Attachment Behavior of *Shewanella putrefaciens* onto Magnetite under Aerobic and Anaerobic Conditions

Jennifer A. Roberts, David A. Fowle, Brian T. Hughes, and Ezra Kulczycki

Department of Geology, University of Kansas, Lawrence, Kansas, 66047, USA

In this study, batch experiments were used to characterize attachment behavior of *Shewanella putrefaciens* strain 200R to ferrihydrite and magnetite. Attachment was quantified in batch experiments with a 0.01 M NaNO₃ solution as a function of pH (ranging from 3 to 10), sorbed anion (PO₄³⁻), and growth conditions (aerobic vs. anaerobic). Electrophoretic mobility data was collected for *S. putrefaciens* cells and magnetite grains and used as a means to interpret the role of electrostatic interaction in attachment studies. Little difference in attachment behavior was observed as a function of growth conditions or surface treatments. The exception was at pH ranging from 2 to 4, under anaerobic conditions, where increased attachment was measured on magnetite surfaces with sorbed PO₄³⁻. This increased attachment was attributed to development of Fe-PO₄ surface complexes or secondary mineral phases, resulting in altered surface interactions between cell and mineral surfaces. Attachment was irreversible and increased with time under anaerobic conditions even under elevated pH conditions unfavourable to electrostatic interactions between cells and mineral surfaces. These results suggest that electrophoretic mobility data in this system is not a good predictor of attachment behavior, while surface charge development via protonation and deprotonation of surface functional groups is consistent with experimental attachment data. In this study, *S. putrefaciens* appears to utilize polymers or pili to remain attached to Fe-oxides and this process may facilitate Fe reduction on these surfaces. Results from this study underscore the need for quantitative bulk measurements of microbial attachment to accurately predict partitioning of dissimilatory iron reducing bacteria between solution and solid phases.

Keywords bacterial attachment, *Shewanella putrefaciens*, magnetite

INTRODUCTION

Microbial attachment to mineral surfaces is a fundamental process responsible for initiating a cascade of metabolic and

weathering reactions. In aqueous environments microbial attachment behavior controls weathering reactions in the vicinity of colonizing cells (e.g. Bennett et al., 2001), mobility of metals via cell wall sorption (e.g., Beveridge and Murray 1980; Beveridge and Fyfe 1985; Beveridge and Doyle 1989; Fein et al. 1997; Fowle and Fein 1999; Fein 2000), and is a primary control on microbial transport and biofouling in aquifers. In general, cells benefit from attachment by gaining access to surface adherent nutrients (Davis and McFeters 1988; Lechevallier and McFeters 1990; Mueller 1996) and refuge from predation by grazers (Harvey 1997). In particular, dissimilatory iron reducing bacteria (DIRB) may require attachment in order to couple the oxidation of organic matter to the reduction of mineral sources of Fe(III) (e.g., Lovley 1987; Nealson and Saffarini 1994). Therefore rates of organic matter oxidation may be limited and controlled by the solubility and bioavailability of Fe(III) reservoirs in subsurface environments (Sulzberger et al. 1989). DIRB have evolved a number of strategies to overcome the inherent insoluble nature of the iron oxides in the environment including the use of chelators and electron shuttles to mobilize Fe(III) at circumneutral pH (Nevin and Lovley 2002). Direct attachment, however, is still accepted as the predominant mechanism for accessing Fe(III) minerals in environmental settings (Lovley et al. 2004).

Dissimilatory Fe(III) and Mn(IV) reduction by *Geobacter* sp. and *Shewanella* sp., appears to be linked to outer-membrane expression of terminal reductases joined to transmembrane and intracellular electron transport components. Gaspard et al. (1998) found that Fe(III) reductase activity is predominantly expressed in the outer membrane fraction of lysed *G. sulfurreducens* cells; 80% of Fe(III) reductase activity was associated with the outer membrane, while 20% was found in the periplasmic region, with no significant activity observed in the cytoplasm (Gaspard et al. 1998). These results are consistent with studies of *Shewanella putrefaciens* in which peripheral proteins secreted to the exterior of the cell membrane exhibit the majority of Fe(III) reductase activity (DiChristina et al. 2002). Both *S. putrefaciens* and *G. sulfurreducens* exhibit 70 to 100 kDa heme-containing proteins located in the outer membranes, further supporting a role for attachment in the reduction of Fe(III). Some DIRB, however, may obviate the need for attachment to satisfy metabolic needs

Received 14 March 2006; accepted 21 July 2006.

The authors gratefully acknowledge the assistance and contributions of Jeremy Fein, Brena S. Mauck, Christina Smeaton, Nathan Yee, and Rachel Mathis. This research was supported by the National Science Foundation, EAR #0230204 (Roberts) and the Geology Associates of the University of Kansas.

Address correspondence to Jennifer A. Roberts, Department of Geology, University of Kansas, Multidisciplinary Research Building, 2030 Becker Dr., Lawrence, KS 66047, USA. E-mail: jenrob@ku.edu

by utilizing nanowires to shuttle electrons from surface adherent cells (Gorby et al. 2006; Reguera et al. 2005).

When taken together these results support a conceptual model wherein direct cell-mineral contact, for at least some portion of cells, is necessary for DIRB to access insoluble Fe(III) oxide and hydroxide phases. Both *Shewanella* sp. and *Geobacter* sp. utilize specialized flagella to attach to Fe-oxide minerals and remain attached in order to obtain mineral-bound Fe(III) to use as a terminal electron acceptor (TEA) (Caccavo and Das 2002; Childers et al. 2002; Lower et al. 2001). Lower et al. (2001) found that *S. oneidensis* responded to goethite surfaces by producing stronger adhesion energies at the cell-mineral interface under anaerobic growth conditions. Preferential attachment to goethite, compared to hydrous ferric oxide and hematite, by *Shewanella alga* was also demonstrated in bulk adhesion studies (Caccavo and Das 2002). While specific adhesion mechanisms for DIRB have been studied in some detail on the molecular scale, comparatively less attention has been given to the quantification of their bulk attachment behavior. Quantitative measurements of DIRB attachment to typical Fe(III) oxides are still needed to predict the spatial distribution of these microbial communities in subsurface environments and may be key in assessing the impact of their metabolic processes in contaminated settings (Mauck and Roberts 2006).

In this study, we examined the attachment behavior of the facultative DIRB, *S. putrefaciens* to magnetite as a function of sorbed PO_4^{3-} . Magnetite is often a secondary product of dissimilatory iron reduction of Fe(III) oxides (e.g., Fredrickson 1998; Sparks et al. 1990; Lovely 1991) and it has been shown previously that *S. putrefaciens* can reduce magnetite with direct contact (Kostka and Nealson 1995; Dong et al. 2000). Magnetite, therefore, may be an abundant attachment surface for DIRB in established iron reducing environments (Emerson 1976; Emerson and Widmer 1978; Karlin and Levi 1983; Karlin et al. 1987; Maher and Taylor 1988; Pye et al. 1990; Baedeker et al. 1992). Though PO_4^{3-} is often scarce in the subsurface, in many environments it occurs sorbed onto Fe oxides (Bostrom et al. 1982; Shaffer 1986; Müller et al. 2002), which may provide DIRB with this essential nutrient in close proximity to TEA. Alternatively, PO_4^{3-} may compete with cells for attachment sites on the oxide surface. Here we used controlled batch attachment experiments containing magnetite with and without sorbed PO_4^{3-} and *Shewanella putrefaciens* 200R grown aerobically and anaerobically to quantify attachment potential.

MATERIALS AND METHODS

Culture Conditions

All media, reagents and electrolytes were made using reverse osmosis deionized (18 M Ω) water. Attachment experiments were performed using *Shewanella putrefaciens* strain 200R grown under aerobic or anaerobic conditions. The cells were cultivated aerobically in 30 g L⁻¹ trypticase soy broth (Becton Dickinson and Company, Sparks, Maryland) at 30°C

with end-over-end mixing and harvested in stationary phase after 13 hours.

Aerobically grown *S. putrefaciens* was converted to anaerobic growth by first washing cells in log-phase growth with an anaerobic growth medium. The anaerobic medium, consisting of 15.5 g L⁻¹ Luria broth (Becton Dickinson and Company, Sparks, Maryland) with the addition of 2 mM ferric citrate as an electron acceptor and 15 mM sodium lactate as an electron donor (Myers and Nealson 1990; Lower et al. 2001), was boiled for 15 minutes, capped, and cooled in an ice bath. The medium was then dispensed in an anaerobic chamber Coy Laboratories Inc., Grass Lake, MI), sealed, and autoclaved. Aerobic cells were pelleted by centrifugation at 2012 $\times g$ for 10 minutes and then resuspended in the anaerobic medium. This process was repeated 5 times and a final suspension was made with anaerobic medium. Fresh media was inoculated with this suspension, incubated in an anaerobic chamber at 25°C and harvested in stationary phase after 18 hours.

S. putrefaciens was prepared for experiments by washing pelleted cells three times with the electrolyte of interest using alternating centrifugation and vortexing (e.g., Yee et al. 2000) with resuspension in fresh electrolyte. This washing step ensures that no growth media remained prior to experimentation.

Mineral Synthesis and Preparation. 2-line ferrihydrite (Fer) was prepared using methods from Cornell and Schwertman (1996). The precipitate was rinsed once with RO water and centrifuged, then placed in a dialysis bag and submerged in RO water for 48 hours. The clean ferrihydrite, (crystal size ~ 3 nm), was freeze-dried (Freeze Dry System, Freezone 4.5, Labconco, Kansas City, MO) before use in batch experiments to promote aggregation of crystals. An industrial magnetite (Mgt) was obtained from PEL Technologies in Canton, Ohio. The bulk material was ground using a ceramic mortar and pestle and sieved to retain the 200–500 μm size fraction. The material was subjected to a rinsing procedure in which it was rinsed with 10% NaOH, rinsed with RO water, rinsed with 10% HNO_3 , rinsed with RO water until the pH was circum-neutral and freeze dried (Freeze Dry System, Freezone 4.5, Labconco, Kansas City, Missouri). Mineralogy of both magnetite phases was confirmed by X-ray diffraction (XRD) (Bruker D8 Discover powder diffractometer; University of Kansas Crystallography Lab). Samples were scanned for two hours from 4° to 70° with an increment of 0.04 at two seconds per step. The materials were also characterized using a scanning electron microscope (LEO 1550 field emission SEM) equipped with an electron dispersive system (EDAX). Surface area of the 2-line ferrihydrite (229 m² g⁻¹) and magnetite (3.10 m² g⁻¹) were measured using a three point BET with N₂ as the adsorbate gas on a Quantachrome Nova Series E.

Aliquots of the sieved and washed magnetite were also prepared with sorbed PO_4^{3-} for batch attachment experiments. The magnetite was reacted with a 300 μM phosphate buffer solution (pH 6.9) for 12 hours, then rinsed with RO water and freeze-dried (Lovley and Phillips 1986). In order to verify that no desorption of the PO_4^{3-} occurred over the course of batch attachment

experiments, experimental blanks were included and dissolved PO_4^{3-} was determined colorimetrically at 690 nm using the stan-
nous chloride method (Greenberg et al. 1992).

Surface Charge Characterization

Electrophoretic mobility of bacterial cells, ferrihydrite, and magnetite were measured at 25°C utilizing a laser-Doppler velocimetric device (Zetasizer 3000, Malvern Instruments, UK). Electrophoretic mobility will vary as a function of pH and ionic strength, therefore a dilute electrolyte with monovalent cations was used for these measurements as well as attachment/detachments experiments to prevent any collapse of the electric double layer or suppression of the electrical field with outer sphere complexation of Ca^{2+} , Sr^{2+} , and Ba^{2+} (e.g., Simoni et al. 2000; Yee et al. 2004). Measurements of bacterial electrophoretic mobility was determined on washed *S. putrefaciens* grown both aerobically and anaerobically and diluted to a solid-solution concentration of 50 mg solid L^{-1} (dry wt.) in 0.01 M NaCl. Electrophoretic mobility of prepared 2-line ferrihydrite and magnetite were determined with suspensions of 10 mg solid L^{-1} in 0.01 M NaNO_3 . The pH of each suspension was adjusted via the addition of small aliquots of standardized HNO_3 or KOH of similar ionic strength and allowed to equilibrate with end-over-end mixing for two hours. Velocity measurements were made in duplicate as a function of pH with an applied field strength of 2500 V m^{-1} .

Batch Attachment/Detachment Experiments

Batch attachment and detachment experiments were performed using *S. putrefaciens* as a function of pH, sorbed PO_4^{3-} and growth conditions. To minimize repetition in the text of this manuscript hereafter we will refer to the following short forms: S200R = *Shewanella putrefaciens* 200R; A = aerobic; An = anaerobic; P = magnetite treated with PO_4^{3-} . Thus S200R A P would indicate the experiment included *S. putrefaciens* grown aerobically and magnetite pretreated with PO_4^{3-} . Cells were harvested in stationary phase and washed (as described above) in 0.01 M NaNO_3 . The wet weight of pelleted cells was determined following the last rinse. Each experiment consisted of 9 polypropylene test tubes containing 10 mL of solution. The solution consisted of 1 g *S. putrefaciens* L^{-1} ($\sim 10^9$ cells mL^{-1}) 0.01 M NaNO_3 and 4 g L^{-1} 2-line ferrihydrite or 50 g L^{-1} prepared magnetite. A standard curve was established using the 1 g S200R L^{-1} 0.01 M NaNO_3 solution, along with a dilution series of 0.75 g L^{-1} , 0.5 g L^{-1} , and 0.25 g L^{-1} . Optical density of each solution was determined 600 nm using a spectrophotometer (Spectronic 2000, Bausch and Lomb 1979). Kinetics of attachment of *S. putrefaciens* were determined at pH 2.5 and 6 measuring optical density at 600 nm every 20 minutes for at least 2 h.

For attachment experiments the pH in each tube was adjusted using 0.1 M NaOH or 0.1 M HNO_3 to a final pH of 2.5, 3, 4, 5, 6, 7, 8, 9, and 10, respectively. The test tubes were rotated end-

over-end for 2 h at 8 rpm. After 2 h a final pH was recorded, and cells and mineral phases were allowed to separate via gravity before the optical density of suspended cells was measured at 600 nm.

Reversibility of attachment was determined in a parallel set of detachment experiments. Approximately 100 mL of 1 g S200R L^{-1} 0.01 M NaNO_3 and 50 g L^{-1} prepared magnetite solution was adjusted to pH 2.5. Ten test tubes were filled with 10 mL each of the pH-adjusted solution. The tubes were rotated end-over-end for 2 h at 8 rpm after which optical density was measured at 600 nm for 2 of the 10 tubes. The remaining 8 tubes were to pH 3, 4, 5, 6, 7, 8, 9 and 10, respectively, using 0.1 M NaOH and 0.1 M HNO_3 . The tubes were rotated for an additional 2 h, and then optical density was measured at 600 nm and final pH was recorded.

Attachment and detachment experiments were conducted with S200R A and An reacted with ferrihydrite, magnetite, and magnetite with sorbed PO_4^{3-} (prepared as described in the previous section). All experiments containing anaerobically grown S200R were conducted in an anaerobic chamber and performed with solutions and test tubes that were allowed to degas overnight in the anaerobic chamber. All attachment and detachment experiments were done in duplicate and, in some cases triplicate. Because of variations in pH between reactors the data is shown in its entirety and not homogenized.

RESULTS AND DISCUSSION

Electrophoretic Mobility Measurements of Bacteria and Minerals

The electrophoretic mobility of magnetite, ferrihydrite, and 200R (A and An) in 0.01 M NaNO_3 electrolyte solutions is shown in Figure 1. The data indicate that the cells (Figure 1B) and magnetite (Figure 1A) are dominantly electronegative in the pH range studied. Ferrihydrite (Figure 1A) is electropositive from pH values ranging from 2–7.8 with the isoelectric point of the mineral at approximately pH 7.8. The magnitude of the electropositive mobility decreases from pH 2.2 to pH 7.8 with a measured change in mobility of 3.27 [(microns/sec)/(volt/cm)]. In contrast, magnetite displays electropositive mobility only at pH values less than 3.0 with a measured isoelectric point at a pH of 3.03. Above the isoelectric point, the magnetite mobility is negative decreasing from 0 to -3.54 [(microns/sec)/(volt/cm)], with increasing pH.

Cells grown both aerobically and anaerobically and suspended in 0.01 M NaNO_3 display a sharp increase in the absolute value of the electrophoretic mobility from pH 2.5 to 5. Under neutral and alkaline pH values, cell mobility levels and remains fairly constant with little difference between 200R A and 200R An. Electrophoretic mobility experiments conducted on the anaerobic cells indicate a charge reversal to positive mobility values at low pH, with an isoelectric point between pH 3 and 3.5. 200R grown aerobically, however, never reaches an electropositive condition between pH 2 and 10.

FIG. 1. (a) Electrophoretic mobility as a function of pH for magnetite (Mgt) and ferrihydrite (Fer) in 0.01 M NaNO_3 . (b) Electrophoretic mobility as a function of pH for *S. putrefaciens* 200R grown aerobically (S200R A) and anaerobically (S200R An) in 0.01 M NaNO_3 .

The pH dependent mobility behavior of mineral and bacterial surfaces results from differences in the concentration and density of negatively charged surface sites. Results for pH_{zpc} values for ferrihydrite from this study are fairly consistent with other published values for a number of iron oxides, which range from pH 6.7 to 8.5 (e.g., goethite, HFO, hematite, Stumm and Morgan 1996). Mobility data for magnetite, however, indicate a far lower pH_{zpc} of 3.03 than previous studies, which found a value closer to circumneutral ($\text{pH}_{\text{zpc}} = 6.5$, Stumm and Morgan 1996). Our previous measurements of the industrial magnetite characterized in this study as well as synthesized magnetite support pH_{zpc} values between 3 and 4.5. Neither phase has discernable impurities according to analysis by XRD and SEM-EDS. Electrophoretic mobility measurements of these phases in a complex electrolyte solution (a groundwater containing divalent cations) results in substantial shielding of the electronegative potential of the mineral surfaces (Roberts et al. 2004).

Growth conditions also appear to impact electrophoretic mobility, with the aerobically grown cells slightly more electronegative than anaerobically grown cells in the range of pH 2–4. 200R mobility data indicate an isoelectric point ranging from pH 2–3.4. At this pH condition, the concentration of negative charged surface sites is equal to the concentration of positively charged amino acid sites, and the net surface charge is equal to zero. As the pH increases from the isoelectric point, the acidic cell wall functional groups progressively deprotonate, generating a net negative charge within the cell wall and an electronegative potential on the cell surface. In addition to surface charge, the magnitude of the electrophoretic mobility is also controlled by the

concentration of electrolyte ions in solution. At high electrolyte concentrations, electrostatic sorption of Na^+ counter-ions can satisfy the excess surface charge and decrease surface electric potential. In contrast, dilute electrolyte concentrations allows the surface electric field to expand which results in increasing electrophoretic velocities.

The Role of Growth Conditions on Attachment Behavior

Figure 2 depicts the attachment of S200R onto ferrihydrite as a function of growth condition and pH. Cells grown both anaerobically and aerobically display similar trends in attachment behavior. Specifically, between pH values 3–5.5, 70–80% of the cells are adhered to the ferrihydrite surface. Although there is a slight decrease in the mass of anaerobic cells attached in this pH range, differences can be attributed to experimental error, which was consistently $\pm 2\%$ but ranged as high $\pm 5\%$. Between pH 5.5 and 6.2 there is a sharp decrease in the abundance of cells attached to the ferrihydrite surface, which is consistent with an attachment edge relating to a change in surface attraction at these values. Based on the electropositive nature of the minerals shear plane and the highly electronegative nature of the cells at these pH values the attachment edge would be expected to shift to slightly higher pH values (Figure 1). It should be noted that there is very little difference between the attraction of anaerobic and aerobic cells at low pH values although the anaerobic cells have positive electrophoretic mobilities at these pH values. We interpret this as a result of hydrophobic attractive forces dominating over electrostatic driving forces under these

FIG. 2. Percent of *S. putrefaciens* 200R grown aerobically (S200R A; gray circles) and anaerobically (S200R B; black circles) attached onto ferrihydrite.

pH conditions. The dominance of hydrophobic interactions in adherence of *Shewanella alga* to amorphous Fe(III) oxide has been demonstrated previously (e.g., Caccavo et al. 1997; Caccavo 1999), and is a likely explanation for the attachment behavior observed here.

Attachment of both S200R A and An onto magnetite and magnetite P is depicted in Figure 3. Attachment behavior of S200R

FIG. 3. Percent of *S. putrefaciens* 200R grown aerobically (S200R A) and anaerobically (S200R An) attached onto magnetite or magnetite with PO_4^{3-} sorbed to the surface (S200R A P; S200R An P). Shaded region depicts a $\pm$ zone of 5% for standard attachment error around S200R A, S200R A P, and S200R An. Note there is little difference between the four treatments. The exception is at low pH under anaerobic conditions with sorbed PO_4^{3-} , where attachment increases to $\sim 45\%$.

on magnetite is similar to ferrihydrite with little difference between the four treatments from pH 5 through 8. Attachment averages $4.1\% \pm 4.8\%$ for S200R A, S200R An, and S200R A P treatments for these pH values. The attached cells are heterogeneously distributed on the magnetite surface when viewed with SEM (Figure 4A), with cells attached to a variety of crystal faces. Some cells appear to be in contact with each other via polymeric material or other pili-like appendages or wires (e.g., Gorby et al. 2006). Attachment of S200R An P is slightly higher than all other treatments at pH 5–8 and is significantly higher at pH values less than 5 with a 10 and 30% greater mass of cells on the surface of magnetite than the other attachment regimes. These results suggest a distinctly different attachment mechanism at pH values when compared to S200R A P. Potential mechanisms for increased attachment can be seen in Figure 4B, which depicts patches of what appear to be mixed Fe- PO_4 coatings (as interpreted from EDS and crystal structure consistent with vivianite) on the surface of the magnetite grains. These crystals are in low abundance and are below the detection limit for characterization by x-ray diffraction. Furthermore, these secondary precipitates were observed only in PO_4 -amended experiments conducted anaerobically. Studies by Parfitt et al. (1975) and Martin et al. (1988) have shown previously that sorption of PO_4^{3-} onto oxides can result in the formation of mixed Fe- PO_4 phases at the sorption interface. This may in fact be facilitated under anaerobic conditions where cells may continue to promote reduction in the absence of an excess of electron donor (e.g., Kenward et al. 2006), in this case promoting reductive dissolution of magnetite and producing Fe(II), which reacts with surface-sorbed PO_4^{3-} .

Reversibility of Attachment

Detachment studies were performed to investigate the reversibility of the attachment of *S. putrefaciens* onto magnetite (Figure 5). Initial attachment was equivalent to the percentage of cells attached at pH 2.5 (Figure 3). Detachment, therefore, is expressed as the percentage of those attached cells, which detach into solution after pH adjustment. Figure 5A depicts the detachment of aerobically grown *S. putrefaciens* off the surface of both magnetite and PO_4^{3-} treated magnetite. Intriguingly there is little to no detachment of the cells off the magnetite surface. This is in direct contrast with studies of Gram-positive bacterial attachment, which demonstrated complete reversibility of *Bacillus subtilis* attachment on quartz and corundum surfaces (e.g., Yee et al. 2000). Although the driving force for detachment is quite high at pH > 7 , only $\sim 10\%$ of the attached cells are released. These results indicate that once attached: (a) the hydrophobic or chemical interactions between the cell and the surface are too strong to overcome by a change in electrostatic repulsion; or (b) once attached the interface between the cell and surface generates a microenvironment that is rich in protons unaffected by the bulk aqueous geochemical parameters (e.g., Glasauer et al. 2001); or, (c) that once attached the cells release

FIG. 4. Two SEM photomicrographs of anaerobically grown *S. putrefaciens* 200R on magnetite with sorbed PO_4^{3-} after a 2-h batch attachment experiment. (a) Potential nanowires (arrows, e.g., Gorby et al. 2005; Reguera et al. 2005) or exudates from lysis are visible and only observed on magnetite with sorbed PO_4^{3-} . Scale bar is 1 μm ; (b) Secondary precipitate coating magnetite surface. Scale bar is 2 μm .

polymer or express pili or other similar attachment appendages that permanently adhere the cell to the mineral surface (e.g., Caccavo and Das 2002; Lower et al. 2001).

Cells grown anaerobically for detachment studies (Figure 5B) show similar behavior with very little detachment at low to mid pH values (although it should be noted that a single experiment

does appear to demonstrate some detachment). In contrast to results with S200R, the cells grown anaerobically exhibit continued attachment at pH > 5 (up to 30% increase in attachment). These data are completely contrary to predictions based on mobility data. The mechanisms for both continued attachment and lack of detachment, however, are revealed when the

FIG. 5. a) Reversibility of attachment onto magnetite (and magnetite with PO_4^{3-} sorbed) for *S. putrefaciens* 200R grown aerobically (S200R A; S200R A P) b.) Reversibility of attachment onto magnetite (and magnetite with PO_4^{3-} sorbed) for *S. putrefaciens* 200R grown anaerobically (S200R An; S200R An P). Shaded region depicts zone that may be considered net zero detachment due to experimental error. Cells were attached at an initial pH of 2.5 for 2h prior to detachment. Detachment data demonstrates the irreversibility of attachment for both aerobically and anaerobically grown cells on both magnetite and magnetite with sorbed PO_4^{3-} . Negative detachment signifies continued attachment to the magnetite surface by anaerobic cells.

FIG. 6. SEM photomicrograph of anaerobically grown *S. putrefaciens* 200R on magnetite with sorbed PO_4^{3-} after a two-hour batch detachment experiment. Note dissolution pits (center) that might physically inhibit detachment. Scale bar is 2 μm .

anaerobic cells are imaged via SEM. Figure 6 depicts anaerobic cells attached to the PO_4^{3-} -coated magnetite surface. Many cells appear to have dissolved significant quantities of the oxide surface forming etch pits in which they are entrapped and clearly unavailable for detachment. Several locations on the magnetite surface exhibit cells that have aggregated within a small area on the surface of the mineral grain. This could be interpreted as the cells “attaching” via hydrophobic interaction to other cells rather than to the electrostatically repulsive mineral surface.

Surface Charge Development and Modeling

Figures 7 and 8 depict modeling of surface charge development on the surfaces of both the bacterial cells and mineral grains. Figure 7 specifically shows the regions in which we would predict significant electrostatic attraction between the cell and mineral surface. Based on electrostatic interactions magnetite-*Shewanella* attachment should occur at a pH value between 6–7 based on the bacterial surface parameters of Haas (2004) for S200R. In contrast, data from Fein et al. (personal communication 2005) predict attachment from pH values 2–7 for a species of the same genus (*Shewanella oneidensis*). The predictions of Fein et al. (personal communication 2005) appear to more closely approximate the attachment phenomena demonstrated in this study. In combination, these data support a surface

speciation model such as the one depicted in Figure 8 where the *Shewanella* surface functional groups as shown in Equation 1:

(>R = a surface functional group within the bacterial cell surface) begin to deprotonate at pH 2 leading to the development of negative surface charge.

Negative surface charge, therefore, promotes the bulk of attachment at low pH values, but also results in continued attachment until the magnetite surface is predominately neutral. Prediction of magnetite surface charge after the adsorption of PO_4^{3-} to the surface (Figure 7) appears to dampen positive charge on the surface via the formation of neutrally charged surface complexes. Surface-sorbed PO_4^{3-} alone would not enhance attachment as observed in this study (Figure 3) unless biotic forces such as chemotaxis are at play. Instead, we propose that under aerobic conditions there is very little influence on the attachment behavior of *Shewanella* sp. due to the presence of surface-sorbed PO_4^{3-} . It is clear, however, from visual inspection that adsorption of PO_4^{3-} under anaerobic conditions leads to surface precipitation of discrete zones of amorphous Fe- PO_4 mineral phases that likely possess positive charge in the pH range of observed enhanced attachment (e.g., phosphates exhibit $\text{pH}_{\text{zpc}} = 6.4\text{--}8.5$, Stumm and Morgan 1996). This in turn may explain enhanced

FIG. 7. Plot depicting calculated surface charge for magnetite (Mgt), magnetite with sorbed PO_4^{3-} (Mgt-P) and ferrihydrite (Fer), *S. putrefaciens* 200R grown aerobically (S200R A) and anaerobically (S200R An), and *S. oneidensis* (SONed; grown anaerobically) due to surface deprotonation effects as a function of pH. Bacterial surface charge calculations were generated using surface titration data from Haas (2004) (*Shewanella putrefaciens* 200R) and Fein et al. (personal communication 2005) (*Shewanella oneidensis*). Magnetite surface charge calculations were generated using data from Missana et al. (2003). All calculations were made using JChess software with a database modified as described above (Van der Lee 1988). Shaded regions depict zone of predicted attachment onto magnetite based on simply electrostatic interactions for two different models of *Shewanella* surface charge.

FIG. 8. Surface speciation diagram of the first function groups for (>RA₁, dashed lines, personal communication, Fein 2005) and the magnetite surface (>Mgt, solid lines, Missana et al. 2003).

colonization of magnetite inclusions within primary silicates by native consortia of iron reducing bacteria, which has been reported previously (e.g., Roberts et al. 2004).

CONCLUSIONS

Results from this study demonstrate that electrophoretic mobility data for the S200R–magnetite system is not a good predictor of bacterial attachment behavior, while surface charge development via protonation and deprotonation of surface functional groups is consistent with experimental attachment data. Neither model, however, accurately predicts the reversibility of attachment. Attachment is not reversible for S200R under aerobic or anaerobic conditions, and increased attachment is observed at circum-neutral pH for S200R grown under anaerobic conditions suggesting that under anaerobic conditions *Shewanella putrefaciens* may utilize polymers or pili (e.g., Lower et al. 2001; Caccavo and Das 2002; Gorby et al. 2006) to remain attached to Fe mineral surfaces as a means of accessing Fe(III) as a terminal electron acceptor. Because clean mineral surfaces are rarely encountered in the environment where sediment grains exhibit coatings and are comprised of mixed mineralogy, our results indicate that one might expect significant discrepancies between laboratory-based attachment studies and “natural” mineral surfaces with inherent heterogeneity.

REFERENCES

- Baedecker MJ, Cozzarelli IM, Evans JR, Hearn PP. 1992. Authigenic mineral formation in aquifers rich in organic material, 7th International Symposium on Water-Rock Interaction, Park City, Utah.
- Bennett PC, Rogers JR, Hiebert FK, Choi WJ. 2001. Mineralogy, mineral weathering, and microbial ecology. *Geomicrobiol J* 18:3–19.
- Beveridge TJ, Doyle RJ, editors. 1989. *Metal Ions and Bacteria*. New York: John Wiley and Sons.
- Beveridge TJ, Fyfe WS. 1985. Metal fixation by bacterial cell walls. *Can J of Earth Sci* 22:1893–1898.
- Beveridge TJ, Murray RGE. 1980. Sites of metal deposition in the cell walls of *Bacillus subtilis*. *J Bacteriol* 141:876–887.
- Bostrom B, Jansson M, Forsberg C. 1982. Phosphorus release from lake sediments. *Archiv für Hydrobiologie Beihefte Ergebnisse der Limnologie* 18:5–59.
- Caccavo F. 1999. Protein-mediated adhesion of the dissimilatory Fe(III)-reducing bacterium *Shewanella alga* BrY to hydrous ferric oxide. *App Environ Microbiol* 65:5017–5022.
- Caccavo FJ, Das A. 2002. Adhesion of dissimilatory Fe(III)-reducing bacteria to Fe(III) minerals. *Geomicrobiol J* 19:161–177.
- Caccavo F, Schamberger PC, Keiding K, Nielsen PH. 1997. Role of hydrophobicity in adhesion of the dissimilatory Fe(III)-reducing bacterium *Shewanella alga* to amorphous Fe(III) oxide. *App Environ Microbiol* 63:3837–3843.
- Childers S, Clufo S, Lovely DR. 2002. *Geobacter metallireducens* accesses insoluble Fe(III) oxide by chemotaxis. *Nature* 416:767–769.
- Cornell RM, Schwertmann U. 1996. The iron oxides: Structure, properties, reactions, occurrences and uses. VCH, Weinheim.
- Davis DG, McFeters GA. 1988. Growth and comparative physiology of *Klebsiella oxytoca* attached to granular carbon particles and in liquid media. *Micro Eco* 15:165–175.
- DiChristina T, Moore C, Haller C. 2002. Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires ferE, a pulE (gspE) homolog of Type II protein secretion. *J Bacteriol* 184:142–151.
- Dong HL, Fredrickson JK, Kennedy DW, Zachara JM, Kukkadapu RK, Onstott TC. 2000. Mineral transformation associated with the microbial reduction of magnetite. *Chem Geol* 169:299–318.
- Emerson S. 1976. Early diagenesis in anaerobic lake sediments: chemical equilibria in interstitial waters. *Geochim Cosmochim Acta* 40:925–934.
- Emerson S, Widmer S. 1978. Early diagenesis in anaerobic lake sediments: II. Thermodynamic and kinetic factors controlling the formation of iron phosphate. *Geochim Cosmochim Acta* 42:1307–1316.
- Fein JB. 2000. Quantifying the effects of bacteria on adsorption reactions in water-rock systems. *Chem Geol* 169:265–280.
- Fein JB, Daughney CJ, Yee N, Davis TA. 1997. A chemical equilibrium model from metal adsorption onto bacterial surfaces. *Geochim Cosmochim Acta* 61:3319–3328.
- Fowle DA, Fein JB. 1999. Competitive adsorption of metal cations onto two gram positive bacteria: Testing the chemical equilibrium model. *Geochim Cosmochim Acta* 63:3059–3067.
- Fredrickson JK, Zachara JM, Kennedy DW, Dong H, Onstott TC, Hinman NW, Li SM. 1998. Biogenic iron mineralization accompanying the dissimilatory reduction of hydrous ferric oxide by groundwater bacterium. *Geochim Cosmochim Acta* 62:3239–3257.
- Gaspard S, Vazquez F, Holliger C. 1998. Localization and solubilization of the iron(III) reductase of *Geobacter sulfurreducens*. *App Environ Microbiol* 64:3188–3194.
- Glasauer S, Langley S, Beveridge TJ. 2001. Sorption of Fe (hydr)oxides to the surface of *Shewanella putrefaciens*: Cell-bound fine-grained minerals are not always formed de novo. *App Environ Microbiol* 67:5544–5550.
- Gorby YA, Yanina S, McLean JS, Rosso KM, Moytes D, Dohnalkova A, Beveridge TJ, Chang IS, Kim BH, Kim KS, Culley DE, Reed SB, Romine MF, Saffarini DA, Hill EA, Shi L, Elias DA, Kennedy DW, Pinchuk G, Watanabe K, Ishii S, Logan B, Nealsen KH, Fredrickson JK. 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *PNAS* 103:11358–11363.
- Greenberg AE, Clesceri LS, Eaton AD. 1992. *Standard Methods for the Examination of Water and Wastewater*. Washington DC: APHA, 809 pp.
- Haas JR. 2004. Effects of cultivation conditions on acid-base titration properties of *Shewanella putrefaciens*. *Chem Geol* 209:67–81.
- Harvey RW. 1997. In situ and laboratory methods to study subsurface microbial transport. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenback LD, Walter MV, editors. *Manual of Environmental Microbiology*. Washington, DC: ASM Press, pp. 586–599.
- Karlin R, Levi S. 1983. Diagenesis of magnetic minerals in recent hemipelagic sediments. *Nature* 303:327–330.
- Karlin R, Lyle M, Heath GR. 1987. Authigenic magnetite formation in suboxic marine-sediments. *Nature* 326:490–493.
- Kenward P, Yee N, Fowle DA. 2006. Microbial selenate sorption and reduction in nutrient limited systems. *Environ Sci Technol* in press.
- Kostka JE, Nealsen KH. 1995. Dissolution and reduction of magnetite by bacteria. *Environ Sci Technol* 29:2535–2540.
- Lechevallier MW, McFeters GA. 1990. Microbiology of activated carbon. In: McFeters GA, editor. *Drinking water microbiology*. New York: Springer Verlag, pp. 104–119.
- Lovley DR. 1991. Magnetite formation during microbial dissimilatory iron reduction. In: Frankel RB and Blakemore RP, editors. *Iron Biominerals*. New York: Plenum, pp. 151–166.
- Lovley DR. 1987. Organic-matter mineralization with the reduction of ferric iron: a review. *Geomicrobiol J* 5:375–399.
- Lovley DR, Holmes DE, Nevin KP. 2004. Dissimilatory Fe(III) and Mn(IV) reduction. In: Poole R, editor. *Advances in Microbial Physiology*. London: Elsevier Ltd. 49:219–286.
- Lovley DR, Phillips EJP. 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *App Environ Microbiol* 51:683–689.
- Lower SK, Hochella MF, Beveridge TJ. 2001. Bacterial recognition of mineral surfaces: Nanscale interactions between *Shewanella* and α -FeOOH. *Science* 292:1360–1363.

- Maher BA, Taylor RM. 1988. Formation of ultrafine-grained magnetite in soils. *Nature* 336:368–370.
- Martin RR, Smart RSC, Tazaki K. 1988. Direct Observation of Phosphate Precipitation in the Goethite/Phosphate System. *Soil Science Society of America Proceedings* 52:1492–1500.
- Mauck BS, Roberts JA. 2006. Mineralogic control on abundance and diversity of surface-adherent microbial communities. *Geomicrobiol J*, in review.
- Missana T, Garcia-Gutierrez M, Fernandez V. 2003. Uranium(VI) sorption on colloidal magnetite under anoxic environment: Experimental study and surface complexation modelling. *Geochim Cosmochim Acta* 67:2543–2550.
- Mueller RF. 1996. Bacterial transport and colonization in low nutrient environments. *Water Res* 30:2681–2690.
- Müller B, Granina L, Schaller T, Ulrich A, Wehrelil B. 2002. P, As, Sb, Mo, and other elements in sedimentary Fe/Mn layers of Lake Baikal. *Environ Sci Technol* 36:411–420.
- Myers CR, Nealson KH. 1990. Respiration-linked proton translocation coupled to anaerobic reduction of manganese(IV) and iron(III) in *Shewanella putrefaciens* MR-1. *J Bacteriol* 172:6232–6238.
- Nealson KH, Saffarini D. 1994. Iron and manganese in anaerobic respiration: Environmental significance, physiology, and regulation. *Ann Rev Microbiol* 48:311–343.
- Nevin KP, Lovley DR. 2002. Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix fermentans*. *Appl Environ Microbiol* 68:2294–2299.
- Parfitt RL, Atkinson RJ, Smart R. 1975. The mechanism of phosphate fixation by iron oxides. *Soil Science Society of America Proceedings* 39:837–841.
- Pye K, Dickson JAD, Schiavon N, Coleman ML, Cox M. 1990. Formation of siderite - Mg-Calcite-iron sulfide concretions in intertidal marsh and sandflat sediments, North-Norfolk, England. *Sedimentology* 37:325–343.
- Reguera G, McCarthy KD, Mehta T, Nicoll JS, Tuominen MT, Lovley DR. 2005. Extracellular electron transfer via microbial nanowires. *Nature* 435:1098–1101.
- Roberts JA, Hughes BT, Fowle DA. 2004. Micro-scale mineralogic controls on microbial attachment to silicate surfaces: iron and phosphate mineral inclusions. In: Wanty RB, Seal RR, editors. *Water-Rock Interaction. Eleventh International Symposium on Water-Rock Interaction WRI-11*. Saratoga Springs, NY: WRI, 1149–1153.
- Shaffer G. 1986. Phosphate pumps, shuttles in the Black Sea. *Nature* 321:515–517.
- Simoni S, Bosma TNP, Harms H, Zehnder AJB. 2000. Bivalent cations increase both the subpopulation of adhering bacteria and their adhesion efficiency in sand columns. *Environ Sci Technol* 34:1011–1017.
- Sparks NHC, Mann S, Bazylinski DA, Lovely DR, Jannasch HW, Frankel RB. 1990. Structure and morphology of magnetite anaerobically-produced by a marine magnetotactic bacterium and a dissimilatory iron-reducing bacterium. *Earth Planet Sci Lett* 98:14–22.
- Stumm W, Morgan JJ. 1996. *Aquatic Chemistry*. John Wiley and Sons, New York.
- Sulzberger B, Suter D, Siffert C, Banwart S, Stumm W. 1989. Dissolution of Fe(III)(hydr)oxides in natural-waters: Laboratory assessment on the kinetics controlled by surface coordination. *Mar Chem* 28:127–144.
- Yee N, Fein JB, Daughney CJ. 2000. Experimental study of the pH, ionic strength, and reversibility behavior of bacteria-mineral adsorption. *Geochim Cosmochim Acta* 64:609–617.
- Yee N, Fowle DA, Ferris FG. 2004. A Donnan Model for metal sorption onto *Bacillus subtilis*. *Geochim Cosmochim Acta* 68:3657–3664.