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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. *Copyright © 2000 Elsevier Science Ltd*

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 (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 intermolecular and intramolecular 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.

2. MATERIALS AND METHODS

2.1. 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 sorptive 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 ms. Induction of the plasmid was accomplished by culturing the cells in 0.02 M

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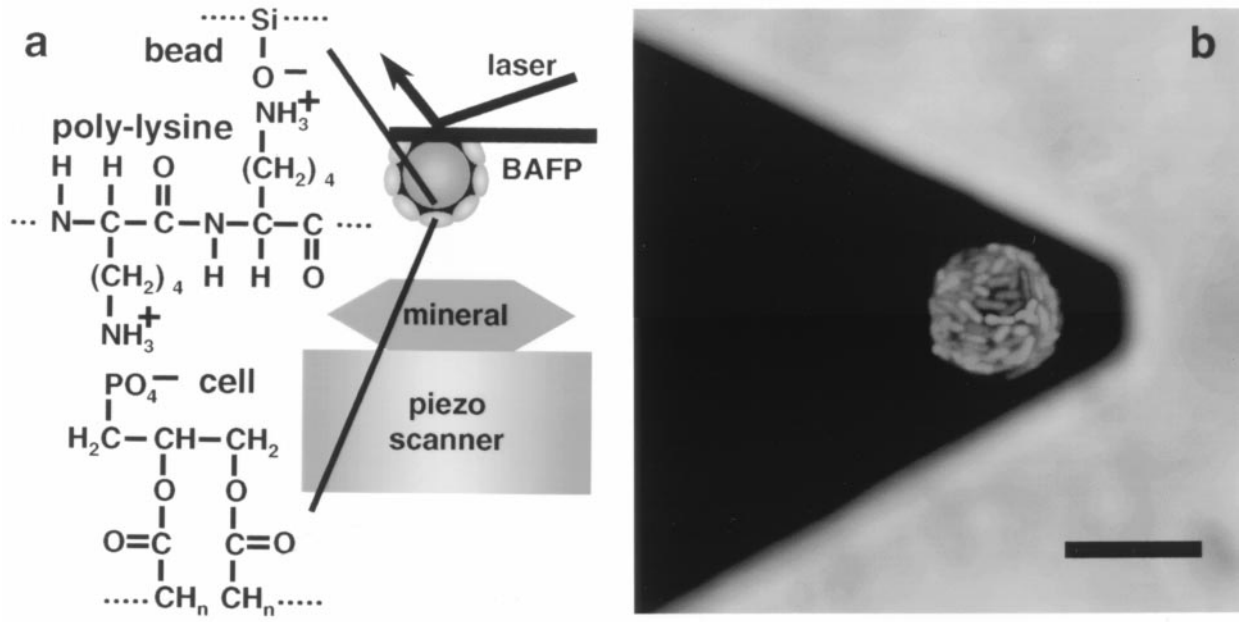

Fig. 1. (a) BFM components highlighting the linkage between bacterial cells and the force-sensing cantilever. Illustrated is one of many possible poly-lysine linkages between negatively charged silanol groups on the bead and negatively charged cell–surface functional groups on biomolecules, such as phospholipids (shown here) or lipopolysaccharides. A piezoelectric scanner was used to translate a mineral to and from a 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 toward 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.

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).

2.2. 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. 1). Glass beads (~5 μm radius; Duke Scientific or Polysciences) were cleaned with hydrofluoric acid (1% solution for 1.5 min) or sodium hydroxide (50% solution for 60 min), 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 8000 × g for 5 min 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, 200× 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 is 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 100×, 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.

2.3. 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 toward, made contact with, and retracted from bacteria on the probe (Fig. 1). The mineral was translated at rates of <3 μm s^{-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 conver-

sion 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 a 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 bacteria have been attached to cantilevers (results not shown), but this linkage is extremely difficult. Recent advances in optical tweezers (Svoboda and Block, 1994) and nanotweezers (Kim and Lieber, 1999) technologies could significantly enhance the linkage of single cells to a cantilever.

3. RESULTS

Net repulsive interfacial forces were observed for muscovite approach curves (Fig. 2a). Conversely, *E. coli*-goethite and -graphite systems exhibited more attractive interfacial forces on approach (Fig. 2b,c). 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 $\sim$400 nm (Fig. 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. 2b,c).

Results from the control experiment between muscovite and a naked poly-lysine coated bead (no bacteria) (Fig. 2d) were very different from those between muscovite and an *E. coli*–coated bead (Fig. 2a). As expected, the positively charged poly-lysine exhibited strong attraction toward 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.

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 Figure 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 $\sim\pm$ 0.2 nN in force and $\sim\pm$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.

4. DISCUSSION

4.1. 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 (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 nanometers 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 nanometers (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).

4.2. Mineralogical and Microbiological Aspects of Forces

Deconvolution of the net force between two surfaces into contributions made by individual interaction mechanisms

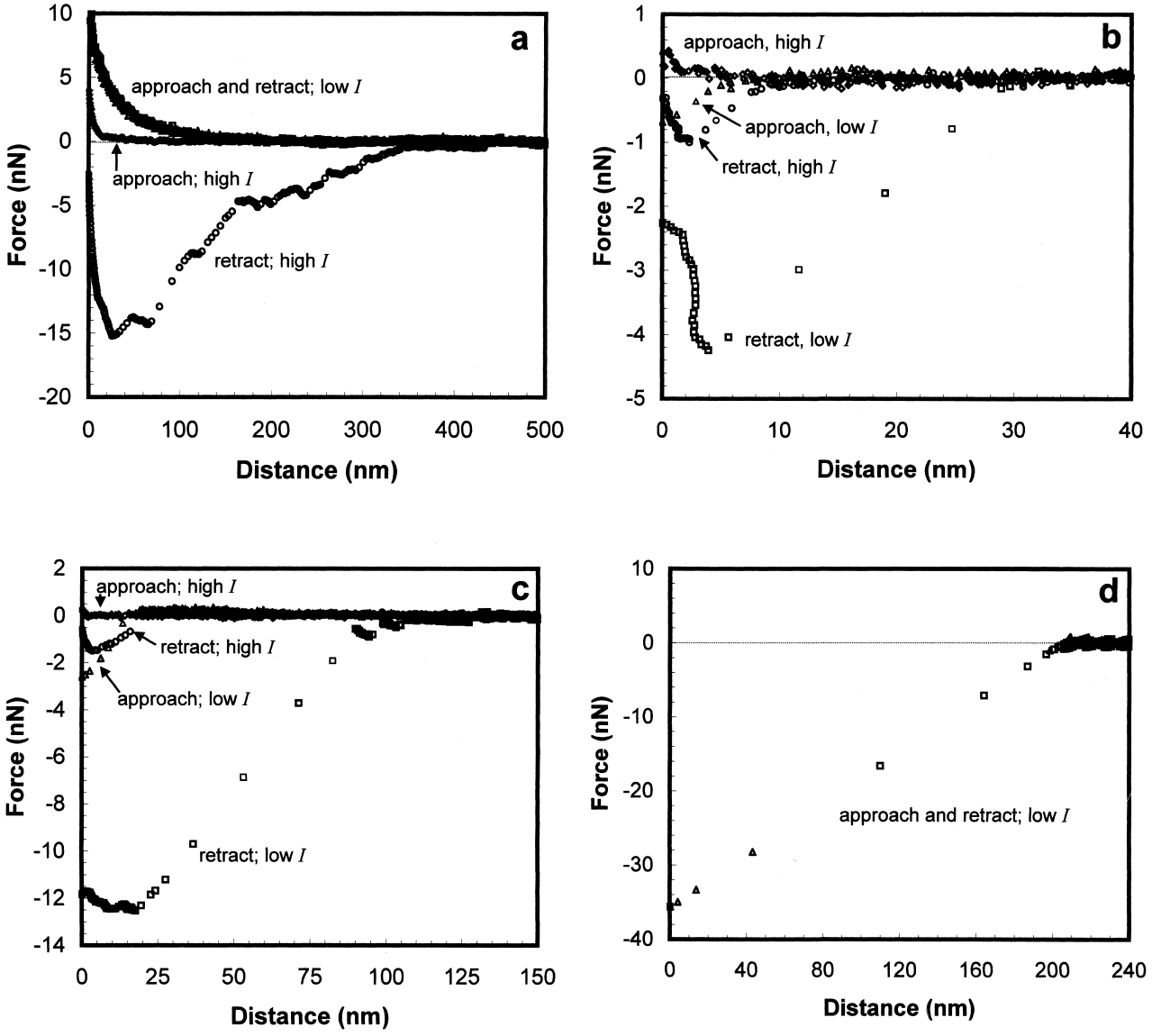

Fig. 2. Force–distance curves for *Escherichia coli* K-12 and (a) muscovite (001) surface, (b) goethite (010) surface, and (c) graphite (001) surface at pH 6, 25°C and varying ionic strengths (I). (d) Control experiment for muscovite (001) surface and naked poly-lysine coated bead. Jump-to- or jump-from-contact 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.

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. Nonetheless, a general interpretation consistent with the characteristics of the force types outlined above can be made from the force–distance curves shown in Figure 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. 2a). The magnitude of repulsion and distance over which the force was operative diminished at high 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. 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 such as 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. 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 to 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. 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. 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).

5. 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 complemented 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 interspecies and intraspecies signaling of microorganisms within a biofilm or other community by probing bacteria–bacteria interactions, host–pathogen recognition, and/or the effects of aqueous biochemical signals 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 BFM 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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