materials and Methods

#### Organisms, media and substrates

A number of different bacterial cultures have been employed to generate biofilms in batch cultures under stagnant conditions on surfaces of 316 stainless steel.

**Pipeline consortium:** Samples of biofilms recovered from corroding cast iron potable water mains were placed in modified Wolfes' media (Kucera and Wolfe, 1957). After 8 weeks incubation at 10°C, 1 ml aliquots were transferred into basic sulphate media (Herbert and Gilbert, 1984) and incubated at 27°C.

**A4 consortium:** A natural consortium obtained from a marine corrosion failure supplied by Dr. V. Chau (British Steel Technical, Swinden Laboratories, UK) was grown in Postgate (1984) medium C at 27°C.

**Alaskan sulphate reducing bacteria (SRB) consortium:** Isolated from a marine corrosion failure grown

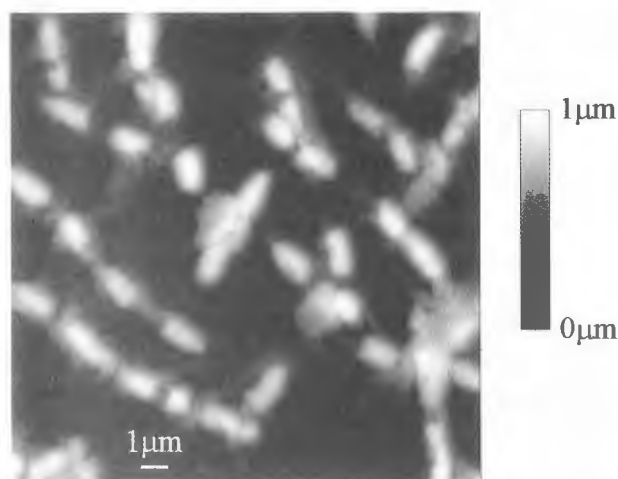
in basic sulphate media at 37°C. For each culture, the cell suspension was adjusted by haemocytometer counts to give an initial inoculum concentration of  $10^7$  cells/ml. Coupons of 316 stainless steel, 1 cm in diameter, were ground (to 1200 grit SiC) and polished (using 1 µm diamond paste). Coupons were placed aseptically in the bacterial cultures and incubated for 7-14 days.

#### AFM Imaging

Imaging was carried out using a NanoScope III (Digital Instruments, Santa Barbara, CA) scanning probe microscope. For *ex situ* imaging, the stainless steel coupons were removed from the bacterial cultures, gently rinsed with sterile distilled water, and allowed to air dry for approximately five minutes. The coupons were imaged in both the contact and tapping AFM modes using microfabricated silicon nitride (nominal spring constant  $k = 0.06$  N/m) and silicon ( $k = 17-60$  N/m) cantilevers, respectively. *In situ* imaging was carried out using the AFM fluid cell and silicon nitride cantilevers.

#### *Ex situ* imaging

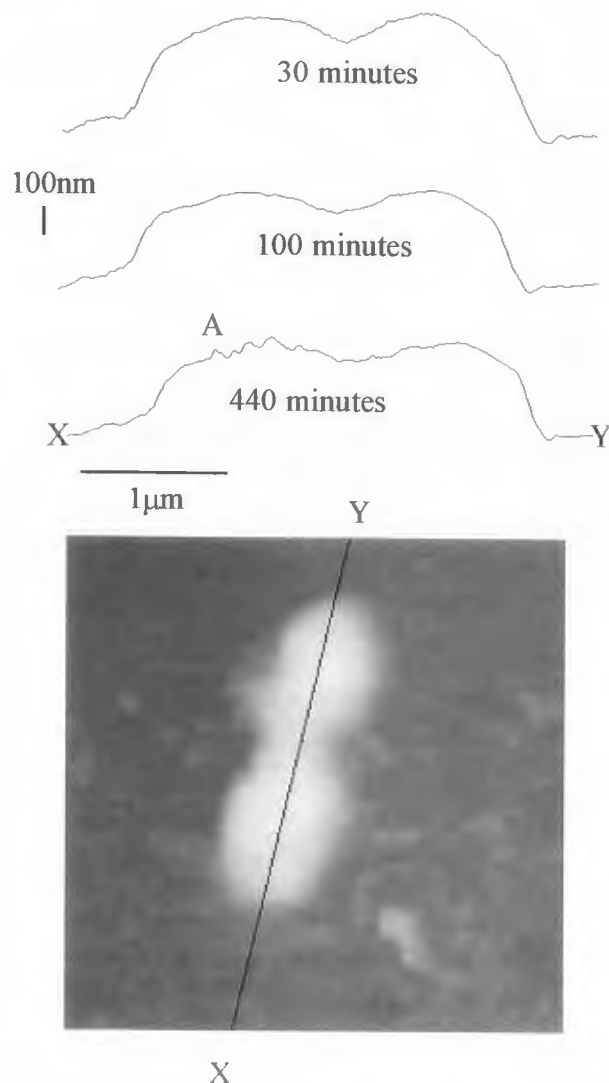
Images of air dried biofilms formed by the pipeline consortium are shown for both the contact (Fig. 1a) and tapping (Fig. 1b) modes of operation. In both cases, imaging is relatively straightforward with features as small as 30 nm distinguishable. There are, however, subtle differences between the two images which suggest that tapping mode is the preferred technique. In the contact mode, image artifacts due to the geometry of the tip are clearly apparent as the images reveal angular sides to the steep walls of bacterial cells. This can be attributed to the fact that the silicon nitride tips used for



**Figure 2.** Contact mode AFM image of an air-dried pipeline consortium biofilm showing EPS surrounding individual bacterial cells.

contact mode have a half cone angle  $\alpha \sim 35^\circ$ , compared with  $\alpha \sim 18^\circ$  for the silicon tips used for tapping mode. Also, the surfaces of the bacteria in the contact mode image have more pronounced undulations than on the tapping mode images. This appearance reflects the dehydration of the bacteria but is likely to be more pronounced with the higher force applied during contact mode imaging. Furthermore, a covering layer of EPS may also be present, which the tip may penetrate in contact mode but not in tapping mode. Imaging on areas of samples with incomplete coverage of biofilm indicate that EPS is present (Fig. 2), extending around individual bacterial cells.

To study the process of dehydration in greater detail, samples of pipeline consortium biofilms were prepared in the manner described earlier allowing an initial five minutes of air drying before mounting the sample on the AFM stage. A suitable area was located and imaged at intervals over a period of 440 minutes using contact mode AFM whilst maintaining a constant force. Figure 3 shows linescans across a bacterium after 30, 100 and 440 minutes of air drying. The bacterium has dried down on to the surface, the peak height reducing from 412 nm after 30 minutes to 318 nm after 440 minutes. However, the magnitude of the undulations on the bacterial surface have not changed significantly. The feature labelled A in the lower linescan was attributed to use of the force calibration mode to adjust the force applied to the surface. In this mode, the tip is periodically indented into the surface by application of a triangular waveform to the z piezo. This demonstrates that the surface of the bacteria are still compliant even after considerable air drying, in agreement with the observations of Fritz *et al.* (1994) who obtained contrast from the



**Figure 3.** AFM cross-sections showing the dehydration of a bacterium. Feature A resulted from periodically indenting the tip into the bacterium.

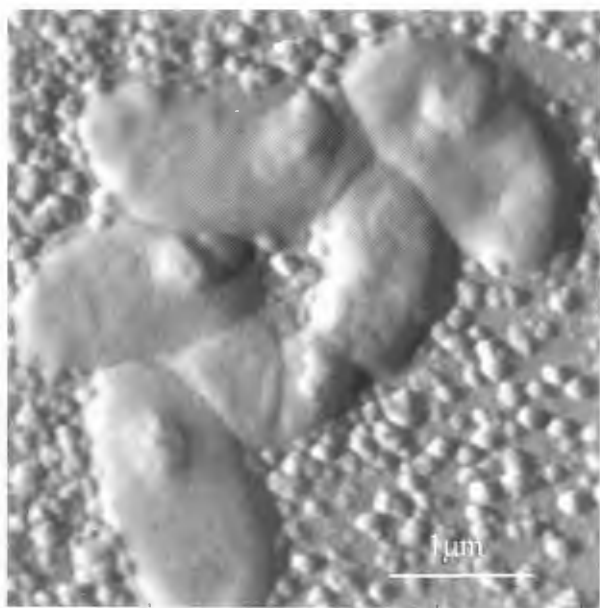
force modulation mode in their study of magnetotactic bacteria.

*Ex situ* imaging also allowed observation of internal bacterial structures. Each cell type dehydrated in a very characteristic manner as shown, for example, by comparison of Figure 1 with Figure 4. Differences between the surface morphologies reflects variations in the hydration state and rigidity of the intracellular structures. To fully exploit this capability of the AFM to probe internal detail, comparison with other microscopies or the use of labelling techniques will be required.

#### *In situ* imaging

To more accurately reproduce the conditions in which biofilms exist in nature, samples were studied immersed in a liquid using the AFM fluid cell. Prior to

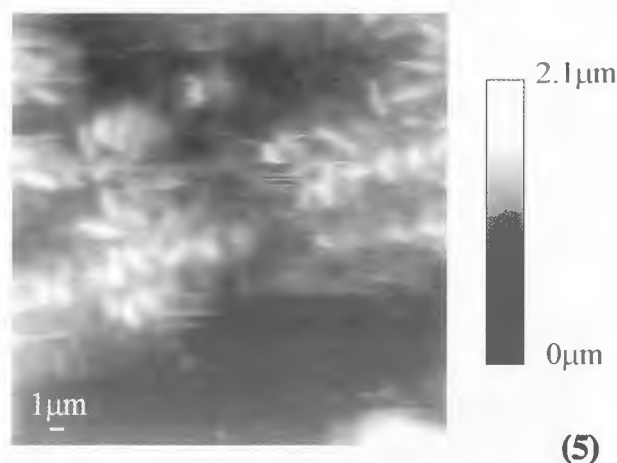




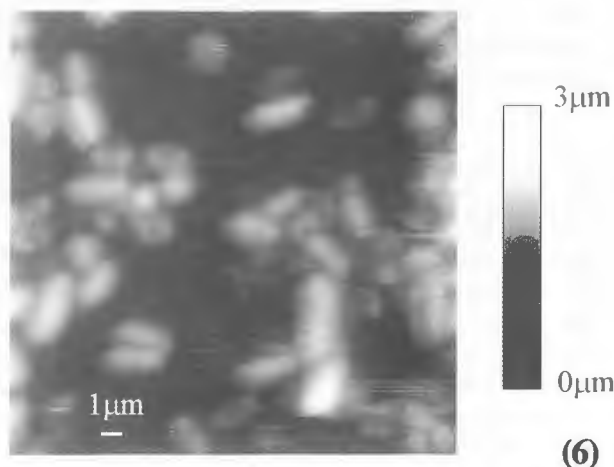
**Figure 4.** A deflection mode image of an air-dried Alaskan consortium SRB biofilm with a different cell surface morphology to that shown in Figure 1.

imaging, samples were lightly rinsed with sterile distilled water as described earlier. A droplet of sterile distilled water was placed on the sample which was then transferred to the AFM stage. In this way, imaging was carried out without any dehydration occurring. As shown in Figure 5 for an A4 consortium biofilm, the resolution is much poorer than in air, and there is considerable streaking across the image. In addition, bacterial cells were removed from the scanned area on a regular basis. These observations are indicative of there being a movement of the sample under the tip. This can be attributed to a much reduced adhesion between the biofilm and its steel substrate. Also, a transient force, described by Henderson (1994) as being caused by poor tracking of the tip when it encounters a steep sided object, is likely to be significant with this type of sample. Despite these imaging difficulties, the use of the AFM tip to move bacteria could be potentially employed as a method of studying the strength of adhesion of a biofilm to its support, which is an important factor in understanding MIC.

One way to improve the adhesion of the biofilm to its substrate is to allow a period of drying prior to imaging under a liquid. Other methods, such as chemically treating the substrate with poly-L-lysine, were also considered, although it could be argued that this will alter the natural biofilm growth process. Figure 6 displays an *in situ* image of a pipeline consortium biofilm which was first air-dried for five minutes. The drying down of the



(5)



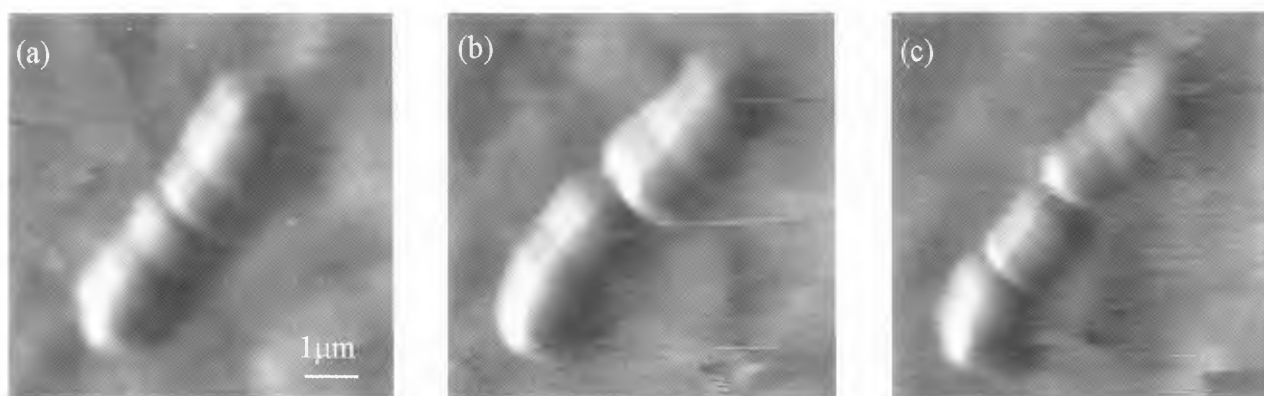
(6)

**Figure 5 (top).** *In situ* AFM image of an A4 consortium biofilm. The streaking in the image suggests movement of the biofilm under the tip.

**Figure 6 (bottom).** An AFM image showing a pipeline consortium biofilm, air-dried for five minutes prior to imaging *in situ*.

biofilm led to a significant improvement in attachment of the cells and therefore in imaging, although possibly at the expense of the hydrated EPS matrix and certain cell types which were not able to withstand the desiccation. Cells, which were rehydrated, were still susceptible to movement by the tip. Surprisingly, some cells could be rehydrated to their original state after being exposed to air for several days.

An alternative method of imaging poorly adhered samples is to use intermittent contact techniques, such as, tapping mode. Recently, tapping mode has been successfully demonstrated in a liquid environment (Hansma *et al.*, 1994). Preliminary investigations using this technique have not shown significant improvements in resolution for fully hydrated biofilms. Allowing a short



**Figure 7.** A sequence of deflection mode AFM images revealing a bacterium in the process of dividing. Images correspond to time  $T = 0$  (a), 100 (b), and 220 minutes (c). Photo Width =  $7 \mu\text{m}$ .

period of dehydration prior to imaging gave a slight improvement in the resolution over contact mode, however, the most noticeable difference was the ability to image for long periods without removing any cells.

One of the rewards of *in situ* imaging using AFM is the ability to visualise dynamic biofilm processes. The sequence of images shown in Figure 7 demonstrates this capability. The images were obtained for an A4 consortium biofilm using contact mode AFM in a sterile distilled water environment without prior dehydration. Imaging was not continuous: the tip was withdrawn between scans and therefore needed to be relocated for each scan. A cell is observed in the process of division. The error signal images clearly show the separation of the two daughter cells after 100 minutes, with a further subdivision of the lower cell in progress after 220 minutes.

### Conclusions

Atomic force microscopy shows great potential as a technique to reveal the complex structure of bacterial biofilms in their natural environment. There are, however, a number of issues to consider when imaging these structures, particularly the state of hydration and the influence of tip-surface interactions. Tapping mode AFM is the technique of choice for *in air* characterisation, revealing fine structure on cell surfaces and potentially EPS. Imaging in a liquid is possible, although care to minimise deformation or movement of cells by the tip is of paramount importance. Fixation with glutaraldehyde has been purposely avoided during these studies to reduce the number of preparative steps prior to imaging. It is likely though to help preserve the bacterial structure during desiccation, as demonstrated by SEM studies, although this may not resolve the problems of accurately imaging EPS. However, as the AFM techniques become less invasive, it may not be necessary to

carry out chemical fixation in order to reveal the intricate structures of biofilms.

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#### Discussion with Reviewer

**M.J. Miles:** Can the types of bacteria be identified from these images?

**Authors:** There is no reason why AFM would not provide some means of bacteria identification from size, shape and even dehydration characteristics, although we have not studied this in detail.