Mineral-Microbe Interactions Probed in Force, Energy, and Distance Nanospace

Steven K. Lower

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Michael F. Hochella, Jr., Chair
Duane Berry
Malcolm Potts
J. Donald Rimstidt
Christopher Tadanier

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(ABSTRACT)

Biological force microscopy (BFM) was developed to quantitatively measure pico- to nano-Newton forces (10^{-9} to 10^{-12} N) as a function of the nanoscale distance (nanometers) between living bacteria and mineral surfaces, in aqueous solution. Native cells were linked to a force-sensing probe, which was used in a force microscope to measure attractive and repulsive forces as a mineral surface approached, made contact with, and subsequently withdrew from a bacterium on the probe. The resulting data were used to interpret the interactive dynamics operative between bacteria and mineral surfaces under environmentally relevant conditions.

BFM was used to study bacterial adhesion to mineral surfaces. In the case of *Escherichia coli* interactions with goethite, graphite, and muscovite, attractive and repulsive forces were detected at ranges up to 400 nanometers, the magnitude and sign depending on the ionic strength of the intervening solution and the mineral surface charge and hydrophobicity. Adhesion forces, up to several nanoNewtons in magnitude and exhibiting various fibrillation dynamics, were also measured and reflect the complex interactions of structural and chemical functionalities on the bacteria and mineral surfaces. In the study of *Burkholderia cepecia* interactions with mica, it was found that the physiological condition of the cell affected the observed adhesion forces. Cells grown under oligotrophic conditions exhibited an increased affinity for the mineral surface as opposed to cells grown under eutropic conditions.

BFM was also used to characterize the transfer of electrons from biomolecules on *Shewanella oneidensis* to Fe(III) in the structure of goethite. Force measurements with picoNewton resolution were made in aqueous solution under aerobic and anaerobic conditions. Energy values (in attoJoules) derived from these measurements show that the affinity between *S. oneidensis* and goethite rapidly increases by two to five times under anaerobic conditions where electron transfer from bacterium to mineral is expected. Specific signatures in the force curves, analyzed with the worm-like chain model of protein unfolding, suggest that the bacterium recognizes the mineral surface such that a 150 kDa putative, iron reductase is quickly mobilized within the outer membrane of *S. oneidensis* and specifically interacts with the goethite surface to facilitate the electron transfer process.

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Chapter 1 - Introduction

The fundamental forces between a bacterium and mineral surface are central to understanding the intricacies of interfacial phenomena such as bacterial attachment to or detachment from minerals, biofilm formation and structure, bacterial affinity for or recognition of specific mineral surfaces, and dispersal of microorganisms in the environment. In nature, a myriad of physical and chemical interactions occur at bacteria-mineral interfaces due to the mosaic of macromolecular structures on bacteria, and the diversity of mineral surface functionality and crystallography. Mineral-microbe interactions are governed by the cumulative effects of fundamental, nano- (10^{-9} N) to pico-scale (10^{-12} N) forces such as van der Waals, electrostatics, solvation interactions, and steric interactions. However, acquiring even an elementary appreciation of these forces presents a daunting challenge, primarily due to the minute scale at which these interfaces must be probed, and the difficulty in developing a technique that preserves the natural intricacies of bacteria surfaces. This dissertation describes the development and use of a new technique, biological force microscopy (BFM), which is capable of quantitatively measuring interfacial and adhesion forces between living bacterial cells and mineral surfaces (or other living cells), in situ.

Chapter two was published in *Geochimica et Cosmochimica Acta*, 2000, 64: 3133-3139. Access to this manuscript may be obtained at http://www.elsevier.com/. In this chapter, BFM is used to measure attractive and repulsive forces in the nanoNewton range between *Escherichia coli* and muscovite, goethite, and graphite surfaces at separations of <1 μm in aqueous solutions (pH 6, 25 °C) of varying ionic strength. The resulting data are used to interpret the intermolecular forces operative between living bacteria and mineral surfaces in natural environments.

Chapter three was published in *Geomicrobiology Journal*, 2001, 18: 63-76. Access to this manuscript may be obtained at http://www.tandf.co.uk/. This chapter presents a detailed description of the use of BFM to accurately measure nanoscale force and distance relationships between a bacterium and mineral surface. The fabrication of biologically-active-force-probes (i.e., force-sensing probes activated with living cells) is also discussed in detail. BFM is used to

measure approach and retraction forces between mica and *Burkholderia spp.* grown under high or low nutrient conditions. The resulting data are used to discuss the role that physiology plays in adhesion strategies.

Chapter four is in review in *Science*, 2001. If accepted, access to this manuscript may be obtained at http://www.sciencemag.org/. In this chapter, BFM is used to probe electron transfer processes between a dissimilatory-metal-reducing-bacterium and iron oxyhydroxides. Force measurements with picoNewton resolution were made with living cells of *Shewanella oneidensis* (also called *S. putrefaciens*), in aqueous solution under aerobic and anaerobic conditions as a function of the nanoscale (in nanometers) distance between the cell and mineral surface. Energy values (in attoJoules = 10^{-18} J) derived from these measurements show that *S. oneidensis* recognizes goethite such that the affinity between the bacterium and mineral rapidly increases by two to five times under anaerobic conditions where electron transfer from bacterium to mineral is expected. Specific signatures in the force curves, analyzed with a theoretical model describing conformational changes in proteins, suggest that a 150 kDa putative, iron reductase is quickly mobilized within the outer membrane of *S. oneidensis* and specifically interacts with the goethite surface to facilitate the electron transfer process.

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Chapter 2 - Measuring interfacial and adhesion forces between bacteria and mineral surfaces with biological force microscopy

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Abstract

Interfacial and adhesion forces between living, unmodified bacterial cells (*Escherichia coli*) and mineral surfaces (muscovite, goethite, and graphite) have been directly measured in aqueous solution using a force microscope. Native cells are linked to a force-sensing probe that is used to characterize interactions as a mineral surface approaches, makes contact with, and withdraws from bacteria on the probe. Attractive and repulsive interfacial forces were detected at ranges up to 400 nanometers separation, the magnitude and sign depending on the ionic strength of the intervening solution and the mineral surface charge and hydrophobicity. Adhesion forces, up to several nanoNewtons in magnitude and exhibiting various fibrillation dynamics, were also measured and reflect the complex interactions of structural and chemical functionalities on the bacteria and mineral surfaces.

Introduction

The fundamental forces between a bacterium and mineral surface are central to understanding the intricacies of interfacial phenomena such as bacterial attachment to or detachment from minerals (van Loosdrecht et al., 1989; Fletcher, 1996; Yee et al., 2000), mineral dissolution and crystal growth (Myers and Nealson, 1988; Hiebert and Bennett, 1992; Schultze-Lam et al., 1992; Roden and Zachara, 1996; Fortin et al., 1997), biofilm formation and structure (Lawrence et al., 1991; Davies et al., 1998), bacterial affinity for or recognition of specific mineral surfaces (Ohmura et al., 1993; Fleminger and Shabtai, 1995; Bhosle et al., 1998; Dziurla et al., 1998; Edwards et al., 1998), and dispersal of genetically engineered microorganisms in the environment (Gannon et al., 1991; Mills and Powelson, 1996; Trevors and van Elsas, 1997). A myriad of physical and chemical interactions occur at bacteria-mineral interfaces in nature, due

to *(i)* the mosaic of spatially discrete macromolecular cell envelope structures on bacteria, *(ii)* the dynamic nature of these structures imposed by various environmental conditions, and *(iii)* the diversity of mineral surface functionality and crystallography. These interactions are governed by the cumulative effects of interfacial forces when bacteria and minerals are separated by some finite distance, and by adhesion forces when in intimate contact (Israelachvili and McGuiggan, 1988; Israelachvili, 1992; Kendall, 1994; Butt et al., 1995; Fletcher, 1996; Gay and Leibler, 1999). However, acquiring even an elementary appreciation of these forces presents a daunting challenge, primarily due to the minute scale at which these interfaces must be probed, and the difficulty in developing a technique that preserves the natural intricacies of the bacteria surface.

Here we introduce and describe a new technique, biological force microscopy (BFM), which is capable of quantitatively measuring interfacial and adhesion forces between native bacterial cells and mineral surfaces, in situ. BFM was inspired by research that uses atomic force microscopy to study inter- and intra-molecular interactions between organic and inorganic surfaces with resolutions as small as a few picoNewtons (Ducker et al., 1991; Tsao et al., 1993; Florin et al., 1994; Frisbie et al., 1994; Lee et al., 1994; Moy et al., 1994; Boland and Ratner, 1995; Dammer et al., 1995; Hinterdorfer et al., 1996; Rief et al., 1997a; Rief et al., 1997b; Wong et al., 1998; Grandbois et al., 1999). In this study we use BFM to measure attractive and repulsive forces in the nN range between *Escherichia coli* and muscovite, goethite, and graphite surfaces at separations of <1 μm in aqueous solutions (pH 6, 25 °C) of varying ionic strength (low $I = 10^{-5}$ M, high $I = 10^{-1}$ M). The resulting data are used to interpret the interactive dynamics operative between living bacteria and mineral surfaces.

Material and Methods

Mineral and Bacteria Specimens

Mineral specimens were selected to span a range of surface charges and hydrophobicities. Their points-of-zero-charge (pzc) and contact angles (θ) are (Stumm and Morgan, 1996; Adamson and Gast, 1997): pzc = 2-3, θ = near 0° for muscovite; pzc = 8-9, θ = near 0° for goethite; and pzc = 7-8, θ = 85-90° for graphite. Muscovite and graphite were cleaved immediately before using them, whereas goethite crystals were cleaned by agitation in ultrapure

water (Milli-Pore), acetone, and ethanol to remove adventitious carbon (Stipp and Hochella, 1991).

E. coli K-12 was selected because of the wealth of structural, physiological, and molecular information on this organism and also because of increasing concerns related to the release of this bacterium into the environment. Wastewater treatment facilities and wastewater reclamation projects, for example, are interested in the interaction of this bacterium with sorbtive mineral phases (Crook et al., 1998). Furthermore, as *E. coli* can develop natural competence (i.e., genetic transformation in natural environments) (Baur et al., 1996), improper disposal of genetically altered strains may pose a significant threat to human health and natural ecosystems (Av-Gay, 1999).

Electroporation was used to transform bacterial cells with a plasmid (pGLO, Bio-Rad) encoding a green fluorescent protein (Dower et al., 1988; Miller et al., 1988). Electro-competent cells were transformed with a Gene-Pulser (Bio-Rad) at 25 μF, 12.5 kV cm^{-1}, 4 msec. Induction of the plasmid was accomplished by culturing the cells in 0.02 M L-arabinose ($C_5H_{10}O_5$). Transformed cells were grown to exponential phase, plated onto agar, and allowed to grow overnight. Several colonies were scraped from the plate, washed in 10^{-5} M sodium chloride, and used in BFM experiments (see below). These transformed bacteria produced an intracellular fluorophore, which allowed us to characterize the distribution of cells on the force sensors without using dyes that would bind to outer surface macromolecules potentially altering cell surface properties (see below).

Design and Characterization of Biologically-Active-Force-Probes

Force-sensing probes, termed biologically-active-force-probes (BAFPs), were fabricated by linking a minute bacteria-coated-bead to a silicon nitride cantilever in a manner that conserved the orientation, structural integrity, and conformation of macromolecules on the bacterial surface (Fig. 2.1). Glass beads (~5 μm radius; Duke Scientific or Polysciences) were

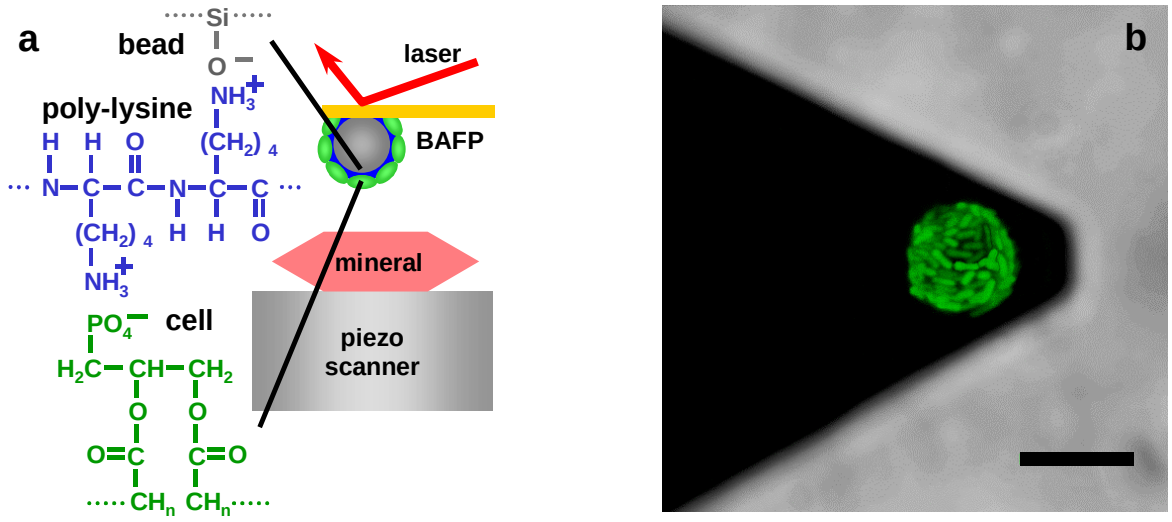

Figure 2.1. (a) Biological force microscopy components highlighting the linkage between bacterial cells and the force-sensing cantilever. Illustrated is one of many possible poly-lysine linkages between negatively charged silanol groups on the bead and negatively charged cell-surface functional groups on biomolecules, such as phospholipids (shown here) or lipopolysaccharides. A piezoelectric scanner was used to translate a mineral to and from a biologically-active-force-probe (BAFP). Forces between bacteria on the probe and the mineral were detected by reflecting a laser off the top of the cantilever and into a photodiode detector. (b) Scanning laser confocal micrograph of a BAFP (perspective is from the mineral surface looking towards the BAFP). Bacteria are fluorescing due to excitation of an intracellular protein. Biological activity of these cells was demonstrated by culturing a colony on an agar plate using the probe as the inoculum. Scale bar 10 µm.

cleaned with hydrofluoric acid (1 % solution for 1.5 minutes) or sodium hydroxide (50 % solution for 60 minutes), rinsed with ultrapure water (Milli-Pore), and functionalized with amino groups by incubation in a 2% solution of 135 kDa poly-D-lysine (Sigma) for ~4 hours. This procedure creates a 1-3 nm thick, positively charged monolayer on glass beads (Pagac et al., 1998a; Pagac et al., 1998b). Amine-functionalized beads were dispersed in a suspension of washed cells and spun at 8,000 x *g* for 5 minutes at 4 °C. A single bead supporting a monolayer of cells was attached to a cantilever using a small amount of epoxy resin, which has previously been found to be inert in aqueous solutions (Pincet et al., 1995; Yoon et al., 1997). This attachment procedure was conducted in solution with the aid of a microscope (Nikon, 200X magnification) and a micromanipulator to translate the cantilever.

By using whole bacteria expressing macromolecules in their natural state rather than individual biomolecules (e.g., exopolysaccharides, proteins) purified from bacterial surfaces, we avoided situations in which the linkage procedure modified the conformation of biomolecules such that they were no longer in a natural state (Stotzky, 1986; Ellen and Burne, 1996; Turner et al., 1996; Ingersoll and Bright, 1997; Turner et al., 1997). Linkage of cells to the cantilever is extremely stable. The strength of bonds between poly-lysine and either glass or bacteria are on the order of 1000 kJ molecule^{-1} (Voet and Voet, 1995; West et al., 1997), which is at least one to four orders of magnitude greater than interfacial and adhesion forces expected to occur between bacteria and mineral surfaces (Israelachvili, 1992).

A BAFP was placed in the fluid-cell used in force measurements and imaged with a scanning laser confocal microscope LSM-510 Axiovert 100M (Zeiss) using a 100X, 1.4 N.A. objective lens. The confocal ability of the microscope, and cellular expression of the intracellular fluorophore encoded by the inserted plasmid, allowed noninvasive characterization of the three-dimensional nature of BAFPs.

Biological Force Microscopy Measurements

BFM measurements were performed in sodium chloride solutions using a NanoScope IIIa Multimode SPM (Digital Instruments). The deflection of a BAFP was monitored as an oriented mineral grain (mounted on a piezoelectric scanner) was indexed towards, made contact with, and

retracted from bacteria on the probe (Fig. 2.1). The mineral was translated at rates of <3 μm sec^{-1} which is within the range of velocities of motile bacteria (Marshall, 1976). Interfacial forces were measured as the mineral approached the bacteria on the probe; whereas adhesion forces were measured upon contact and subsequent retraction of the mineral from the bacteria. Mineral samples were driven to the same contact force to normalize the effect that loading can have on measured forces during retraction (Weisenhorn et al., 1992). To ensure reproducibility, measurements were taken as solution in the fluid-cell was cycled between low *I* and high *I* four to five times per mineral.

Force-distance curves were constructed from photodiode-voltage versus piezo displacement data (i.e., "force curves") (Ducker et al., 1991; Ducker et al., 1992). Diode response (in volts) was converted to cantilever deflection (in meters) using the diode/displacement conversion factor defined by the region of constant compliance (i.e., slope on the force curve where diode response becomes a linear function of piezo displacement). Hooke's Law, $F = k_{sp}\, d$, where d is cantilever deflection and k_{sp} is the cantilever spring constant (0.17 N m^{-1}; determined according to Cleveland et al., 1993), was then used to obtain the force of interaction (in Newtons). The distance-axis origin was defined as the point of intimate contact (i.e., beginning of the region of constant compliance). This conversion method is appropriate when the cantilever is the most compliant component of the system (Hutter and Bechhoefer, 1993). A region of constant compliance with 1:1 correspondence between probe deflection and piezo displacement was observed in all approach-retraction cycles in this study. Recent elasticity measurements of various bacterial surface macromolecules suggest that this situation is valid for most bacteria (Xu et al., 1996; Yao et al., 1999). However, for cells that are more compliant than the cantilever or have fragile appendages, it will be necessary to use a method that does not require bacteria-mineral contact (D'Costa and Hoh, 1995; Sader et al., 1999).

The results presented herein illustrate the interaction between a mineral surface and an aggregate of cells rather than single bacterium. The surface properties of an aggregate of cells may differ from those of a single cell. It should be noted, however, that force measurements made with force microscopes are presumed to involve the interaction of only a few tens to hundreds of square nanometers (Butt et al., 1995). These dimensions are closer to the size of a

single bacterium than the entire aggregate of cells on the cantilever. Single bacterium have been attached to cantilevers (results not shown), but this linkage is extremely difficult. Recent advances in optical tweezer (Svoboda and Block 1994) and nanotweezer (Kim and Lieber 1999) technologies could significantly enhance the linkage of single cells to a cantilever.

Results

Net repulsive interfacial forces were observed for muscovite approach curves (Fig. 2.2a). Conversely, *E. coli*-goethite and -graphite systems exhibited more attractive interfacial forces on approach (Fig. 2.2b, 2.2c). For these latter systems, jump-to-contact events were observed at distances of <20 nm because the force gradient between bacteria and mineral surface exceeded the cantilever spring constant. Ionic strength effects were noted for all mineral-bacteria systems. In general, the magnitude of interfacial forces and distances over which they were operative diminished at high *I*.

Adhesion forces of varying magnitude were observed for each bacteria-mineral system upon retraction and were sensitive to solution *I*. At high *I* the muscovite system showed adhesion and the retraction curve displayed a large number of discrete "pull-off" events to separations of ~400 nm (Fig. 2.2a). At low *I* the muscovite system did not exhibit attractive adhesion forces. Goethite and graphite systems were markedly different as they exhibited decreased adhesion force at high *I* and displayed jump-from-contact events because the cantilever spring constant exceeded the force gradient between bacteria and mineral surface (Fig. 2.2b, 2.2c).

Results from the control experiment between muscovite and a naked poly-lysine coated bead (no bacteria) (Fig. 2.2d) were very different from those between muscovite and an *E. coli* coated bead (Fig. 2.2a). As expected, the positively charged poly-lysine exhibited strong attraction towards the (001) surface of muscovite which is negatively charged at pH 6. Hysteresis between the approach and retraction curves was absent for the interaction between muscovite and poly-lysine. These observations indicate that the poly-lysine linker did not affect forces measured between the bacteria and mineral.

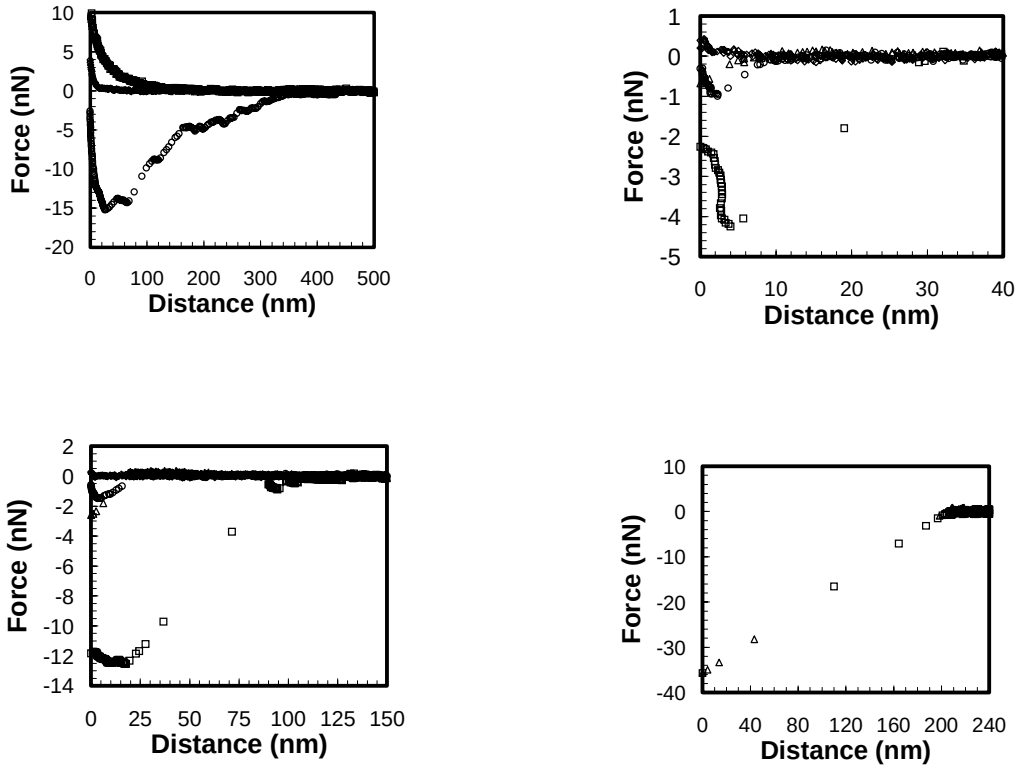

Figure 2.2. Force-distance curves for *Escherichia coli* K-12 and (a; top left) muscovite (001) surface, (b; top right) goethite (010) surface, and (c; bottom left) graphite (001) surface at pH 6, 25ºC and varying ionic strengths (*I*). (d; bottom right) Control experiment for muscovite (001) surface and naked poly-lysine coated bead. Jump-to- or jump-from-contact events result in linear segments with a slope corresponding to the cantilever spring constant (0.17 N m^{-1}). Curve convention: (triangles) $I = 10^{-5}$ M, approach; (squares) $I = 10^{-5}$ M, retraction; (diamonds) $I = 10^{-1}$ M, approach; (circles) $I = 10^{-1}$ M, retraction. Force sign convention: (+) repulsive; (–) attractive. Note differences in axes scales.

The bacteria-mineral data presented above were performed by applying the same BAFP to muscovite, goethite, graphite, and then once again to muscovite. Only minor variation was observed between the initial and replicate muscovite experiments indicating that bacterial surfaces were not significantly altered by repeated contact with mineral surfaces. Results shown in Fig. 2.2 are representative of 300-400 force-distance curves collected using three separate *E. coli* BAFPs. While results were highly reproducible (e.g., errors in measurement of muscovite-bacteria approach curves are ~± 0.2 nN in force and ~± 2.0 nm in distance), some variation was observed particularly in retraction curves. The dynamic nature of the macromolecular cell envelope mosaic (Beveridge, 1999) and specific cell orientation on a BAFP are likely to account for the variation.

Discussion

Categories of Interfacial and Adhesion Forces

All intermolecular forces ultimately depend on the distribution of electrons surrounding interacting particles or surfaces (Israelachvili, 1992). Unfortunately, theoretical and experimental studies of the electronic structures of complex biological and mineralogical surfaces are still in their infancy (e.g., Becker et al., 1996; Beveridge, 1999; Rosso et al., 1999). Therefore, the fundamental interactions responsible for interfacial and adhesion forces have been operationally classified into a number of force categories which differ in magnitude, sign, and operative range.

Van der Waals forces and hydrophobic interactions are generally considered attractive and predominate from contact to tens of nm separation, whereas electrostatic forces may be attractive or repulsive and tend to predominate at greater separations (Israelachvili and McGuiggan, 1988; Israelachvili, 1992; Butt et al., 1995; Fletcher, 1996; Yoon et al., 1997). The magnitude and operative distance of electrostatic forces decrease with increasing *I*, commonly termed the electrostatic double-layer effect. Polymer interactions or bridging forces, involving long-chain organic molecules, represent the combined effects of several forces including hydrophobics, electrostatics and van der Waals. These interactions may be attractive or repulsive depending on ionization of functional groups and typically extend outwards to

hundreds of nm (van Loosdrecht et al., 1990; Israelachvili, 1992; Biggs, 1995; Jucker et al., 1998). Forces attributable to ionic-polymers decrease with increasing *I*, whereas neutral-polymers are unaffected by different *I* (Frank and Belfort, 1997).

At intermediate distances (0.5 to 5 nm), repulsive forces related to solvation/hydration and steric/entropic effects become operative and may be influenced by *I* dependent conformation changes in polymeric surface molecules or solution charge screening (Israelachvili and McGuiggan, 1988; Israelachvili, 1992; Butt et al., 1995). At extremely small separations (< 1 nm), attractive, specific interactions between surfaces such as hydrogen bonding, cation bridging, and receptor-ligand interactions may occur (Israelachvili and McGuiggan, 1988; Israelachvili, 1992; Butt et al., 1995; Fletcher, 1996; Kinloch, 1996).

Mineralogical and Microbiological Aspects of Forces

Deconvolution of the net force between two surfaces into contributions made by individual interaction mechanisms would require detailed spectroscopic, microscopic, and surface structural information. Additionally, it is beyond the scope of this paper to compare the measured interfacial and adhesion forces to those predicted by models such as DLVO (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) and JKR (Johnson et al., 1971), respectively. None-the-less, a general interpretation consistent with the characteristics of the force types outlined above can be made from the force-distance curves shown in Fig. 2.2.

For the interaction between *E. coli* and muscovite, both of which are negatively charged at pH 6, repulsive interfacial forces observed for the approach curves are consistent with electrostatic forces (Fig. 2.2a). The magnitude of repulsion and distance over which the force was operative diminished at high ionic strength (*I*) as expected due to compression of the electrostatic double layers around the bacteria and muscovite (Israelachvili, 1992) and the flattened conformation of negatively charged polymers on the bacteria surface (Frank and Belfort, 1997; Jucker et al., 1998).

An adhesion force was observed for the *E. coli*-muscovite system at high *I* as the retraction curve displayed a large number of discrete “pull-off” events to separations of ~400 nm

(Fig. 2.2a). This behavior is indicative of formation of strong specific interactions after contact. The discrete pull-off events and plateau beginning at a separation of ~ 150 nm are reminiscent of fibrillation or stringing – formation and subsequent rupture of discrete adhesive fibers or fibrils – which may occur upon separation of two surfaces bonded by a polymeric adhesive film (Kendall, 1994; Gay and Leibler, 1999). This phenomenon occurs particularly if one or both of the surfaces are molecularly smooth (e.g., muscovite) allowing intimate contact to be established (Kendall, 1994; Kinloch, 1996).

Adhesion at high *I* is likely due to interactions of cell-surface macromolecules such as lipopolysaccharides and fimbriae/pili with the mineral surface (Fletcher, 1996; Neidhardt, 1996). Pull-off events observed at distances of less than ~50 nm are consistent with the formation of hydrogen bonds between lipopolysaccharide hydroxyl groups and surface hydroxyl or structural water molecules on muscovite (Jucker et al., 1997). Proteinaceous structures like fimbriae/pili would be expected to interact for up to ~500 nm through either electrostatic or hydrogen bonding interactions between charged amino acid residues and mineral surface charges particularly at high *I* due to charge screening of the intervening solution (Fletcher, 1996; Frank and Belfort, 1997).

For the interaction between *E. coli* and goethite, interfacial attraction was expected because the mineral and bacteria are oppositely charge at pH 6. While attraction was observed upon approach (Fig. 2.2b), it was relatively weak. Adsorbed anions (e.g., phosphate), from an undetermined source, were detected on crystals taken from the same batch as that used in force measurements (x-ray photoelectron results not shown). Adsorption of anions is known to depress the pzc of iron-oxides causing them to be less positive (McBride, 1994) and therefore have a weaker interaction with negatively charged bacteria. Decreased interfacial attraction upon approach at high *I* is consistent with the withdrawal of negatively charged bacterial polymers from solution (Fletcher, 1996; Frank and Belfort, 1997). Attractive adhesion forces, observed upon retraction, were relatively weak and short range (Fig. 2.2b). For goethite, a relatively rough, growth surface was used rather than a smooth, cleavage surface as was the case for the other two minerals. Therefore intimate contact, a prerequisite for strong adhesive bonds (Kinloch, 1996), could not be established resulting in weak adhesion forces.

Hydrophobic interactions appropriately account for the attractive interfacial behavior observed upon approach of *E. coli* to the hydrophobic and sparingly charged graphite surface (Fig. 2.2c). Relatively strong, longer-range adhesion was observed upon retraction. This is consistent with the formation of hydrophobic bonds between nonpolar residues on *E. coli* and the hydrophobic graphite surface (Fletcher, 1996; Yoon et al., 1997). The decrease in attractive interactions at high I is consistent with the influence of solution electrolyte concentration on hydrophobic forces (Tsao et al., 1993).

Conclusion

BFM provides a holistic approach for directly measuring interfacial and adhesion forces between microorganisms and solid surfaces in situ. This technique has the potential to be highly versatile based on the unique manner in which BAFPs are fabricated. Activated beads tailored to accommodate the vast biochemical diversity of biological surfaces can be synthesized with a variety of linkers presently used in various chromatographic techniques (e.g., ligand-receptor groups and silanized hydrocarbons functionalized with ionizable or hydrophobic groups). Furthermore, by changing the cantilever spring constant (i.e., using levers of different geometry or composition) a wide range of interfacial and adhesion forces can be probed using one BAFP protocol.

BFM could be used as a method for studying fundamental processes associated with the mineral-microbe interface, particularly when it is used to complement other techniques. Several examples are discussed below. (1) Quantitative evidence of bacterial recognition of mineral surfaces could be addressed with BFM. (2) By using BFM with microbial mutants lacking specific surface biomolecules, one could assess the roles of proteins, lipopolysaccharides, and extracellular polysaccharides in attachment or detachment processes. (3) BFM could also be used to explore mineralogical control on the genetic expression of microbial surface structures. Such studies could be complimented by noninvasive imaging techniques such as confocal microscopy and scanning near-field optical microscopy, fluorophores that target specific molecules or biochemical structures, and/or fluorescence resonance energy transfer. (4) Mineral surfaces with and without organic conditioning films could be probed to assess the adhesive

properties of humic or fulvic acids. (5) BFM could be used to study inter- and intra-species signaling of microorganisms within a biofilm or other community by probing bacteria-bacteria interactions, host-pathogen recognition, and/or the effects aqueous biochemical signals have on quorum sensing phenomena. (6) BFM could provide insight into enzyme activity or conformation changes in cell surface proteins involved in oxidation/reduction reactions at mineral surfaces. In so doing, BFM may contribute to a better understanding of electron transfer at the microbe-mineral interface. (7) Forces measured with BFM could be compared to force models (e.g., DLVO and JKR) to gain a fundamental understanding of attachment and detachment phenomena. Techniques like biological force microscopy have the potential to open a new door in mineral-microbe research, one in which researchers are able to gain a fundamental insight into the nanoscale world that exists at the interface between microorganisms and minerals in nature.

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Chapter 3 - Dynamics of the mineral-microbe interface: Use of biological force microscopy in biogeochemistry and geomicrobiology

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Abstract

At the most fundamental level, inter- and intra-molecular forces delineate the interface between a microorganism and a mineral surface. A new technique, termed biological force microscopy (BFM), is described which can be used to directly probe the dynamics of the mineral-microbe interface. BFM quantifies attractive and repulsive forces in the nanoNewton range between living microbial cells and mineral surfaces in aqueous solution. Native bacterial cells are linked to a force-sensor that is used in a force microscope to measure bacteria-mineral interactions as a function of the distance between the mineral surface and the cells on the sensor. The magnitudes and ranges of the measured forces reflect the chemical and structural intricacies of the mineral-microbe interface. BFM is presented with potential applications to studies assessing the role microbes or biomolecules play in geochemical and mineralogical processes.

Introduction

Mineral-microbe interactions have been occurring for at least 3 billion years on and within the earth. Thousands of mineral species with enormous variability in surface chemistry and structure may interact with any of millions of bacterial species that display diverse surface mosaics and physiologies. Minerals and microorganisms are intimately linked such that one often cannot exist without the other in nature. Microbial processes play roles in the cycling of elements and sorption of metals (Ehrlich 1990; Fein et al. 1997; Langley and Beveridge 1999; Marshall 1996; Stotzky 1986), the dissolution of minerals (Banfield and Hamers 1997; Barker et al. 1998; Edwards et al. 1998a; Edwards et al. 1998b; Forsythe et al. 1998; Grantham et al. 1997; Hersman et al. 1995; Hersman et al. 1996; Krumbein et al. 1991; Lovley and Phillips 1986; Lovley and Phillips 1987; Myers and Nealson 1988; Robert and Berthelin 1986; Roden and

Zachara 1996; Schrenk et al. 1998; Stone 1997; Welch and Ullman 1993; Welch and Vandevivere 1994), and mineral crystallization (Beveridge and Doyle 1989; Fortin and Beveridge 1997; Fortin et al. 1997; Pentecost and Bauld 1988; Schultze-Lam et al. 1996; Schultze-Lam et al. 1992; Warren and Ferris 1998). Conversely, mineralogical processes influence the distribution, activity, and diversity of microbes (Barker et al. 1997; Bennett et al. 1996; Fletcher 1996a; Fredrickson et al. 1995; Marshall 1996; Rogers et al. 1998; Schrenk et al. 1998; Stotzky 1986), the expression of genes (Arredondo et al. 1994; Dziurla et al. 1998; Fletcher 1996a; Gehrke et al. 1998), community structure and development (Brown et al. 1997; Kennedy and Gewin 1997; Lawrence et al. 1991; Thorseth et al. 1995; Wolfaardt et al. 1994), and transfer of genetic material (Holben 1997; Trevors and van Elsas 1997).

The thread linking these unimaginably complex interactions is the fact that mineral-microbe processes are dependent upon the intimate juxtaposition of a living and nonliving entity, that is, the *interface* between a microbial cell and a mineral surface. A fundamental appreciation of this interface is dependent upon our understanding and characterization of the symphony of inter- and intra-molecular forces between microbes and mineral surfaces in nature. Despite the vast amount of work on microbial affects on mineralogical processes and vice versa, the interface remains largely unexplored due primarily to the fact that it is difficult to directly probe this minute and dynamic space.

Atomic force microscopy – and variations thereof – is an elegant tool for measuring inter- and intramolecular forces between organic and inorganic surfaces (Figure 3.1) (for review see Butt et al. 1995; Cappella and Dietler 1999; Lower and Maurice 2001). Recently, we created biological force microscopy (BFM) as a method to *directly* probe interfacial and adhesion forces between bacteria and mineral surfaces in aqueous solution (Lower et al. 2000). Living cells are linked to a sensor that is used to quantitatively measure attractive and repulsive forces in the nanoNewton range between bacteria and mineral surfaces at distances between zero (i.e., contact) and 1 µm. Measured forces reflected the complex interactions of structural and chemical functionalities on the bacteria and mineral surfaces. Herein, we demonstrate and

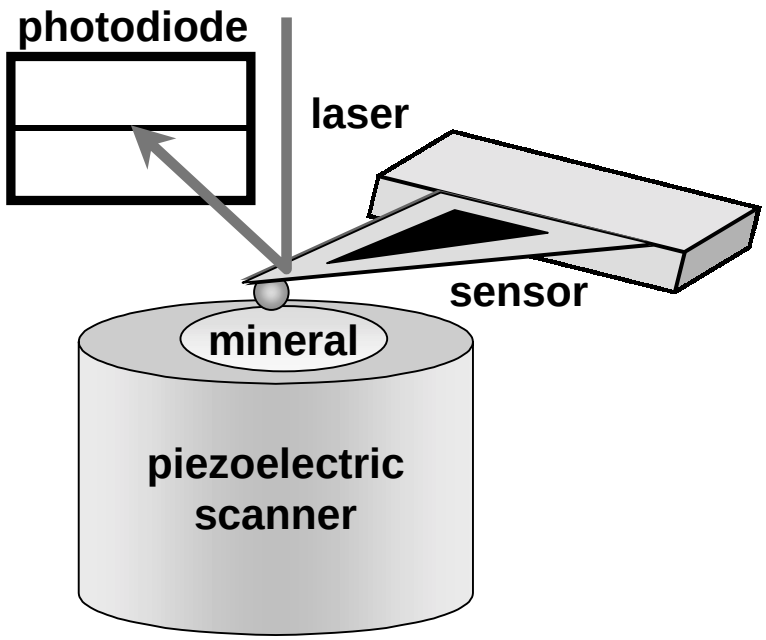

Figure 3.1. Schematic diagram showing the key components of a force microscope. The mineral and force sensor are within a fluid cell (not shown) containing aqueous solution. Force measurements are made by recording the deflection of a sensor (i.e., cantilever) in response to attractive or repulsive forces between itself and the sample (a mineral in this case). The sample, mounted on a piezoelectric scanner, indexes towards, makes contact with, and retracts from the sensor. Deflection of the sensor is detected by reflecting a laser off the top of the sensor and into a split segment photodiode. In this study, bacteria (shown as a sphere) have been linked to the sensor thereby creating a biologically-active-force-probe (BAFP) used to measure interfacial and adhesive forces between bacteria and mineral surfaces, *in-situ*.

discuss this technique and suggest potential applications of BFM to studies assessing the role that microbes or biomolecules play in geochemical and mineralogical processes.

Materials and Methods

Mineral and Bacteria

Freshly cleaved muscovite ($KAl_2(AlSi_3O_{10})(OH)_2$) and a Gram-negative soil bacteria were used for all experiments. The bacteria strain was obtained from a chemostat inoculated with an iron-oxide rich soil from Pandapas Pond, Jefferson National Forest, Virginia (Tadanier et al. 2000). Through comparison of 500 bp of 16S rRNA sequence, this bacterium has been aligned at the species level (0.67% difference) with the pseudomonad *Burkholderia cepacia* (MIDI Labs, Newark, DE). *Burkholderia* sp. were cultured on agar plates under oligotrophic (glucose concentration = 0.6 mM) or eutrophic conditions (glucose concentration = 2.8 mM) and used in BFM experiments as described below (see Tadanier et al. 2000 for complete description of growth media).

Force Sensor Preparation and Characterization

Biologically-active-force-probes (BAFPs) can be created by linking bacteria either directly to a silicon or silicon nitride force sensor (i.e., cantilever), or indirectly by linking a monolayer of bacteria to a small glass bead that is then fixed to the end of a cantilever. Direct-linkage of bacteria to the cantilever was accomplished by first placing a cantilever in a 1-5% solution of the polycationic molecule, poly-D-lysine (135 to 150 kDa; pH near neutral). Bacteria were linked to the poly-lysine functionalized cantilever by lowering it into a colony of live bacteria with the aid of a microscope (Nikon, 200x magnification) and a micromanipulator to translate the cantilever. For the indirect-linkage method, small glass beads (Polysciences or Duke Scientific, radii 3-7 μm, cleaned with a solution of 1% hydrofluoric acid or 10% sodium hydroxide) were activated with a 1-5% solution of poly-lysine. The activated beads were placed in a suspension of *Burkholderia* sp. and centrifuged at 8000 x *g* for 5 minutes. A single bacteria-coated-bead was attached to a cantilever using a small quantity of epoxy resin, which has

previously been found to be inert in aqueous solutions (Pincet et al. 1991; Yoon et al. 1997). This attachment procedure was conducted in solution using a micromanipulator.

Prior to BFM measurements, scanning laser confocal microscopy (Zeiss LSM 510) was used to characterize the three-dimensional nature of a BAFP. A probe was placed in the fluid cell used for force measurements and imaged with a 100x, 1.4 N.A. objective. This imaging procedure was facilitated by transforming bacteria with a plasmid (pGLO, Bio-Rad or pSMC2, provided by G. A. O'Toole, Dartmouth University) that encoded an intracellular green fluorescent protein. The bacteria fluoresced when excited by light at 458 nm or 488 nm. Fluorescence emitted by the epoxy resin revealed that it was confined to the region between the cantilever and glass bead (i.e., resin was not in a position that would alter the interaction between the bacteria and mineral during BFM experiments).

Cantilever spring constants (N m^{-1}), essential for measuring force magnitudes with a force microscope (see below), can vary substantially from the nominal value listed by the manufacturer (Cleveland et al. 1993; Senden and Ducker 1994). Spring constants were determined by attaching known masses to the end of a cantilever and recording the change in cantilever resonant frequency (Cleveland et al. 1993). A linear relationship was observed between added mass and resonant frequency with the slope being the spring constant (0.17 N m^{-1}). These measurements were accomplished using ten cantilevers from the same wafer as that used to create BAFPs. The variability of spring constants from cantilever to cantilever within the same wafer has been determined to be very small (Senden and Ducker 1994). This was confirmed by the linear fit of our data and the reproducibility of the resonant frequency of unloaded cantilevers (13.7 kHz ±0.2).

Force Measurements

BFM measurements were performed using a NanoScope IIIa Multimode SPM (Digital Instruments) in aqueous solution (pH 6, ionic strength 10^{-5} M, 25 °C). A piezoelectric scanner was used to translate the mineral towards and away from bacteria on a BAFP at rates of 0.1 to 3 μm sec^{-1}. For comparison, this is within the range of velocities of motile bacteria (Marshall 1976). Interfacial forces were measured as the mineral approached the bacteria on the probe;

whereas adhesion forces were measured upon contact and subsequent retraction of the mineral from the bacteria. Mineral samples were driven to the same contact force to normalize the effect that loading can have on adhesion forces during retraction (Weisenhorn et al. 1992).

Force measurement data are collected as cantilever deflection (diode voltage) and corresponding piezo displacement, typically termed a force curve (Figure 3.2A). This data must be manipulated to produce the familiar force-distance curve, which describes interfacial and adhesion forces (Figure 3.2B). Distance (i.e., separation between bacteria and mineral) is calculated by correcting the recorded piezo position (i.e., displacement) by the measured deflection of the cantilever. For example, if the mineral attached to the piezo scanner moves 10 nm towards bacteria attached to the cantilever, and the bacteria are repelled 2 nm due to repulsive forces, then the actual mineral-bacteria distance (or separation) changes by only 8 nm. The distance axis origin is chosen as the point on the force curve where sensor deflection becomes a linear function of piezo displacement (the sensor is in contact with the sample).

Force (F) is determined using Hooke's Law, $F = k_{sp}\, d$, where d is cantilever deflection (meters) and k_{sp} (N m^{-1}) is the cantilever spring constant. In order to use Hooke's Law, cantilever deflection measured by the photodiode in volts must be converted to meters. A diode/displacement conversion factor (also called "optical lever sensitivity") is defined from the slope of the force curve region where the cantilever is in contact with the sample on the piezo (Figure 3.2Aiii, called the region of constant compliance). The reciprocal of this conversion factor (in nm V^{-1}) can be used to convert measured cantilever deflection in volts to meters. A zero force reference value is determined as the force curve region where sensor deflection is independent of piezo displacement (Figure 3.2Ai, the sensor and sample are not interacting because they are far apart).

It is important to note that using the constant compliance region of the force curve to convert photodiode response into force will overestimate the force of interaction if the bacteria are more compliant than the cantilever. Recent measurements of the elasticity of bacterial surface macromolecules suggest that bacteria are less compliant (i.e., stiffer) than cantilevers having spring constants smaller than 10 N m^{-1} (Xu et al. 1996; Yao et al. 1999).

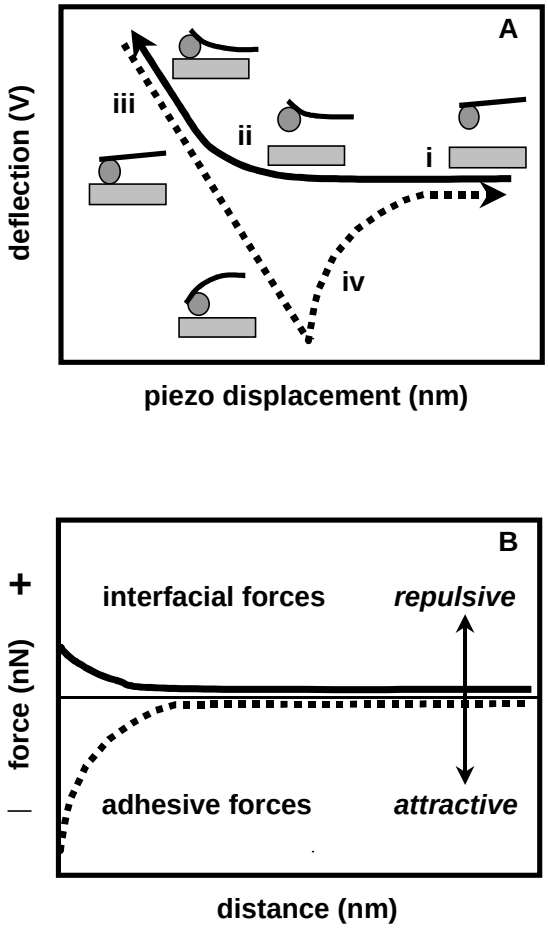

Figure 3.2. Schematic diagrams of a force curve (A) and force-distance curve (B). When the sensor and sample are far apart (i) they exhibit no interaction (region of zero force). As the sample approaches the sensor, intermolecular forces between the bacteria and mineral cause the sensor to deflect upwards (ii) due to repulsive forces shown here. Eventually the probe makes contact with the sample (iii) and their movement becomes coupled (region of constant compliance). The sample is then retracted from the probe (iv) until the sensor and sample return to their original positions thereby completing one cycle (an entire cycle requires nano- to milliseconds). Hysteresis, shown here, may occur upon retraction due to adhesion forces. See text for discussion on converting cantilever deflection and piezo displacement into force and distance, respectively. Interfacial forces are measured on approach and adhesion forces are measured upon retraction; repulsive forces are positive and attractive forces are negative.

In instances where bacteria (or biomolecules) linked to the cantilever are more compliant than the cantilever or for cells with fragile appendages, other methods must be used to accurately convert the measured deflection of the cantilever (in volts) into a force of interaction (in Newtons) (D'Costa and Hoh 1995; Sader et al. 1999). For example, because the optical lever sensitivity is strongly dependent upon the shape of the laser spot on the photodiode detector, the "photodiode shift voltage" can be used to convert volts of cantilever deflection into meters of deflection (D'Costa and Hoh 1995). Photodiode shift voltage is measured as the difference in output voltage when the photodiode detector is shifted approximately 318 μm (one full turn of the positioning screw) on either side of the zero setting. Figure 3.3 illustrates the strong correlation between photodiode shift voltages and optical lever sensitivities measured by directing the laser to different positions on the cantilever. Once this correlation is established for a given instrument, piezoelectric scanner, fluid cell, and cantilever (e.g., 200 μm long, V-shaped, silicon nitride cantilevers), the optical lever sensitivity can be accurately determined without pressing the cantilever against any other surface. This method ensures that forces can be determined regardless of the compliance of the cantilever relative to any microorganism attached to it, and also ensures the preservation of fragile macromolecules on microorganisms attached to the cantilever.

Results and Discussion

Characterization of Biologically-Active-Force-Probes

BAFPs were created by either attaching a single bacterium to a force sensor (Figure 3.4A) or by attaching a single bacteria-coated-bead (Figure 3.4B) to a force sensor. Fluorescent dyes (ViaGram, Molecular Probes) and plating techniques revealed that cell membranes on these bacteria are intact and the cells are viable. Both the direct and indirect method of attaching bacteria to a cantilever preserves the orientation and structural integrity of macromolecules on the cell surface. This is important because the native conformation of macromolecules on a bacteria surface is critical to their function, activity, and role in nature. By using whole bacteria expressing macromolecules in their natural state rather than individual biomolecules (e.g., exopolysaccharides, proteins) purified from bacterial surfaces, we avoid situations in which the linkage procedure modifies the conformation of biomolecules such that they are no longer in a

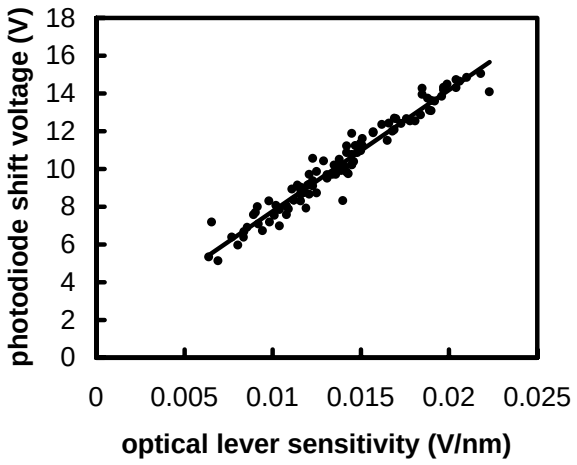

Figure 3.3. Variation of photodiode shift voltage as a function of optical lever sensitivity using a J-type piezoelectric scanner and eight silicon nitride cantilevers (200 μm in length). All measurements were performed on muscovite and hematite in a fluid cell containing aqueous solutions ranging in ionic strength from zero (i.e., Milli-Q water) to 10^{-2} M. The slope of the regression line is 642 nm and the intercept is 1.3 V ($R^2 = 0.95$). With this correlation, the optical lever sensitivity can be determined in a matter of seconds by simply using the photodiode shift voltage. Furthermore, this method is valid regardless of whether the cantilever is the most compliant component of the system.

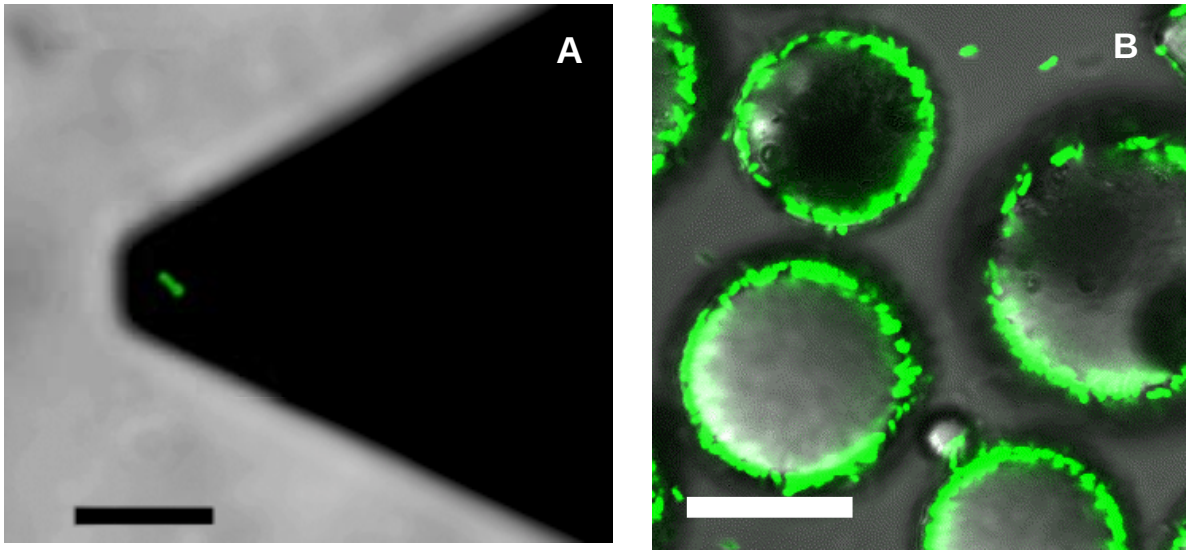

Figure 3.4. (A) Scanning laser micrograph of a BAFP created by directly linking a single cell of *Burkholderia* sp. to a force-sensing cantilever. (B) Confocal micrograph focused on the mid-plane of bacteria-coated beads revealing a layer of cells. Focusing up and down on the beads reveal that many are evenly coated with cells. Beads showing the most uniform coverage are attached to cantilevers, thereby creating BAFPs. These cells have been transformed with a plasmid encoding a green fluorescent protein and are emitting "natural" fluorescence. This fluorescent protein, being intracellular, together with the confocal abilities of the laser scanning microscope allow noninvasive characterization of the orientation and distribution of cells on the force sensor, *in-situ*, without affecting the surface chemistry of the microbes. Plating experiments in which BAFPs were used as the inoculum, revealed that the bacteria on the sensors are viable and presumably active during force measurements. Scale bars = 10 μm.

natural state (Ellen and Burne 1996; Ingersoll and Bright 1997; Stotzky 1986; Turner et al. 1996; Turner et al. 1997).

As both Gram-negative and Gram-positive bacteria are typically negatively charged at most pH, poly-lysine with its high pK_a is a more-or-less universal linker molecule. However, due to the large diversity in bacterial surfaces, situations will likely arise in which other linker molecules are necessary. The versatility of our linkage protocol allows for modification on a case-by-case basis. For example, we have also fabricated BAFPs using aminopropyltriethoxysilane and polyethyleneimine with species of *Shewanella* and *Pseudomonas*. Additionally, methods used to attach proteins or antibodies to small glass spheres or sensors (e.g., Caruso et al. 1998; Florin et al. 1994; Frey and Corn 1996; Rezania et al. 1999; Turner et al. 1997) could be applied to our protocol. For example, lysozyme, which has been genetically altered such that it possesses an active binding site but an inactive catalytic site (Voet and Voet 1995), is an attractive choice because of its high affinity for bacterial cell walls. Antibodies specific for cell surface receptors may also be effective linkers.

A critical component to consider when designing BAFPs is the relative difference between those forces binding the cells *to* the sensor, and interfacial and adhesive forces that will be probed *by* the cells *on* the sensor. Forces holding bacteria to the sensor must be greater than the forces that are actually probed, otherwise the bacteria will be stripped from the cantilever during force measurements. Electrostatic attachment of bacteria to sensors via poly-lysine is very strong. Approximations using Coulomb's law and molecular dynamic calculations show that the energy associated with amine-silanol (i.e., the poly-lysine glass bead linkage or poly-lysine cantilever linkage) and amine-carboxylic pairs (i.e., the poly-lysine bacteria linkage) (Pagac et al. 1998a; Pagac et al. 1998b; Voet and Voet 1995; West et al. 1997) are at least one to four orders of magnitude stronger than potential intermolecular forces between a bacteria and mineral (Israelachvili 1992). Organosilane linkages like that of aminopropyltriethoxysilane form covalent bonds with silanol groups (Plueddemann 1991) and should therefore be even stronger than poly-lysine linkages.

Comparison of the direct and indirect linkage protocols suggests that the latter is more desirable. Direct linkage of a single cell to a cantilever (Figure 3.4A) is very difficult. Techniques such as optical tweezers (Svoboda and Block 1994) or nanotweezers (Kim and Lieber 1999) could significantly enhance the linkage of a bacterium to a cantilever, but such techniques are not at present easily applicable to such procedures. Another practical reason for selecting the indirect protocol is the need to use cantilevers with appropriate spring constants in order to probe the entire range of potential forces between microbes and minerals. Cantilevers of different composition (e.g., glass, silicon, silicon nitride, and gold coated cantilevers) are often used to vary the spring constant. Gold coated and uncoated silicon nitride cantilevers, for example, have very different surface properties and silicon nitride itself can vary from Si_3N_4 to $Si_{15}N_4$ (Weisenhorn et al. 1992). These compositional differences would require use of more than one linker molecule to directly attach one particular strain of bacteria to different cantilevers. Conversely, indirect linkage allows the use of one protocol to attach bacteria to glass (or latex) beads of uniform composition, which can then be attached to cantilevers of different compositions.

Forces of interaction between bacteria and mineral surfaces in-situ

The interactions between muscovite and poly-lysine (Figure 3.5A) and muscovite and the soil *Burkholderia* sp. (Figures 3.5B and 3.5C) were studied with BFM at pH 6, ionic strength 10^{-5} M, 25 ˚C. As expected, the positively charged poly-lysine exhibited a strong attraction towards the (001) surface of muscovite which is negatively charged (Figure 3.5A). Hysteresis was not observed for the interaction between muscovite and poly-lysine. In contrast, the *Burkholderia* sp. exhibited a repulsive interaction with muscovite beginning at ~80 nm (Figure 5B, approach). However, once contact was made this repulsive force was overcome and the bacteria exhibited an attractive adhesion towards the mineral (Figure 5B, retraction).

The repulsive interfacial forces between the *Burkholderia* sp. and muscovite illustrated in Figure 3.5B (approach) are consistent with the negative surface charges of both the bacteria and mineral at circum-neutral pH. The distance at which repulsion occurs is slightly smaller than that expected based solely on electrostatic interactions (Debye length thickness is ~100 nm at this ionic strength). Other forces associated with hydrophobic, steric, and entropic effects likely

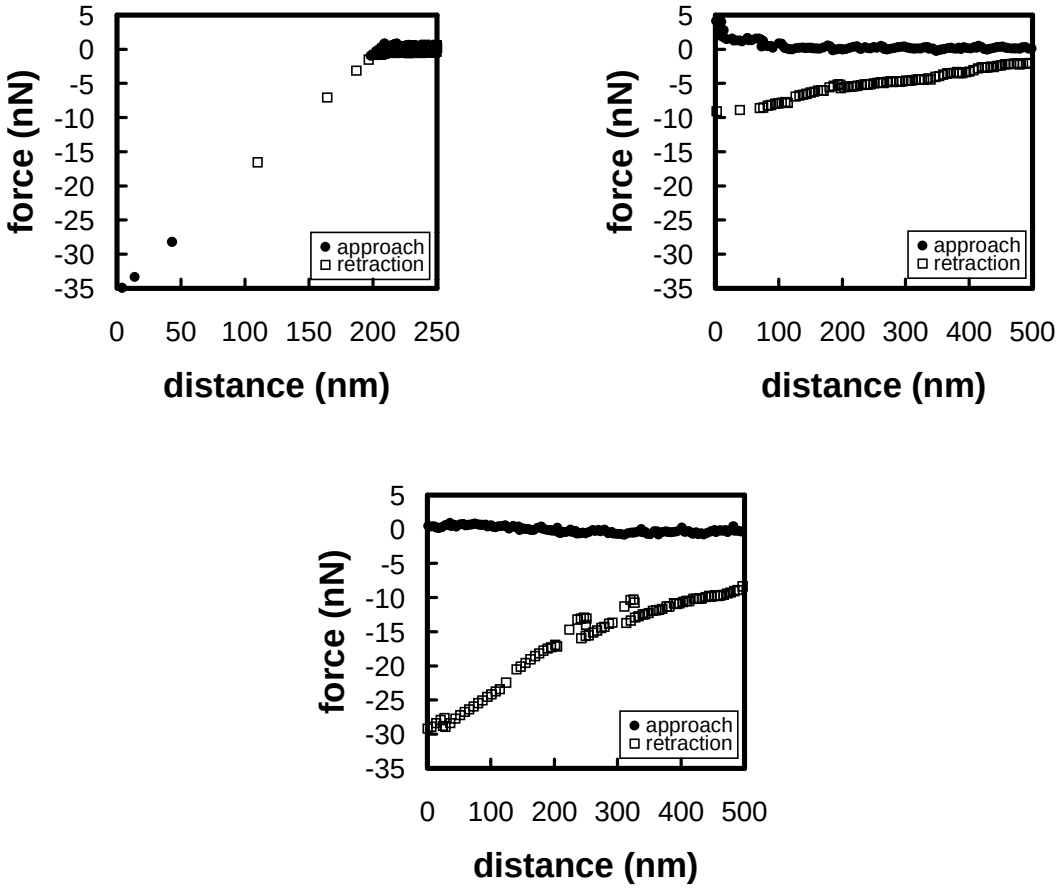

Figure 3.5. Biological force microcopy (BFM) force-distance curves between the (001) surface of muscovite, and a poly-lysine functionalized cantilever (A; top left), versus *Burkholderia* sp. grown in a eutropic environment (B; top right), versus *Burkholderia* sp. grown in an oligotropic environment (C; bottom). Solution conditions were pH 6, ionic strength 10^{-5} M, 25 °C. The force of interaction is plotted as a function of the distance between the two surfaces (i.e., separation between poly-lysine or bacteria on the sensor and the mineral surface). Note difference in x-axis scale. Curves begin on the right and proceed towards the left as the mineral is brought towards the sensor (solid circles, approach curve). The sensor makes contact with the mineral surface and subsequently withdraws from the muscovite (open squares, retraction curve) to complete one cycle.

play a key role in bacteria-mineral interactions due to the relatively long polymers on bacterial surfaces (Israelachvili and McGuiggan 1988; Israelachvili 1992).

The adhesive behavior between *Burkholderia* sp. and muscovite is drawn out for hundreds of nanometers (Figure 3.5B, retraction). This is likely due to stretching and fibrillation of biomolecules (e.g., lipopolysaccharides or flagella) as a result of attractive forces such as hydrogen bonds between polymers on the bacteria and surface hydroxyls or structured water molecules on muscovite (Jucker et al. 1997).

When the same *Burkholderia* sp. was grown in nutrient poor media it exhibited very strong affinity for muscovite (Figure 3.5C). Interfacial repulsion was not detected (Figure 3.5C, approach) and attractive adhesion forces were very large and long range (Figure 3.5C, retraction). This phenomena is consistent with the observation that many bacterial species alter their surface properties under oligotrophic versus eutropic environments (e.g., Bengtsson 1991). In fact, many bacteria show greater affinity for mineral surfaces under oligotrophic rather than eutropic conditions (Fletcher 1996b; Marshall 1996). This cycling between attached and planktonic states is believed to be a strategy bacteria use to proliferate in nature where many environments have eutropic/oligotropic sequences.

Potential Applications of Biological Force Microscopy to Geomicrobiology Studies

BFM could provide a unique perspective into several aspects of geomicrobiology (e.g., mineral dissolution, crystal growth, and biofilm formation), particularly if used to complement other techniques. Listed below are several examples presented in a question and answer format.

1. Do bacteria “recognize” particular minerals or crystallographic orientations? Probe different minerals or different faces on the same mineral with one BAFP.
2. Do bacteria show an increased affinity for particular minerals under oligotrophic or anaerobic conditions? Change solution conditions within fluid cell while collecting BFM measurements.

3. How does inter- and intra-species communication affect biofilm development? Use a BAFP to measure interactions with various species of bacteria that are immobilized on a substrate.
4. What intermolecular forces (e.g., van der Waals, electrostatics, hydration, hydrophobic, entropic) are involved in bacterial attachment and detachment processes? Compare forces measured with BFM to those calculated from theoretical models (e.g., DLVO theory).
5. What cell surface macromolecules mediate bacterial attachment to mineral surfaces? Are conditioning films required for attachment? Create BAFPs using various mutants that differ in surface macromolecules and use these in BFM on minerals with and without sorbed organic molecules.
6. How do bacteria alter their cell surface in response to changing environmental conditions? Do mineral surfaces induce genetic expression of particular macromolecules on a cell surface? If so, how much time is necessary for production of a protein or extracellular polysaccharide? What is the distribution of a particular macromolecule on the cell surface? Combine BFM measurements with biomolecule sensitive fluorophores, scanning laser confocal microscopy, and/or scanning near-field optical microscopy.
7. Is direct bacteria-mineral contact necessary for oxidative or reductive dissolution of sulfides, iron oxides, or manganese oxides? Use BFM to measure bacterial affinity for various minerals under aerobic vs anaerobic conditions. Combine BFM with surface sensitive spectroscopies.
8. How does the conformation of a protein change when electrons are shuttled to/from a mineral surface (e.g., in the case of *Shewanella*-iron oxide interactions)? Combine BFM measurements with fluorescence resonance energy transfer, scanning laser confocal microscopy, and/or proteins labeled with fluorescent tags.
9. Do organic acids exhibit an increased affinity for particular mineral faces? How strong are the bonds between a siderophore or organic ligand and mineral surface? A force sensor could be functionalized with specific biomolecules and used to probe different faces on the same mineral.

Tools such as BFM offer biochemists, biophysicists, geochemists, environmental engineers, microbiologists, and mineralogists a unique insight into the fundamental intricacies of the ubiquitous interfaces between microbes and minerals.

Acknowledgments

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Chapter 4 - Bacterial recognition of mineral surfaces: Nanoscale interactions between *Shewanella* and α-FeOOH

In review in
Science

Abstract

Biological force microscopy has been used to quantitatively measure the minute forces that characterize interactions between *Shewanella oneidensis*, a dissimilatory metal reducing bacterium, and the mineral goethite, α-FeOOH, both commonly found in Earth near-surface environments. Force measurements with sub-nanoNewton resolution were made with living cells, in aqueous solution under aerobic and anaerobic conditions as a function of the nanoscale distance between the cell and mineral surface. Energy values derived from these measurements show that the affinity between *S. oneidensis* and goethite rapidly increases by two to five times under anaerobic conditions where electron transfer from bacterium to mineral is expected. Specific signatures in the force curves suggest that a 150 kDa putative, iron reductase is quickly mobilized within the outer membrane of *S. oneidensis* and specifically interacts with the goethite surface to facilitate the electron transfer process.

Introduction

The Fe(II)-Fe(III) redox cycle represents a major energy flux at the earth's surface. A critical component of this system is the reduction of iron-containing minerals by biological processes (*1, 2*). *Shewanella* - a Gram-negative, dissimilatory metal reducing bacterium found in soils, sediments, surface waters, and groundwaters - is able to conserve energy for growth by using oxygen or ferric iron as a terminal electron acceptor (*3, 4*). In many natural environments oxygen is in limited supply but Fe(III) is present as a major element in the crystal structure of minerals. Hence in many cases, dissimilatory metal reducing bacteria oxidize various carbon substrates by reductively dissolving Fe(III)-containing minerals, the most ubiquitous in the near-surface environments being iron oxyhydroxides (e.g., ferrihydrite, goethite, and hematite) (*5-12*).

This impacts a wide array of processes including the biogeochemical cycle of iron and phosphorus, oxidation of natural and anthropogenic carbon sources, biocorrosion, and the release of heavy metals associated with iron oxyhydroxides.

The dissimilatory reduction of iron-containing minerals presents a rather unique situation because unlike oxygen, Fe(III) in a solid form cannot diffuse across the cell wall to the plasma membrane which, in most bacteria, houses the proteins involved in electron transfer, proton translocation, and subsequent generation of ATP. Microorganisms like *Shewanella* seem to be able to generate two energized membranes using a unique system of proteins that shuttle electrons from an energy source in the cytoplasm, across the plasma membrane and periplasmic space, to the outer membrane (*13, 14*). Once in the outer membrane, iron reductases appear to transfer electrons directly to ferric iron in the crystal structure of minerals causing a weakening of the iron-oxygen bond and reductive dissolution of the mineral (*6, 15-18*). A great deal of research has focused independently on either the microbiological or mineralogical aspects of the interaction between *Shewanella* and iron oxyhydroxides. However, virtually no studies have been conducted on the interface between the bacterium and iron containing minerals. It is this interface, delineated by chemical and structural features on the surface of the organism (e.g., concentration and localization of cytochromes and reductases, physical structure of the outer membrane) and the mineral surface (e.g., density and coordination of iron-oxide moieties, surface microtopography, crystallographic orientation), which likely has a major impact on the kinetics and extent of bacterial reduction of Fe(III)-minerals.

Methods and Materials

In this study, we used biological force microscopy (*19, 20*) to probe the interface between a living cell of *Shewanella oneidensis*, formerly classified as *S. putrefaciens* MR1 (*21*), and the (010) surfaces of goethite (α-FeOOH) and diaspore (α-AlOOH; isostructural with goethite; surface properties such as charge and hydrophobicity are very similar to goethite) at the nanoscale level in anaerobic and aerobic solutions. *S. oneidensis* were grown under anaerobic conditions using lactate as the carbon and energy source and Fe(III)-citrate as the electron acceptor (*22*). To preserve the natural, complex biomolecular network on the bacteria surface,

fully functional cells (0.5 x 2 μm) were linked in their native state to a small bead (5 μm radius) situated on the end of a silicon nitride cantilever thereby creating a biologically-active-force-probe (*22*). A commercial force microscope (Digital Instruments, Santa Barbara, California) was used to measure the deflection of a biologically-active-force-probe by reflecting a laser off the top of the cantilever and into a photodiode-detector while an oriented mineral crystal, mounted on a piezoelectric scanner, was translated towards (i.e., approach data), made contact with, and was subsequently retracted from (i.e., retraction data) a bacterium on the probe. Photodiode response (in volts) was converted to cantilever deflection (in meters) using a conversion factor (in meters $volt^{-1}$) determined from the photodiode shift voltage (*23*). Cantilever deflection was multiplied by the cantilever spring constant (k_{sp} = 0.07 N m^{-1}, determined as described previously (*24*)), to calculate the force of interaction (in Newtons). Displacement of the mineral on the piezoelectric scanner was converted to separation between the bacteria and mineral by correcting for cantilever deflection (*25*) and selecting the point of contact using jump-to-contact and jump-from-contact events for approach and retraction curves, respectively. For retraction curves it is more appropriate to refer to extension rather than separation because polymers on the cell surface form a bridge with the mineral.

Results and Discussion

In a typical experiment with goethite, approach and retraction forces were measured after varying the contact time between *S. oneidensis* and the mineral in anaerobic solutions supplemented with lactate as an energy source (Fig. 4.1a). An aerobic solution supplemented with lactate was then injected into the fluid-cell of the force microscope and allowed to incubate for 30-45 min, after which forces were again measured as a function of the contact time between the bacteria and mineral (Fig. 4.1b). To characterize the complex physical and chemical intricacies involved in these reactions, the work or energy of each curve was determined by integrating force with respect to distance (Table 4.1). In general the retraction curves may provide greater insight into the transfer of electrons from biomolecules on *S. oneidensis* to iron atoms in goethite because direct cell-mineral contact is a prerequisite for this reaction (*6, 17, 26, 27*). However,

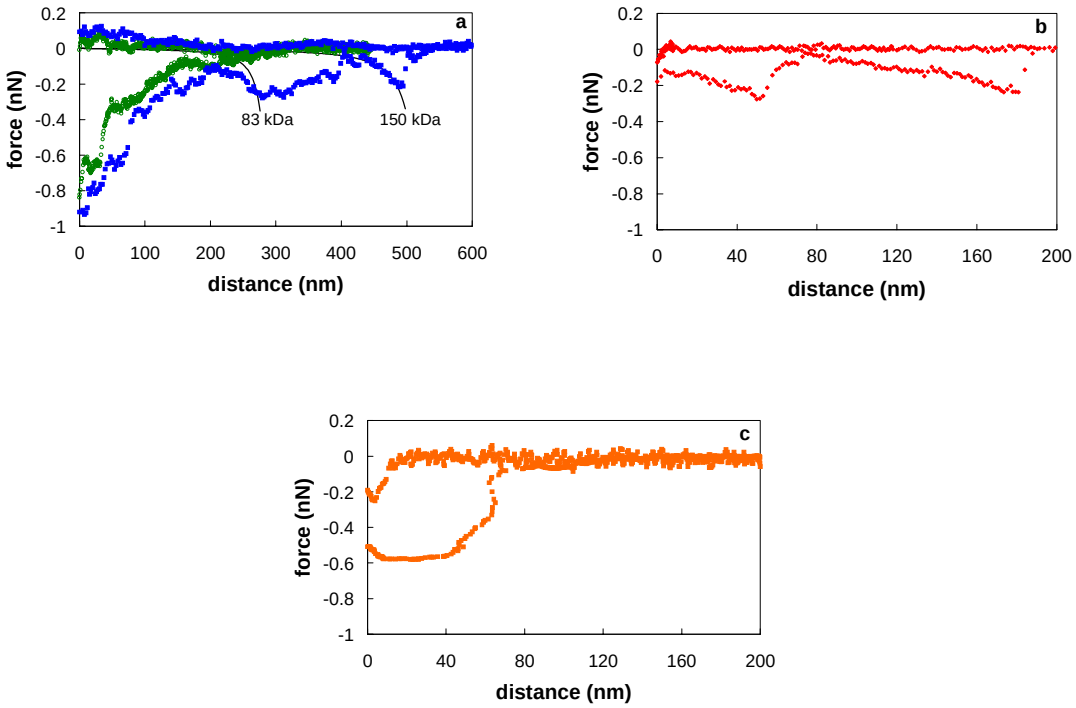

Figure 4.1. Representative force-distance curves between *Shewanella oneidensis* and goethite (α-FeOOH) or diaspore (α-AlOOH) at 25 °C, pH 6, 10 mM sodium chloride, and 15 mM lactate in solution. For each approach-retraction cycle in this figure, the upper data set corresponds to approach values, whereas the lower data set corresponds to retraction values. Positive values are repulsive and negative values are attractive. (a) Goethite-*S. oneidensis* interactions in which the cell and mineral remained in contact for 0-5 min (green circles) or 30-45 min (blue squares) under anaerobic conditions. Black curves correspond to the worm-like chain model prediction for the 83 kDa and 150 kDa outer membrane, putative iron reductases. (b) Goethite-*S. oneidensis* interactions in which the cell and mineral remained in contact for 0-5 min under aerobic conditions. There was no significant difference between the 0-5 min and 30-45 min contact times under aerobic conditions. (c) Diaspore-*S. oneidensis* interactions under anaerobic conditions. There was no significant difference between the aerobic and anaerobic experiments between diaspore and *S. oneidensis*.

Table 4.1. Force and energy measurements for the interactions between *Shewanella oneidensis* and goethite (α-FeOOH) or diaspore (α-AlOOH) in anaerobic or aerobic solutions. In the case of goethite experiments, the results are reported as a function of the amount of contact time between the bacteria and mineral prior to biological force microscopy measurements. Force values (in picoNewtons = 10^{-12} N) are the maximum magnitude recorded in approach or retraction curves and as such represent only one datum point for each. Energy values (in attoJoules = 10^{-18} J) are calculated by integrating force with respect to distance and as such incorporate the entire data set. These values represent the average and standard deviation results of over 500 force measurements conducted with four *S. oneidensis* biologically-active-force-probes. Positive numbers are repulsive; negative numbers are attractive.

		Approach		Retraction	
	Mineral	Force (pN)	Energy (aJ)	Force (pN)	Energy (aJ)
Anaerobic	Goethite (0-5 min)	59 ± 36	3.5 ± 1.9	-747 ± 115	-59.5 ± 8.7
	Goethite (35-45 min)	104 ± 49	6.7 ± 2.5	-795 ± 145	-136.7 ± 20.7
	Diaspore	-237 ± 68	-3.2 ± 2.8	-397 ± 164	-39.2 ± 7.4
Aerobic	Goethite (0-5 min)	-54 ± 22	-0.5 ± 0.4	-248 ± 109	-26.2 ± 6.1
	Goethite (35-45 min)	-35 ± 14	-1.0 ± 0.4	-253 ± 95	-27.1 ± 9.6
	Diaspore	-271 ± 61	-3.1 ± 1.3	-433 ± 127	-41.2 ± 4.2

the approach curves give an indication of the intermolecular forces that will effect whether *S. oneidensis* is able to bridge the solution gap between itself and the mineral.

For the approach curves, repulsion between *S. oneidensis* and goethite under anaerobic conditions was greater after the bacteria and mineral remained in contact for 30-45 min (Fig. 4.1a; Table 4.1). Infusion of an oxygenated solution caused this repulsive interaction to decrease to the point where it became slightly attractive (Fig. 4.1b; Table 4.1). The point-of-zero-charge of *Shewanella* is 5-6 (determined under aerobic conditions) (*28*) and 7-8 for goethite (*29*). Based on electrostatic considerations alone, a slight attraction would be expected at a pH of ~6. However, there is a high degree of electrochemical heterogeneity on the surface of *Shewanella* and its surface charge is dependent upon the concentration and localization of at least five different types of functional groups which are arrayed over the cell surface (*28*). Under aerobic conditions, amine-groups are embedded within the outer membrane and underlying peptidoglycan layer such that they do not significantly contribute to the surface charge (*28*). Anaerobic conditions induce the expression of a significant quantity of proteins in the outer membrane of *S. oneidensis* (*15-17*). The amine-groups on these proteins would likely lead to an increased positive charge on the cell surface such that repulsion would be expected between *S. oneidensis* and goethite in anaerobic solutions, as observed in approach curves.

Retraction curves represent in many cases a complex dynamic of adhesion involving, among other things, bond breaking, intermolecular forces, and extension of outer membrane proteins that form a bridge between a bacterium and the mineral surface. Under aerobic conditions, *S. oneidensis* exhibited a stronger overall adhesion to diaspore relative to goethite (Fig. 4.1b and 4.1c, Table 4.1). Under anaerobic conditions *S. oneidensis* exhibited a greatly increased affinity for goethite, as indicated by the retraction curves, whereas the attractive energy between diaspore and *S. oneidensis* was indifferent to oxygen concentrations in solution (Fig. 4.1a and 4.1c, Table 4.1). Furthermore, biologically-active-force-probes fabricated with non-viable cells exhibited an attraction towards goethite but this affinity did not change with oxygen concentration in solution. Taken together, this indicates that the adhesion between *S. oneidensis* and goethite is due, at least in part, to processes associated with dissimilatory iron reduction. The retraction energies between *S. oneidensis* and goethite were 2-5 times stronger under anaerobic as opposed to

aerobic conditions (Table 4.1). Under anaerobic conditions, this attraction increased with the amount of contact time for goethite (Table 4.1) but not for diaspore. This provides direct evidence suggesting that the bacteria are able to quickly recognize the mineral surface such that they produce and/or localize biomolecules at the interface with goethite. This type of recognition between a microorganism and mineral surface has been previously suggested but never quantified (*30, 31*). Clearly, this could be one of the functions of the fluidity of the outer membrane allowing the bacterium to react rapidly to the mineral surface and to congregate reactive membrane macromolecules at sites of contact. Dynamic modeling of outer membrane lipopolysaccharides suggests molecular motion over microsecond timeframes (*32*), and it is possible that cytochromes and reductases could also have rapid motional attributes.

Anaerobic retraction curves between goethite and *S. oneidensis* exhibit a strong force of interaction within the first few hundred nanometers followed by a jagged plateau (Fig. 4.1a). In many cases, the retraction curves terminate in this saw-tooth-like discontinuity (Fig. 4.1a), which we suggest is a signature of the unfolding of outer membrane proteins that form a bridge between a living bacterium and the mineral surface. The saw-tooth like patterns in the retraction curves were analyzed to gain more definitive evidence for the presence of outer membrane proteins that may be involved in the direct transfer of electrons from the cell to the mineral. A total of four putative iron-reductases ranging in size from 53 to 150 kDa have been isolated from the outer membrane of *S. oneidensis* (*17, 18*). According to the worm-like chain model (*33*) which can be used to calculate the force (F) needed to stretch a polypeptide chain in a solvent to a length x, $F(x) = (kT/b)\ [0.25\ (1 - x/L)^{-2} - 0.25 + x/L]$, where k is the Boltzmann's constant, T is the temperature, b is the persistence length (i.e., length of the stiff segment of the chain) which is 0.38 nm for the distance between two C_α atoms in a polypeptide (*34-36*), and L is the contour length (i.e., length of the completely stretched chain) calculated for each putative iron reductase assuming an average molecular weight of 110 Da per amino acid residue (*18, 37*) and an average molecular length of 0.4 nm per amino acid residue (*35*).

Of the four putative iron reductases, the 83 kDa protein was emphasized in previous reports (*17, 18*); however recent studies involving mutants incapable of producing this protein provide evidence that it is not an iron reductase (C. Myers, personal communication). Predictions based

on the worm-like chain model, when compared to our anaerobic retraction curves (Fig. 4.1a), are consistent with this. Of the remaining three putative iron reductases, the worm-like chain model prediction for the 150 kDa protein fit 82% of the retraction curves for *S. oneidensis* and goethite under anaerobic conditions and longer contact time (Fig. 4.1a). Evidence of this protein was not found in the retraction curves of the aerobic-goethite nor the diaspore experiments. This strongly suggests that the 150 kDa protein in the outer membrane of *S. oneidensis* specifically interacts with the surface of goethite fulfilling some aspect of the electron transfer reaction.

We have shown that under anaerobic conditions *S. oneidensis* responds to the surface of goethite by rapidly developing stronger adhesion forces at the interface. We interpret these data to indicate that following recognition of goethite as a terminal electron acceptor, *S. oneidensis* quickly produces and/or mobilizes proteins (i.e., the 150 kDa putative reductase and perhaps others) that specifically interact with the mineral surface. This is an example of using nanomechanical measurements/properties to help isolate likely biochemical and mineralogical mechanisms that are operative under environmentally relevant conditions. Further, besides an improved fundamental understanding of bacterial interactions with solid surfaces, the ability to quantitatively probe and characterize these specific interactions in dimensional, force and energy nanospace could lead to advances in nanotechnology. For example, one may now be able to systematically tailor the exquisite, natural specificity between biomolecules, as produced by living microorganisms, and material surfaces for the purposes of chemical and/or structural nanoscale modification of the cell, the material surface, or both.

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22. *S. oneidensis* (ATCC 700550) was transformed with plasmid pSMC2 which codes for a constitutive, intracellular green fluorescent protein (*38*). Transformed cells were grown in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Michigan) to mid-logarithmic phase at room temperature as previously described (*4*) in defined medium supplemented with 15 mM lactate and 2 mM ferric citrate (*4*). Cells were rinsed three times in an anerobic solution of 10 mM sodium chloride. Washed cells were linked to glass beads functionalized with 5-10% 3-aminopropyltriethoxysilane. A single bacteria coated bead was then attached to a cantilever with epoxy (*19*). Scanning laser confocal microscopy was used to verify bacterial coverage of the probe by imaging the green fluorescent signal emitted from cells. Confocal measurements as described previously (*19*) suggest that a single bacterium made contact with the mineral during force measurements. Viability of cells on the probe was assessed by inoculating an agar plate with a biologically-active-force-probe subsequent to force measurements.

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40. J. Banfield provided constructive comments. We thank G. O'Toole for providing plasmid pSMC2. S.K.L. acknowledges the support of J Tak. Mineral samples provided by the Virginia Tech Geological Sciences Museum. Financial support from the U.S. Department of Energy and the National Science Foundation.

Curriculum Vitae

STEVEN K. LOWER
Department of Geological Sciences
Virginia Polytechnic Institute and State University (Virginia Tech)
Blacksburg, VA 24061-0420
Tel: 540-231-6521 (ext. 8575), Fax: 540-231-3386
e-mail: slower@vt.edu

Education

Ph.D. GEOLOGICAL SCIENCES, BIOCHEMISTRY, AND MICROBIOLOGY (8/97-12/00) Departments of Geological Sciences and Biochemistry, Virginia Tech, Blacksburg, VA.

Course emphasis: biochemistry, genetics, geochemistry, microbial ecology, microbial physiology, microbiology, mineralogy, molecular biology, surface chemistry.
Techniques utilized: atomic force microscopy (AFM), chromatography, chemostats, cloning with plasmid and phage vectors, confocal laser scanning microscopy (CLSM), electrophoresis, fluorescent in-situ hybridization (FISH), gene expression, polymerase chain reaction (PCR), scanning electron microscopy (SEM), Southern blot analysis, temperature gradient gel electrophoresis (TGGE), Western blot analysis.
Doctoral research: Development of biological force microscopy. Fundamental nanoscale interactions between a bacterium and mineral surface (e.g., Shewanella-iron oxides; Thiobacillus-sulfides; Pseudomonas-silicates). Macroscale interactions between a community of microorganisms and minerals (e.g., soil microbial community and iron oxides supporting sorbed nutrients and/or metals).
Advisor: Dr. Michael F. Hochella, Jr.

M.S. GEOCHEMISTRY (1/95-5/97) Department of Geology, Kent State University, Kent, OH.

Course emphasis: clay mineralogy, environmental organic chemistry, hydrogeochemistry, mineral-water interface chemistry, optical petrography, soil chemistry, statistics, time series analyses, x-ray crystallography.
Techniques utilized: atomic absorption spectrophotometer (AA/graphite furnace), atomic force microscopy (AFM), high-performance liquid chromatography (HPLC), inductively coupled plasma spectrometer (ICP), scanning electron microscopy (SEM), transmission electron microscopy (TEM), total organic carbon analyzer; x-ray diffraction (XRD).
Thesis research: Mechanisms of lead sorption to calcium-phosphate minerals and the use of apatite to remediate heavy metal contaminated environments. Additional studies include: sorption of dissolved organic matter onto aluminum/iron oxides; dissolution of aluminum/iron oxides by organic acids; pollution associated with urban stormwater runoff.
Advisor: Dr. Patricia A. Maurice

B.S. GEOLOGY AND BIOLOGY (9/90-12/94) Departments of Geology and Biology, Kent State University, Kent, OH.

Course emphasis: anatomy, aquatic biology, biology, botany, chemistry, climatology, ecology, field camps (biology and geology), forestry, geochemistry, geographic information systems, geomorphology, hydrogeology, mathematics, mineralogy, petrology, physics, physiology, stratigraphy, structural geology, zoology.
Independent studies: Characterization of soil samples with x-ray diffraction; development of a constructed wetland for wastewater treatment; preservation of a natural bog.

Employment

GRADUATE RESEARCH ASSISTANT (6/99-present) Department of Geological Sciences, Virginia Tech, Blacksburg, VA.
Researcher. Responsibilities include primary doctoral research, scanning probe microscopy and laser scanning microscopy maintenance, PC hardware and software administration.

GRADUATE TEACHING ASSISTANT (8/97-5/99) Department of Geological Sciences, Virginia Tech, Blacksburg, VA.
Head TA for Elements of Geology class. Responsibilities include design and organization of laboratory and field exercises and examinations.

GRADUATE RESEARCH ASSISTANT (1/95-8/97) Department of Geology, Kent State University, Kent, OH.
Researcher. Responsibilities include primary thesis research and operation/maintenance of x-ray diffraction unit, scanning probe microscopes, and atomic absorption spectrophotometer (graphite furnace). Assess effectiveness of detention basins to attenuate chemical pollutants in urban stormwater runoff. Responsibilities include operation of automated sampling equipment, chemical analysis of water samples for heavy metals and nutrients, and statistical analyses of data.

GRADUATE TEACHING ASSISTANT (8/96-12/96) Department of Geology, Kent State University, Kent, OH.
Head TA for Mineralogy class. Responsibilities include design and organization of laboratory exercises and examinations.

FIELD SCIENTIST (4/91-8/94) Environmental Associates, Akron, OH.
Natural Resource specialist. Responsibilities include development of municipal stromwater pollution prevention plans to mitigate environmental degradation of surface waters.

FIELD SCIENTIST (4/91-8/94) Buckeye Bison, North Canton, OH.
Wildlife manager overseeing a herd of bison. Responsibilities include maintenance of food source, relocation of bison to prevent overgrazing, implementation of immunization program, production of topographic maps showing distribution of various soil and plant types.

Society Memberships

GEOCHEMICAL SOCIETY

MINERALOGICAL SOCIETY OF AMERICA

AMERICAN SOCIETY FOR MICROBIOLOGY

Honors and Awards

C. G. TILLMAN AWARD FOR EXCELLENCE IN TEACHING, Dept. of Geological Sciences, Virginia Tech.

WATER RESOURCE RESEARCH INSTITUTE FELLOWSHIP, Depts. of Biology, Chemistry, Geology, and Geography, Kent State University.

B. J. WALDRON FIELD CAMP AWARD, Dept. of Geology, Kent State University.

OHIO BOYS STATE DELEGATE, Northwest High School.

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