Geobiology (2008), 6, 254–262 DOI: 10.1111/j.1472-4669.2008.00161.x

In search of the microbe/mineral interface: quantitative analysis of bacteria on metal surfaces using vertical scanning interferometry

M. S. WATERS,[1] C. A. STURM,[2] M. Y. EL-NAGGAR,[3] A. LUTTGE,[2] F. E. UDWADIA,[4] D. G. CVITKOVITCH,[5] S. D. GOODMAN[6] AND K. H. NEALSON[3]

[1]*Department of Molecular Biology, University of Southern California, Los Angeles, CA, USA*
[2]*Department of Earth Science, Rice University, Houston, TX, USA*
[3]*Department of Geobiology, University of Southern California, Los Angeles, CA,USA*
[4]*Department of Aerospace Engineering, University of Southern California, Los Angeles, CA, USA*
[5]*Department of Dentistry, University of Toronto, Toronto, Ontario, Canada*
[6]*Department of Dentistry, University of Southern California, Los Angeles, CA, USA*

ABSTRACT

To understand the development of biofilms on metal surfaces, analysis of initial bacterial attachment to surfaces is crucial. Here we present the results of a study, using *Shewanella oneidensis* MR-1 as a model organism, in which vertical scanning interferometry (VSI) was used to investigate the initial stages of cell attachment to glass, steel and aluminium surfaces. It was found that while VSI gave unambiguous results with opaque surfaces, when reflective surfaces were used, an artifact sometimes appeared, with the bacteria appearing as rod-shaped pits rather than as cells on the surface. When the bacteria were altered to increase opacity, this artifact disappeared, and upon further investigation, it was found that the observational artifact was the result of a conflict between light reflected from the bacteria and the light reflected from the bacteria–metal interface. These results suggest that not only can bacteria be measured on surfaces using VSI, but with some modifications to the analytical software, there may be a unique window for studying the bacterial/substrate interface that can be used for quantitative observations. Imaging and characterization of the bacteria–substrate interface *in vivo* (previously invisible) will provide new insights into the interactions that occur at this important juncture.

Received 24 January 2008; accepted 25 March 2008

Corresponding author: M. S. Waters. Tel.: (213) 821-2512; fax: 213-740-0011; e-mail: mswaters@email.usc.edu.

PROLOGUE

It is appropriate at this point to take a moment and ask why it is we are presenting this paper in this venue. It is in the memory of a great man and a great scientist, who had among his many loves, the study of the bacterial surface, and its interaction with the environment, be it ions in solution, inert plastic, or active metals or minerals. Terry Beveridge inspired us all with his curiosity, his enthusiasm, and his expertise: and, most of all, his humanity. He would have looked at what we are presenting here, nodded in appreciation, and then gently reminded us of how we might have done it just a wee bit better! It is an honor to have the chance to say good bye to Terry through participation in this volume, though it would be far better to be saying hello to his ever-friendly smile.

INTRODUCTION

Most of us in the world of microbial physiology have been trained to study microbes living in uniform suspensions – batch cultures, chemostats, etc., in which the properties of the individual cells are thought to be generally uniform, and the properties of the culture can be thus equated to the sum of the properties of the individuals. As time has gone on, it has become apparent that many of the most important activities in which microbes are involved have to do with their interaction with solid surfaces, and that once upon a surface in a biofilm-type of environment, the cells are no longer the same as they were in suspension (Hamilton & Characklis, 1989). That is, the complexity of the system has increased, and the properties of any one cell may not (in fact, almost certainly DO not)

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

reflect the properties of the community. We thus see that on surfaces and in biofilms, microbes: (i) become remarkably different with regard to growth rates; (ii) become resistant to toxic metals and/or antibiotics; (iii) display differences in morphology and/or motility; and (iv) probably change in a myriad of other ways.

With these thoughts in mind, it is clear that one of the central challenges in microbial eco-physiology is that of understanding the interface between the microbe and the surface(s) on which it sits. Initial biofilm attachment is traditionally defined by three sequential stages: adsorption (reversible), adhesion (irreversible), and colonization (Marshall *et al.*, 1971; Characklis, 1981; Van Loosdrecht *et al.*, 1990; Cunliffe *et al.*, 1999). Reversible bacterial adsorption is commonly influenced by environmental and substratum conditions and can happen within a few seconds to a couple of hours. The attached bacteria then undergo additional developmental changes that result in irreversible adhesion, usually in about 1–3 h, depending on environmental (Characklis, 1981; Van Loosdrecht *et al.*, 1990; Cunliffe *et al.*, 1999; Palmer, 1999). Once bacteria have adhered to a surface, division, colonization and biofilm formation follow, with a variety of results, depending on the nature of the surface and the biofilm-forming population (Characklis, 1981).

It is a complex matrix with which one deals when thinking of this problem – even a single microbial cell is a complex creature with many different components on its exterior, and each surface is different, ranging from inert surfaces where cells may take refuge, to surfaces that can act as energy sources (electron donors) or oxidants (electron acceptors). Each surface should be expected to be different from other types with respect to interactions with microbes.

There are many ways to address the problem of the microbe–surface interface, and the processes that occur at this interface. We will deal here with one of them, vertical scanning interferometry (VSI), a technique that utilizes an optical microscope with Mirau interference optics. VSI is a nondestructive and minimally invasive approach that provides a large field of view, a high vertical resolution at the Angstrom to nanometer scale: all this with fast data acquisition, on the order of seconds. The impact of this approach is that with a standard microscopic approach modified to do scanning interferometry, one can achieve, in the *x*- and y-directions a resolution of 1 μm or less, and in the z-direction a resolution of a few nanometers or less (theoretically, less than 1 nm!), thus allowing remarkable resolution of surfaces, and the ability to accomplish things such as real-time observation of mineral dissolution (Lasaga & Lüttge, 2001; Luttge & Conrad, 2004; Davis & Luttge, 2005; Luttge *et al.*, 2005; Luttge, 2006). Using the VSI system, it is possible to easily locate areas of interest using low-power objectives, and switch to higher power objectives to study them at high resolution in a noninvasive and nondestructive fashion. It is this combination of scale interchangeability with the noninvasive and nondestructive manner of the instrument that affords the special advantages of the VSI system.

Some of the first VSI studies of microbes on surfaces were done utilizing carbonate minerals, in particular calcite, a particular crystalline form of calcium carbonate (Lasaga & Lüttge, 2001). The advantage of this mineral is that, when cleaved, the resulting two new surfaces are mirror-images of each other, thus providing an excellent situation with regard to comparing biotic versus abiotic dissolution. When such minerals were examined using the VSI, it was revealed that dissolution of carbonates proceeded via the formation of surface-active dissolution pits, which deepen and spread, eventually coalescing and leading to complete dissolution of the carbonates. In subsequent studies, the effect of bacteria on carbonate dissolution was tested (Luttge & Conrad, 2004; Davis & Luttge, 2005). It was revealed that a strain of *Shewanella oneidensis* called MR-1 was able to attach rapidly and specifically to areas on the carbonate surface that were in the nascent stages of 'dissolution pit' formation. Attachment at these active sites then impeded further dissolution of the carbonate mineral. Neither the mechanism of attachment nor the mechanism of inhibition of dissolution is known, but the implications with regard to carbonate chemistry are potentially of great importance, and the mechanism(s) are under study now. Similar effects have been seen with other carbonate minerals (Luttge *et al.*, 2005). Over time, we have noted in studies of carbonates that the bacteria on the surfaces of carbonate minerals were easily visualized using surface profiling microscopy techniques such as atomic force microscopy (AFM) and, to a lesser degree VSI, and furthermore that with time, the bacteria appeared to dissolve a small amount of the carbonate, presumably via the production of CO_2 and the local dissolution of the carbonate matrix (Fig. 1A. Both the size of the microbes on the surface and the formation of the existence of the local dissolution were confirmed by AFM as shown in Fig. 1B (Davis *et al.*, 2007)).

With regard to these issues, we describe here the use of VSI to study the attachment of microbes to surfaces, one interesting artifact that appears to offer a special advantage for the study of the microbe/mineral interface. Our results show that transparent microbes on reflective surfaces generate observational artifacts that when corrected may allow VSI imagery to provide a noninvasive, high-resolution method for observing microbial attachment and surface dissolution. We have targeted the visualization of bacteria and the bacteria/substratum interface during the initial steps of attachment to glass and metal surfaces. During these analyses, we found that the reflective properties of the surface produce unique signals that can be distinguished from the signals from the upper cell surface. Uncoupling these signals will ultimately allow us to study in a noninvasive way, the initial steps in bacterial attachment and ultimately, adhesion and surface modifications.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

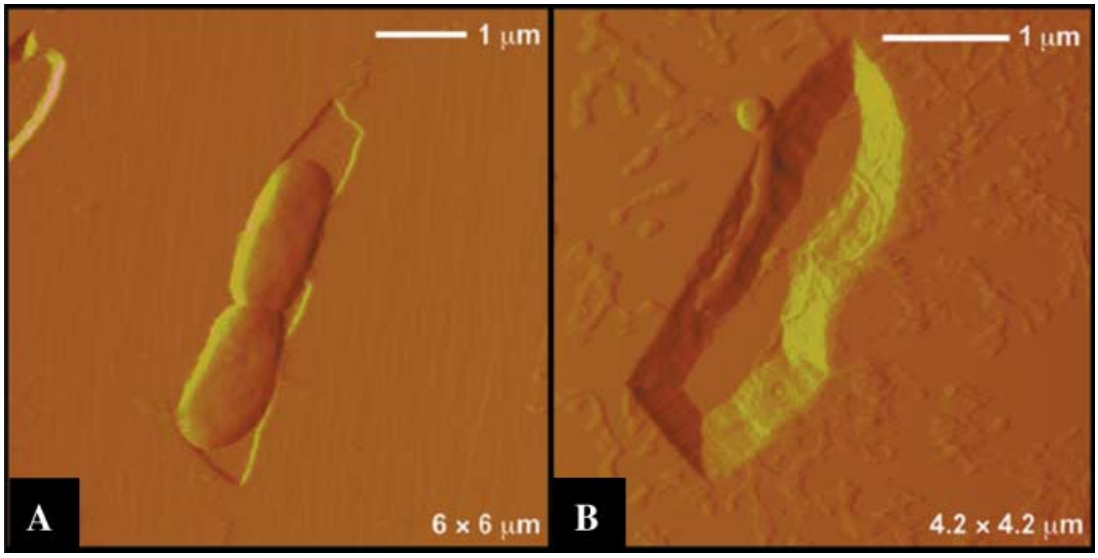

Fig. 1 Atomic force microscopy images of calcite in the context of *Shewanella oneidensis* MR-1 attachment. (A) Two MR-1 cells attached to the calcite with a macrostep emerging from underneath the microbes. (B) Microbial trench lined with organics and characterized by a smooth floor. From Davis *et al*., 2007, with permission from authors.

MATERIALS AND METHODS

Bacterial growth and attachment

In these experiments *S. oneidensis* MR-1 wild-type (Myers & Nealson, 1988) was used with and without plasmid p519nGFP (Stretton *et al.*, 1998), which constitutively expresses green fluorescent protein (GFP). MR-1 (p519nGFP) was grown with the addition of 50 μg/mL kanamycin at all times to prevent loss of the plasmid.

For this paper, bacterial cells were applied to surfaces in two ways: (i) via natural bacterial attachment to the surface of interest in a Palmer flow cell (Palmer, 1999) or alternate culture chamber, or (ii) by placing a small drop of diluted planktonic cells directly onto the surface and allowing it to desiccate to a thin film. For both natural attachment and direct surface application studies, strains were grown to stationary phase in 5 mL of nutrient-rich Luria-Bertani (LB) broth (Bertani, 1951) from frozen glycerol stocks at 30 °C overnight shaking at 120 r.p.m., then inoculated 1 : 100 into 10% carbon LB (1% NaCl, 0.1% Bacto tryptone, 0.05% Bacto yeast extract, lowered from 1% tryptone and 0.5% yeast extract) and grown at 25 °C at 120 r.p.m. for 1, 2, 3, 8 or 16 h. The carbon source of the medium was decreased to help stimulate attachment. Once bacteria were attached to the surface of glass, steel or aluminium, samples were rinsed gently with filtered MilliQ (Millipore, Billerica, MA, USA) water to remove salts and unattached cells. From this point on, 'attachment' will refer interchangeably to either naturally self-adhered biofilm cells or planktonic cells dropped onto a surface, as no differences relevant to the subject of this manuscript were observed throughout the course of this study.

Preparations for measurement of bacteria and comparative substances on substrata

To ensure accurate bacterial measurement on metals, major microtopography (~1.5 μm) was removed by polishing until the surface was a mirror, whose vertical undulations measured a maximum of 40 nm from the highest to the lowest point in any location. Interferometric images of surfaces were acquired before and after attachment of bacteria or other materials, and after the surface was wiped clean. Upon initial attachment (within the first 3 h), the sample was removed from the culture chamber, rinsed, and air-dried either completely (control for comparison) or until almost all water had evaporated. The surface was then optically scanned using the VSI as described in detail below. All VSI measurements were made from either completely or partially desiccated samples. In the latter condition, live/dead staining confirmed that bacteria were mostly living, however, VSI bacterial profiles were unable to distinguish between live and dead cells or wet and dry cells, at our potential for resolution. To control for potential imaging artifacts that may be generated by VSI, MR-1 was compared to hematite, which is similar in size and reflectivity, but nonbiological and opaque. Bacteria and hematite were coincidentally imaged by applying a mixture of colloidal hematite and bacterial culture to the imaging surface, as described above.

All bacterial images were acquired either in the media or after air-drying, with no further sample manipulation. Surfaces intended for bacterial attachment/quantification were measured by VSI before attachment, after attachment, and after bacteria were removed using a soft cloth. To show that images were coincident or the product of *S. oneidensis* MR1, bacteria were

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

assessed by fluorescent compound microscopy, by virtue of a constitutively expressing GFP containing strain. Ethidium bromide (a DNA intercalating stain) mixed with dimethyl sulfoxide (DMSO) (allows the ethidium bromide to access the DNA inside the cell), which makes bacteria fluoresce red under 510–560 nm (green light) excitation, was used to verify that green fluorescence seen under 450–490 nm (blue light) excitation was exclusive to bacteria, and was not obfuscated by background fluorescence.

Clear cellulose fibers (tapered in thickness) were used to assess VSI imaging artifacts that may be associated with transparent material of different sizes. Thicknesses and appearance of the fibers on polished steel were confirmed by taking advantage of its ability to autofluoresce under blue and green light excitation.

All VSI imaging techniques and sample preparations/analyses were carried out using ~1 cm^2 mild steel, 304 stainless steel, aluminium coupons and glass slide sections. Bacteria attached or settled to a surface that had been previously measured by VSI. After the sample was desiccated, the surface was measured again, then cross-linked with glutaraldehyde (see Fig. 7), or stained (data not shown) with Crystal Violet (peptidoglycan stain), Sudan Black (lipid specific stain), Gram's Iodine (saccharide specific stain), and/or Ruthidium Red (EPS specific stain) to increase cell surface opacity.

Bacterial measurements and morphologies were confirmed by atomic force microscopy (AFM) and environmental scanning electron microscopy (ESEM), and translucency was defined by scanning near-field optical microscopy. AFM and SNOM measurements were made simultaneously using a WiTec alpha-SNOM instrument (WiTech, Ulm, Germany) and a silicon AFM cantilever with a hollow aluminum pyramid as a tip, through which a 650-nm excitation laser is transmitted for near field imaging. This technique allows us to measure the transmission of light through a bacterium with 100 nm resolution.

Data analysis and image generation

All VSI images were acquired with a MicroXAM™ Interferoetric Surface Profiler from ADE™ Phase Shift (San Jose, CA, USA). Acquired data were processed and analysed using MapVue AE version 2.13.1 from ADE™ Phase Shift and Scanning Probe Image Processor, SPIP™ version 4.1.8.0 from Image Metrology. Percent reflectivity of a surface was defined by the range of the intensity profile for a coordinate from the most intense black fringe to the most intense white fringe of an individual sample and then averaged across at least 10 randomly selected points. Compared samples were observed under the same emitted light intensity with the possibility of total light reflection being equal to 100%. Image organization was accomplished using Adobe® Photoshop® version 7.0.

RESULTS

Imaging of *Shewanella oneidensis* MR1 on glass

Fluorescent and phase contrast compound microscopy has been used for many years to rapidly and noninvasively identify single bacterial cells on a variety of surfaces (Meyer & Dworkin, 2007), but these approaches lack the capacity to measure surfaces in 3D and are low in resolution compared to other techniques. Conversely, AFM is adept at generating high-resolution bacterial surface profiles (Bolshakova *et al.*, 2004), but finding the area of interest in a large sample can make preparation and imaging quite time-consuming. Furthermore, AFM can be somewhat invasive to the sample, and typically lacks the ability to gather a broad spectrum view without interacting with the sample. VSI bridges these two techniques nicely, as it possesses the unique capacity to rapidly and noninvasively generate 3D images, i.e. high-precision height maps of a bacterium with minimal sample preparation, at a resolution and data acquisition rate that falls between AFM and fluorescent microscopy. Initial experiments were done with glass surfaces to ensure that measurements acquired by VSI were accurate. AFM and fluorescent microscopy were used to confirm the reliability of our findings. Average VSI measurements of bacteria on glass was directly comparable by phase contrast microscopy (Fig. 2), which corresponded to previously reported measurements of the bacterium (Baumann *et al.*, 1972; Myers *et al.*, 1988; Luttge & Conrad, 2004).

Imaging of *Shewanella oneidensis* MR1 on polished steel

In order to clearly distinguish and characterize images/measurements of bacteria on steel, we first removed the prominent image convoluting microtopography of the steel. Given that light intensity is critical to data acquisition by a VSI, it was first necessary to compare the reflectivity of unpolished steel versus mirror polished steel. Under the same emitted light intensity, reflected light intensity profiles (Fig. 3B,D,E) revealed on average that ~10% more light was reflected back from the surface of mirror polished steel, with an average feature range normal to the surface of 40 nm from the highest point to the lowest point when compared to unpolished steel with an average feature range of 1.2 μm (Fig. 3A,C).

When *S. oneidensis* MR-1 was deposited on a mirror polished steel surface, acquired interferometric data of bacteria applied to glass and mirror polished steel (prepared from the same culture at the same time) varied dramatically. On glass, *S. oneidensis* MR-1 appears as a rod-shaped structure setting above the surface to which it was applied; however, on a mirror polished surface bacteria appear as rod-shaped pits (Fig. 4A) and restored when wiped clean (Fig. 4C). As controls, the surface displays both a bump and a pit present in the original surface of the steel, neither of which are altered throughout the experiments. Repetition of all of the above experiments

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

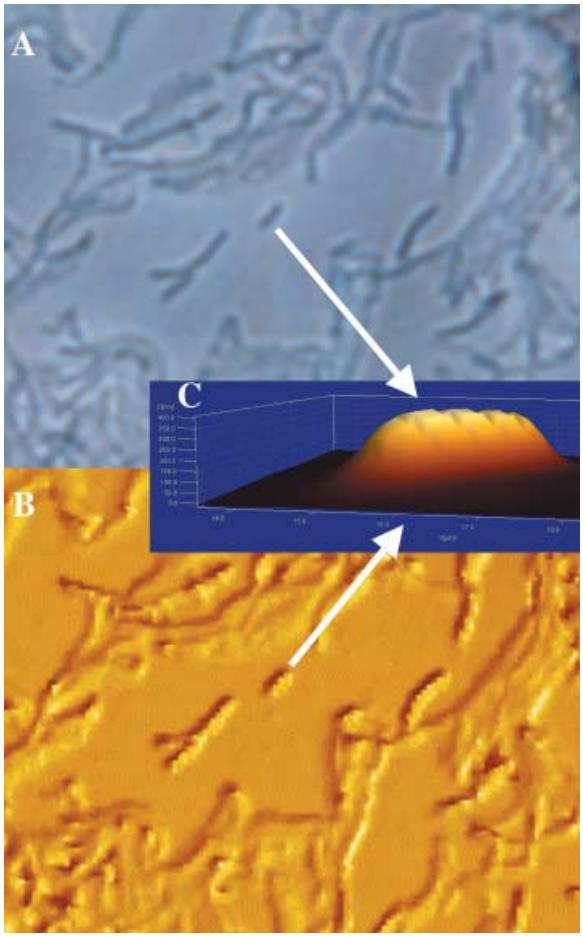

Fig. 2 *Shewanella oneidensis* MR1 on glass phase contrast and vertical scanning interferometry (VSI) comparisons. (A) Phase contrast image of bacterium attached to glass. (B) Comparative 3D VSI image. (C) Zoomed in 3D VSI image of individual bacterium.

using aluminium coupons yielded the similar results (data not shown).

In VSI, reflected light translates directly to data interpreted by software to highlight topographical differences in material, based on the distance traveled from the surface to the interferometer with respect to a reference mirror. This information is compiled into a two-dimensional height map, where height of each coordinate is defined by a gradient scaled color code. The distance of the highest intensity light fringe reflected from each x-y coordinate determines the height set per pixel, with reference to a neighbouring coordinate. Current VSI analytical software makes the assumption that the surface scanned is generally opaque and/or thick (relative to vertical scanning range). Bacteria, however, are small, quite translucent and have a somewhat Lambertian (diffusely reflective) surface. Comparatively, the reflection returned by the bacteria was ~60% less reflective than the specular surface on which it set (Fig. 4D–G). This suggested that we were accessing data from both the bacterium and the bacteria substratum interface.

To ensure that the apparent rod-shaped pit images generated by the VSI were indeed the result of bacteria, a constitutive GFP expressing plasmid, p519nGFP (Stretton *et al.*, 1998) was inserted into wild-type *S. oneidensis* MR-1. Under blue light excitation all of the areas on the mirror-polished steel that appeared as pits fluoresced green, confirming that they were *S. oneidensis* MR-1 containing p519nGFP (Fig. 4B). This result was confirmed by ethidium bromide staining.

Examination of other translucent and opaque objects on metal surfaces

To explore whether this phenomenon would be observable for all objects in contact with a specular surface, cultures of *S. oneidensis* MR-1 (p519nGFP) were mixed with hematite, similar in size to the bacteria, but opaque to white light. VSI measurements confirmed that only bacteria were represented inversely. Hematite appeared as structures sitting atop the surface features of mirror-polished steel (Fig. 5).

Because interferometric images are generated entirely based on reflected light, differences in surface reflectivity and opacity were examined between hematite and *S. oneidensis* MR-1. Tandem AFM/SNOM measurements confirm bacterial translucency, as described in the Materials and Methods section

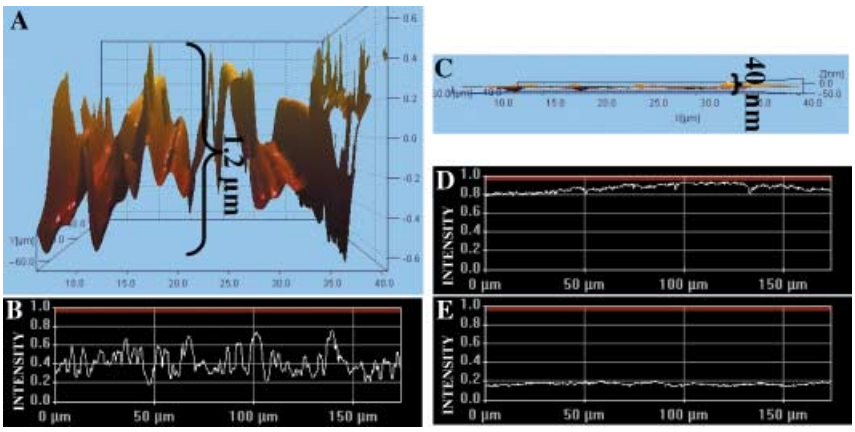

Fig. 3 Comparisons of polished and unpolished steel. (A,C) 3D vertical scanning interferometry image of unpolished and polished steel, respectively, equally scaled to each other for comparison. (B) Range of black and white fringe intensity of unpolished steel. (D,E) Range of white and black fringe intensity of polished steel, respectively.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

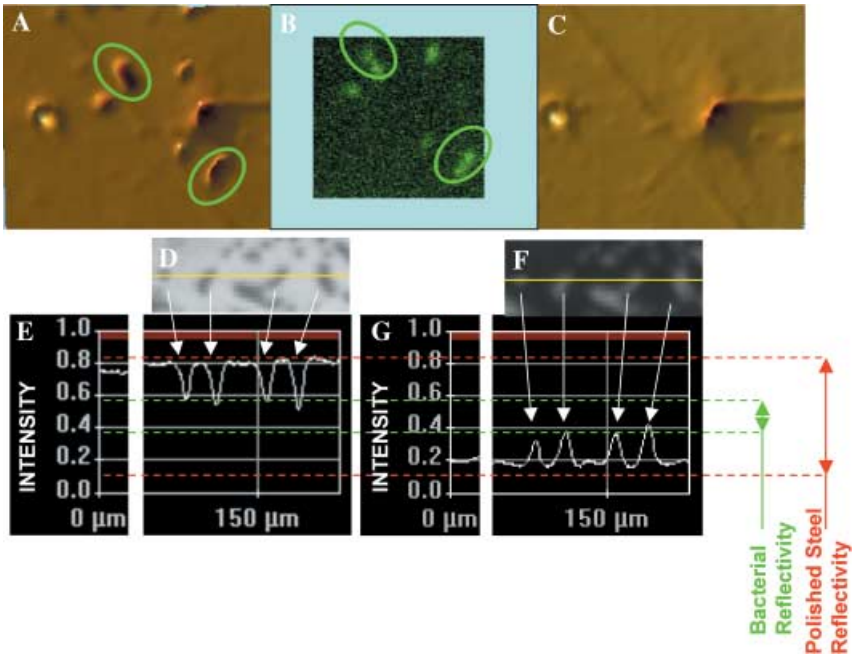

Fig. 4 Vertical scanning interferometry (VSI) images of *Shewanella oneidensis* MR1 on polished steel. (A) 3D VSI image of polished steel with bacteria attached (green circles). (B) Overlapping GFP expression from the same bacteria (green circles). (C) Overlapping 3D VSI image of polished steel after the bacteria have been wiped away. (D,F) Interferometric image of attached bacteria in white and black fringes. The yellow line is where the reflected light intensity is read. (E,G) Range of white and black fringe intensity of bacteria. White arrows point fringes in the interferogram to corresponding the light intensity profile peaks and valleys. The distance between green lines denotes reflectivity of bacteria. The distance between red lines denotes reflectivity of polished steel.

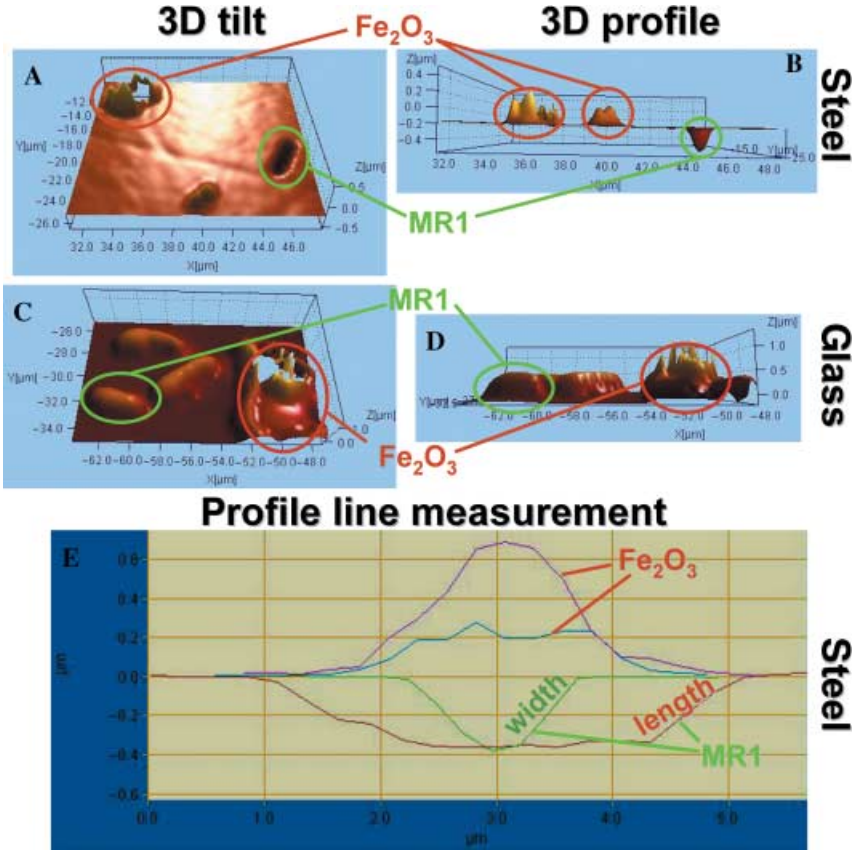

Fig. 5 Comparative vertical scanning interferometry (VSI) images of *Shewanella oneidensis* MR1 and hematite on polished steel. (A,B) 3D VSI image of polished steel with bacteria (green circles) and hematite (red circles). (C,D) 3D VSI image of glass with bacteria (green circles) and hematite (red circles). (E) Line measurements from an interferometric image across the length and width of an individual bacterium and two different hematite particles for comparison on the same polished steel surface.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

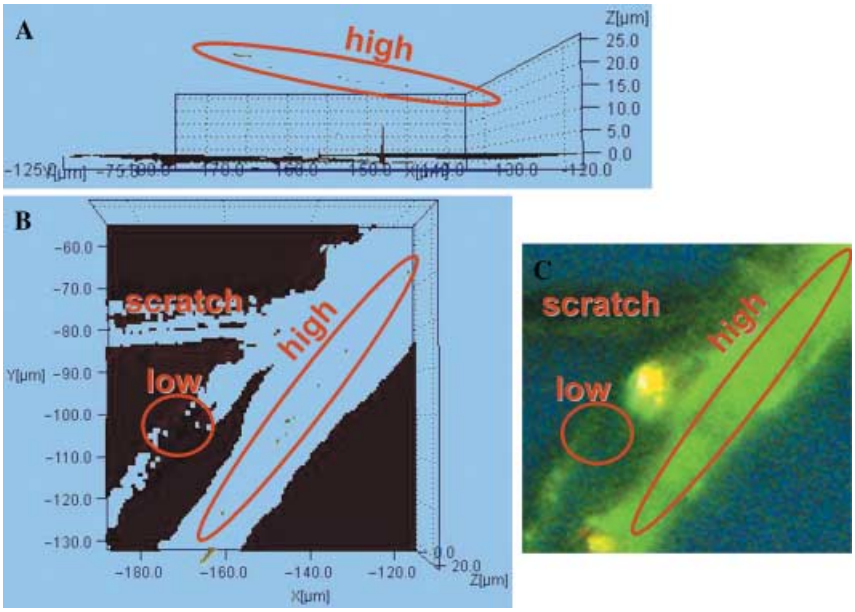

Fig. 6 Vertical scanning interferometry (VSI) images of a tapered cellulose fiber on polished steel. (A) 3D profile of VSI image of cellulose measurement on polished steel. (B) 3D top view of VSI image of cellulose measurement on polished steel, displaying inversed and normal measurements on the same surface from the same fiber. (C) Overlapping autofluorescent image of cellulose fiber.

above. Upon comparison of reflected light intensity, virtually the same amount of light was reflected from bacteria on average as hematite (data not shown).

To determine if the effective range of this optical artifact was consistent with descriptions of thin film optics (Land, 1972), a tapered, dry cellulose fiber was subjected to VSI characterization on mirror polished steel. Dry cellulose is a transparent, nonconductive, polysaccharide consisting of glucose moieties with β1–4 linkages. Height maps of the thinnest section of the cellulose fiber appeared as false impressions in the steel, while the thickest areas appeared to set as a structure above the mirror polished steel surface as expected (Fig. 6).The lack of any organized structure excluded the possibility that apparent image inversion was an effect of membrane-specific diffraction in bacterial samples. Again, experimental repetition on an aluminium surface yielded identical results.

Restoration of bacterial VSI image by staining

To confirm that the second, more intense set of fringes observed were a result of light reflected from the steel through the bacteria and were responsible for the image inversion, the opacity of *S. oneidensis* MR1 (previously measured by VSI) was increased with EPS and membrane stains. Upon staining, bacterial reflectivity was increased, enabling the bacteria to be measured as structures sitting above the steel surface (Fig. 7).

DISCUSSION AND CONCLUSIONS

Studies of metallic surfaces

One of the areas of research of our laboratories is the study of attachment of microbes to surfaces of different types, and the activities that the microbes exert once attached, including mineral dissolution, mineral formation, corrosion, corrosion inhibition, and other activities. It was thus of some concern to us that the VSI system, which worked so well on carbonate and glass surfaces, generated an optical artifact on polished metal surfaces.

However, in following up on the nature of this artifact, it now appears that it may be turned to our advantage. The apparent image-inversion effect described here turns out to be the result of conflicting interferometric information that is received (i) from the top membrane surface of the actual bacteria themselves and (ii) from the substrate surface underneath the bacteria. Current VSI software which is only equipped to distinguish data from a single surface misinterprets this effect. Here we show the image inversion observed is (at least) an effect of the transparency (Fig. 7) and short height (Fig. 6) of the bacteria in combination with the high reflectivity of the surface on which the bacteria sit (Fig. 4). This evidence points to thin film optics as the reason for the development of this artifact, in that some light is reflected from the top of the bacteria, but a greater portion of the light travels through the bacteria to the reflective surface. Some of the light that hits the surface of the steel is reflected back through the bacteria and to the interferometer. Simply put, if more reflected light equals more information about a surface and each pixel is defined by the distance at which maximum reflectivity is acquired, at each coordinate (as interpreted by the software) weakly reflective surfaces would be masked by their more intense mirror images, defined by the light returned that passed through the bacteria, reflected from the substratum and returned to the interferometer (Fig. 8). A similar observation was made recently when thin, transparent films were measured by VSI (Luttge *et al.*, 2005), consistent with our observations made here.

The ability to access information from the mineral–microbe

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

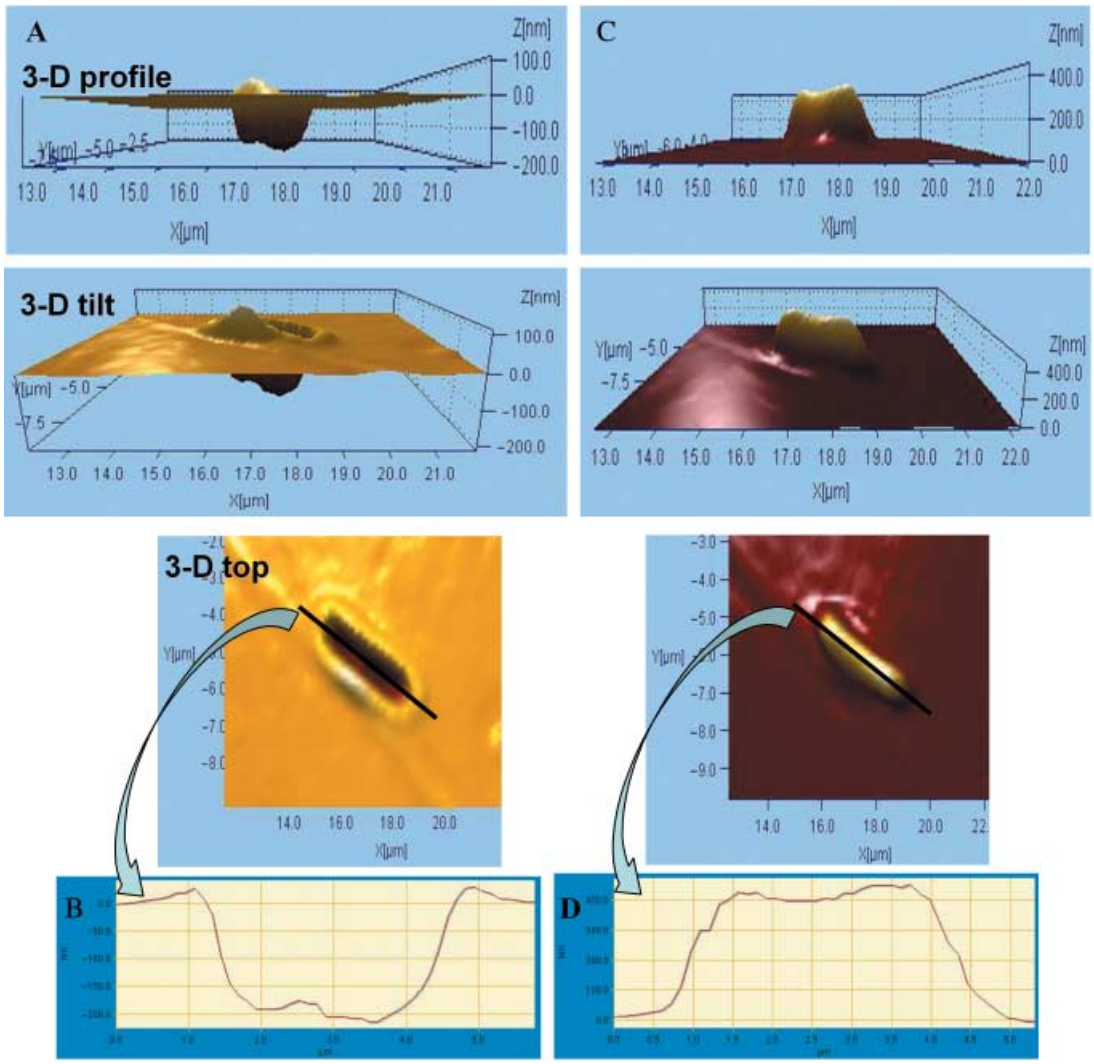

Fig. 7 Vertical scanning interferometry (VSI) images of normal and cross-linked *Shewanella oneidensis* MR1 on polished steel. (A) 3D VSI images of a transparent bacterium on polished steel. (B) Line measurement across a transparent bacterium on polished steel. (C) 3D VSI images of the same transparent bacterium made more reflective on polished steel. (D) Line measurement across the same transparent bacterium made more reflective on polished steel.

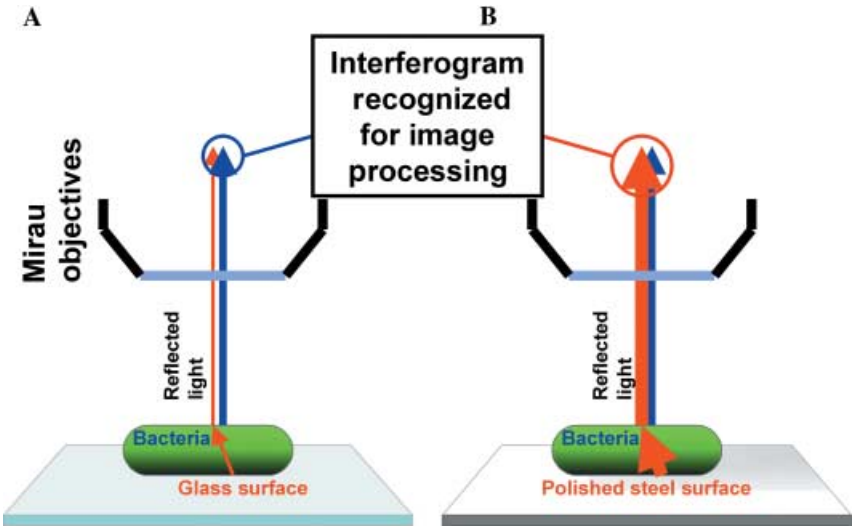

Fig. 8 Software recognition of surface profile based on height and signal intensity. Depicted here is a diagram of two vertical scanning interferometry (VSI) scans of a transparent bacterium on two different substrates. (A,B) illustrate reflected light data from the bacterial surface (blue arrow) and the substrate (red arrow) that corresponds to a single pixel on the XY axis, where a thicker arrow = greater light return. In (A), the reflected light from the bacteria is dominant and therefore is selected by the software, which then derives an accurate image. In (B), the reflected light from the lower mirrored steel surface overwhelms the light reflected from the higher bacterial surface, leading to a convoluted calculation of the pixel's point in 3D space.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

interface opens up a powerful opportunity to noninvasively directly monitor physical and biochemical changes to a surface directly beneath bacteria at the scale of a single microbe. Recognition and deconvolution of data from both the surface of the bacteria and the underlying substrate may be accomplished, in part, by altering VSI software recognition programs so they are capable of separating and distinguishing reflective peaks at different heights, so it is capable of assigning 2 pixels in the Z-axis for a single XY coordinate. Accommodation of multiple points of data input for a single XY coordinate offers the potential to utilize a rapidly made series of measurements that could track substrate dissolution or deposition at a location that has been invisible. Quantitatively discerning specifics about a microbial–surface interaction without significant sample disturbance, currently only offered by VSI, holds great potential for development of new technologies and re-examines what we think we know about prokaryotic physiology.

ACKNOWLEDGEMENTS

This work was supported by the Office of Naval Research award no. N00014-06-1-0115 and MURI award# FA9550-06-1-0292 to KHN, a grant from the AFOSR (DoD).

REFERENCES

Baumann L, Baumann P, Mandel M, Allen RD (1972) Taxonomy of aerobic marine eubacteria. *Journal of Bacteriology* **110**, 402.

Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* **62**, 293.

Bolshakova AV, Kiselyova OI, Yaminsky IV (2004) Microbial surfaces investigated using atomic force microscopy. *Biotechnological Progress* **20**, 1615.

Characklis WG (1981) Fouling biofilm development: a process analysis. *Biotechnology and Bioengineering* **14**, 1923.

Cunliffe D, Smart CA, Alexander C, Vulfson EN (1999) Bacterial adhesion at synthetic surfaces. *Applied and Environmental Microbiology* **65**, 4995.

Davis K, Luttge A (2005) Quantifying the relationship between microbial attachment and mineral surface dynamics using vertical scanning interferometry (VSI). *American Journal of Science* **308**, 727.

Davis KJ, Nealson KH, Luttge A (2007) Calcite and dolomite dissolution rates in the context of microbe–mineral surface interactions. *Geobiology* **5**, 191.

Hamilton W, Characklis WG (1989) *Relative Activities of Cells in Suspension and in Biofilms.* John Wiley, New York, NY.

Land MF (1972) The physics and biology of animal reflectors. *Progress in Biophysics Molecular Biology* **24**, 75.

Lasaga AC, Lüttge A (2001) Variation of crystal dissolution rate based on a dissolution stepwave model. *Science* **291**, 2400–2404.

Luttge A (2006) Crystal dissolution kineetics and Gibbs free energy. *Journal of Electron Spectroscopy and Related Phenomena* **150**, 248–259.

Luttge A, Conrad P (2004) Direct observation of calcite dissolution. *Applied and Environmental Microbiology* **70**, 1627–1632.

Luttge A, Zhang L, Nealson K (2005) Mineral surfaces and their implications for microbial attachment: results from Monte Carlo simulations and direct surface observations. *American Journal of Science* **308**, 766–790.

Marshall KC, Stout R, Mitchell R (1971) Mechanisms of the initial events in the sorption of marine bacteria to surfaces. *Journal of General Microbiology* **68**, 337.

Meyer P, Dworkin J (2007) Applications of fluorescence microscopy to single bacterial cells. *Research in Microbiology* **158**, 187.

Myers CR, Nealson KH (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science* **240**, 1319.

Palmer R Jr (1999) *Microscopy Flowcells: Perfusion Chambers for Real-Time Study of Biofilms.* Academic Press, San Diego, CA.

Stretton S, Techkarnjanaruk S, McLennan AM, Goodman AE (1998) Use of green fluorescent protein to tag and investigate gene expression in marine bacteria. *Applied and Environmental Microbiology* **64**, 2554.

Van Loosdrecht MCM, Norde W, Lyklema J, Zehnder AJB (1990) Hydrophobic and electrostatic parameters in bacterial adhesion. *Aquatic Science* **52**, 1015.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd