Role of Hydrophobicity in Adhesion of the Dissimilatory Fe(III)-Reducing Bacterium *Shewanella alga* to Amorphous Fe(III) Oxide

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Received 27 May 1997/Accepted 7 August 1997

The mechanisms by which the dissimilatory Fe(III)-reducing bacterium *Shewanella alga* adheres to amorphous Fe(III) oxide were examined through comparative analysis of *S. alga* BrY and an adhesion-deficient strain of this species, *S. alga* RAD20. Approximately 100% of *S. alga* BrY cells typically adhered to amorphous Fe(III) oxide, while less than 50% of *S. alga* RAD20 cells adhered. Bulk chemical analysis, isoelectric point analysis, and cell surface analysis by time-of-flight secondary-ion mass spectrometry and electron spectroscopy for chemical analysis demonstrated that the surfaces of *S. alga* BrY cells were predominantly protein but that the surfaces of *S. alga* RAD20 cells were predominantly exopolysaccharide. Physicochemical analyses and hydrophobic interaction assays demonstrated that *S. alga* BrY cells were more hydrophobic than *S. alga* RAD20 cells. This study represents the first quantitative analysis of the adhesion of a dissimilatory Fe(III)-reducing bacterium to amorphous Fe(III) oxide, and the results collectively suggest that hydrophobic interactions are a factor in controlling the adhesion of this bacterium to amorphous Fe(III) oxide. Despite having a reduced ability to adhere, *S. alga* RAD20 reduced Fe(III) oxide at a rate identical to that of *S. alga* BrY. This result contrasts with results of previous studies by demonstrating that irreversible cell adhesion is not requisite for microbial reduction of amorphous Fe(III) oxide. These results suggest that the interaction between dissimilatory Fe(III)-reducing bacteria and amorphous Fe(III) oxide is more complex than previously believed.

Dissimilatory iron-reducing bacteria (DIRB) gain energy to support anaerobic growth by coupling the oxidation of organic matter to the reduction of ferric iron. This metabolism can greatly influence the geochemistry of nonsulfidogenic anaerobic soils and sediments (17, 19, 30, 32) and may also provide a mechanism for both intrinsic and engineered bioremediation of contaminated environments (18, 19). Ferric iron is essentially insoluble at neutral pHs (solubility product constant [K_{sol}] < 10^{-38}) (16), and amorphous Fe(III) oxide is the predominant form of Fe(III) reduced in these environments (25, 36).

Previous studies with both enrichment cultures and pure cultures of DIRB have suggested that physical contact between the cell and Fe(III) minerals is necessary for dissimilatory Fe(III) mineral reduction. When amorphous Fe(III) minerals were separated from DIRB cells by placing the oxide in dialysis tubing, no Fe(III) reduction was observed. Cell-free filtrates from growing cultures of *Pseudomonas* sp. strain 200 (2), *Geobacter metallireducens* (21), and *Shewanella alga* BrY (9) did not reduce Fe(III), suggesting that a soluble, iron-reducing intermediate was not produced. Adhesion of *S. alga* BrY cells to Fe(III) bound in anaerobic activated sludge flocs was found to be a prerequisite for Fe(III) reduction (10).

A number of studies examining the relationship between Fe(III) mineral surface area and Fe(III) reduction rate have provided indirect evidence for the importance of cell-mineral

contact in microbial Fe(III) reduction. Roden and Zachara recently demonstrated that the initial rates and long-term extents of Fe(III) reduction of a variety of Fe(III) oxides by *S. alga* BrY were linearly correlated with the amount of available oxide surface area (34). This same study also found a progressive saturation of reduction sites on the Fe(III) oxides that was akin to ligand adsorption. Arnold et al. reported similar results for hematite reduction by *Pseudomonas* sp. strain 200 (2). These studies suggest that the concentrations of surface sites on Fe(III)-bearing minerals that are available for enzymatic contact are limited by the surface area of the mineral being reduced.

While the necessity of cell-mineral contact for Fe(III) reduction is apparently well-established, the mechanisms by which DIRB adhere to Fe(III) minerals are relatively unknown. In one study, an Fe(III)-reducing *Pseudomonas* sp. that attached to and corroded steel oil pipelines (27) was shown to modify the physicochemical environment of the metal by reducing a protective, insoluble Fe(III) surface film. Corrosion resulted from depolarization of the metal anode (28, 29), and microscopic studies demonstrated that the *Pseudomonas* sp. colonized the surfaces of the steel coupons by means of exopolysaccharide excretion (27). Scanning electron micrographs of *Pseudomonas* sp. strain 200 growing on hematite or goethite also showed that this organism attached to the Fe(III) minerals by what appeared to be extracellular polymers (2).

The purpose of this study was to identify factors that govern the adhesion of the dissimilatory Fe(III)-reducing bacterium *S. alga* to amorphous Fe(III) oxide. This task was accomplished through a comparative examination of the adhesion characteristics, the physicochemical cell surface characteristics, the bulk

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cell chemistry, and the chemistry specific to the cell surfaces of *S. alga* BrY and an adhesion-deficient strain of this species.

MATERIALS AND METHODS

Culture conditions and cell preparation. *S. alga* BrY is a gram-negative rod that was isolated from anaerobic sediments of the Great Bay estuary, New Hampshire (9, 35). *S. alga* RAD20 is an adhesion-deficient strain, the isolation of which is described below. Both *S. alga* strains were grown aerobically in 100 ml of tryptic soy broth (30 g/liter; Difco Laboratories, Detroit, Mich.) at 25°C on a rotary shaker at 150 rpm for 15 h. Cells were harvested by centrifugation (6,000 × g, 4°C, 20 min) during the late exponential or early stationary growth phase. Optimal Fe(III) reductase activity is expressed at this stage of growth (14). Cells were washed once and resuspended in sodium bicarbonate buffer (2.5 g/liter of distilled H₂O, pH 7.0) that had been made anaerobic by boiling and cooling under a stream of O₂-free gas containing 80% N₂ and 20% CO₂ (vol/vol) (4, 5, 8). Stock cultures of both *S. alga* strains were maintained on tryptic soy agar slants at 4°C. Working stock cultures of both *S. alga* strains were maintained on tryptic soy agar plates at ambient temperature.

Adhesion assay. Amorphous Fe(III) oxide was synthesized as described previously (21). The adhesion buffer was made by adding 1.0 ml of anaerobic oxide to 9.0 ml of anaerobic sodium bicarbonate buffer under a constant stream of O₂-free gas containing 80% N₂ and 20% CO₂ (vol/vol). Tubes containing the anaerobic adhesion buffer were sealed with thick butyl rubber stoppers and aluminum crimps and autoclaved at 121°C for 20 min. The adhesion assay was initiated by adding 0.1 ml of the washed-cell suspension to the sterile adhesion buffer (final cell concentration of 1×10^8 to 2×10^8 cells ml⁻¹). After vigorous mixing, the tubes were incubated on their sides at 150 rpm and 25°C for 1 h. The number of total cells was then determined by placing a 0.1-ml aliquot of the adhesion assay buffer into a mixture of 4.8 ml of sterile hydroxylamine hydrochloride (0.25 M in 0.25 M HCl) and 0.1 ml of 30% glutaraldehyde to fix the cells and dissolve the Fe(III) oxide. After a 1-h extraction at room temperature, the cells were counted as described below. The number of unadhered cells was determined by centrifuging a 1.0-ml aliquot of the adhesion assay buffer at $71 \times g$ for 5 min. An aliquot of the supernatant (0.1 ml) was then added to a mixture of 4.8 ml of sterile distilled H₂O and 0.1 ml of 30% glutaraldehyde and mixed, and the cells were counted as described below.

The number of adhered cells was calculated by subtracting the number of unadhered cells from the number of total cells. Adhesion of cells to the glass tubes and cell lysis were accounted for with controls of adhesion assay buffer without amorphous Fe(III) oxide. The adhesion assay was effective in separating unadhered cells from cells bound to the amorphous Fe(III) oxide. Only 0.88% ± 0.31% of the Fe(III) oxide remained in the supernatant after centrifugation. In control tubes, 100% of the cells remained in the supernatant after centrifugation. All adhesion assays were performed in triplicate.

Selective adhesion to amorphous Fe(III) oxide. In order to determine if *S. alga* BrY selectively adhered to amorphous Fe(III) oxide, the adhesion assay was performed in adhesion buffer that contained either 0.1 g of amorphous Fe(III) oxide or 0.15 g of SiO₂ (Aldrich Chemical Co., Inc., Milwaukee, Wis.). These concentrations gave a total available surface area of approximately 4 m² for both minerals, based on the fact that the surface areas of the amorphous Fe(III) oxide and SiO₂ were 600 m² g⁻¹ (34) and 390 ± 40 m² g⁻¹ (Sigma Chemical Co., St. Louis, Mo.), respectively. Hydrophobic interaction assays were conducted as described above, with the exception that the adhesion assay buffer contained 1.0 g (wet weight) of washed phenyl Sepharose CL-4B or Sepharose CL-4B (Sigma Chemical Co.). All treatments were done in triplicate.

Physicochemical characterization of cell surfaces. The surface charge and the hydrophobicity of a cell surface can control bacterial adhesion to a substratum (6). To measure surface charge, the zeta potential of 1:100 dilutions of *S. alga* cell suspensions (final concentration of 1×10^6 to 2×10^6 cells ml⁻¹) was determined at pH 7.0 with a Zetamaster (Malvern Instruments). Zeta potential values were determined from averages of 10 independent measurements. The hydrophobicity of *S. alga* cells was determined by contact angle as described by Mozes and Rouxhet (24). Contact-angle values were determined from averages of five independent measurements. Cells were collected on 0.45-μm-pore-size HVLP filters (Millipore, Bedford, Mass.). Filters were then placed on an agar surface (1% [wt/vol] in a 10% [vol/vol] glycerol solution) and incubated for 1.5 h. A microscope equipped with a gonidometer was then used to measure the contact angle of a drop of distilled H₂O placed on top of the filter. The isoelectric point (pI) of *S. alga* BrY cells was determined by measuring the zeta potential as a function of buffer pH. The pH of the bicarbonate buffer was adjusted with 0.5 N HCl. Cell size was determined with a Zetamaster.

Cell surface-specific chemical analysis. Silver coupons (99.9% silver foil; Johnson Matthey, Alfa Aesar, Ward Hill, Mass.) were cleaned by ultrasonication for 5 min in a mixture of hexane, chloroform, tetrahydrofuran, and methanol. Following cleaning, the coupons were oxidized in concentrated HNO₃ and then rinsed in nanopure water. Isolated colonies of either *S. alga* BrY or *S. alga* RAD20 were transferred to the coupons and dried in air overnight.

The instrument used to collect the time-of-flight secondary-ion mass spectrometry (TOF-SIMS) spectra was a Charles Evans & Associates model T-2000 toroidal reflectance ion flight tube TOF-SIMS. The primary ion source was an 11 keV cesium ion source with an average ion current of 2 pA (measured on Si) for

150 s, with a 156-ps pulse width and a 10-kHz repetition rate, corresponding to 5.7×10^{13} ions/cm², within a static SIMS ion dosage. The ion beam was rastered by using a scatter raster pattern in a 65- by 65-μm area. Charge compensation was accomplished with a 24-V, 4-nA (pulsed-current) beam of electrons, pulsed out of phase with an ion gun every five ion gun cycles. A 3-kV sample bias was placed at the sample stage to act as the secondary ion extraction field. Ions were detected in ion-counting mode. A 1-kV postaccelerating voltage was applied at the detector. Positive and negative ion spectra were obtained.

A Physical Electronics Laboratories model PHI-5600 spectrometer was used to collect electron spectroscopy for chemical analysis (ESCA) spectra. A non-monochromatic Mg Kα source (Kα designates the core energy level from which the X ray is derived) was operated at 300 W (15 kV, 20.0 mA). The samples were analyzed at a 45° take-off angle that equates to a sampling depth of approximately 60 Å. The entrance aperture at the hemispherical energy analyzer was set at 4, which equates to an elliptical sampling area with 800 μm as the long-axis dimension. Pass energies for the analyzer were set at 93.9 eV for wide-region survey scans and 58.70 eV for elemental multiplex scans (C1s, O1s, N1s, S2p, P2p, Na1s, Cl2p, and Si2p). Charge neutralization was accomplished with a beam of 5-eV electrons from an electron source. The operating pressure inside the main chamber was typically 5×10^{-8} torr. PHI ACCESS software was used to collect and display the data, calculate the atomic concentrations from peak areas, and measure binding energy positions for oxidation state identification. Binding energies are referenced to carbon in the hydrocarbon state set at 285.00 eV for sample-charge-correction purposes. Curve fitting of the carbon 1s region was performed with a linear background with four mixed Gaussian-Lorentzian bands.

Relationship between adhesion and amorphous Fe(III) oxide reduction. The necessity for cell-Fe(III) oxide contact for Fe(III) reduction was examined with cell suspensions of *S. alga* BrY (final concentration of 5×10^8 cells ml⁻¹) or *S. alga* RAD20 (final concentration of 5×10^8 cells ml⁻¹). Adhesion-Fe(III) reduction experiments were conducted in adhesion assay buffer in which the N₂-CO₂ headspace was replaced with a headspace of 100% hydrogen, which served as the sole electron donor for Fe(III) reduction. Soluble ferric pyrophosphate (Pfaltz and Bauer, Inc., Waterbury, Conn.) was substituted for amorphous Fe(III) oxide in positive controls. Samples were removed from the tubes over time with syringes and needles (8) and assayed for cell adhesion and Fe(II). All experiments were done in triplicate.

Analytical techniques. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time. The amount of Fe(II) solubilized after 15 min of extraction in 0.5 N HCl was determined with Ferrozine as previously described (21). The amount of hydroxylamine-reducible Fe(III) was determined as described previously (20). Cell numbers were determined by a modification of the epifluorescence microscopy technique (15) as previously described (21). Neutral hexoses were determined by the phenol sulfuric acid method (13) with glucose as the standard. Uronic acids were quantified by the *m*-hydroxydiphenyl method (7) with glucuronic acid as the standard. Protein was determined by adding 0.5 ml of sample to 0.5 ml of 1 N NaOH, heating at 90°C for 10 min, and assaying colorimetrically by the Micro protein assay (Pierce Chemical Co., Rockford, Ill.), with bovine serum albumin as the standard.

RESULTS

Isolation of an adhesion-deficient strain of *S. alga*. In order to isolate an adhesion-deficient strain of *S. alga*, a 0.1-ml aliquot of an overnight *S. alga* BrY culture was added to adhesion assay buffer. The tube was shaken for 1 h at 25°C and 150 rpm, and then a 1.0-ml aliquot was removed and centrifuged at $71 \times g$ for 5 min. A 0.1-ml aliquot from the resulting supernatant was then used as an inoculum for a fresh culture. This cycle was repeated 20 times, and the ability of the adhesion-deficient strain to adhere to amorphous Fe(III) oxide was compared to that of *S. alga* BrY every five cycles. The results of isolation of the adhesion-deficient strain are shown in Fig. 1. This process gradually selected for a strain of *S. alga* that exhibited a reduced ability to adhere to amorphous Fe(III) oxide. After 15 cycles, only 40% of the cells of this strain were able to adhere to the Fe(III) oxide while almost 100% of the *S. alga* BrY cells adhered. However, no further loss in adhesion ability was observed after five additional adhesion cycles. An aliquot of the supernatant from the 20th adhesion cycle was streaked onto a tryptic soy agar plate, and an isolated colony from this plate was used to prepare stock cultures of the adhesion-deficient strain. These cultures were used as inocula for all subsequent experiments. The adhesion-deficient strain was designated *S. alga* RAD20.

FIG. 1. Isolation of an adhesion-deficient strain of *S. alga*. Levels of adhesion to amorphous Fe(III) oxide by *S. alga* BrY (●) and the adhesion-deficient strain (■) are compared over the course of the isolation process. Error bars represent the standard deviations from the means ($n = 3$).

Adhesion characteristics. The kinetics of *S. alga* BrY and *S. alga* RAD20 adhesion to amorphous Fe(III) oxide are shown in Fig. 2. *S. alga* BrY showed a strong affinity for the Fe(III) oxide, as nearly 100% of the cells adhered immediately and remained adhered for the duration of the experiment. *S. alga* RAD20 also showed an affinity for the Fe(III) oxide, since almost 90% of the cells initially adhered. However, after 15 min of incubation, less than 50% of the cells remained adhered, and after 60 min of incubation, cell adhesion stabilized to approximately 40%.

The experiment examining the selectivity of *S. alga* BrY adhesion showed that cell adhesion to amorphous Fe(III) oxide ($97.3\% \pm 0.36\%$ of the cells adhered) was more than an order of magnitude greater than adhesion to SiO_2 ($9.0\% \pm 15\%$ of the cells adhered). More than 50% of *S. alga* BrY cells also adhered to the hydrophobic interaction resin phenyl Sepharose, while less than 20% adhered to the resin lacking the hydrophobic group.

FIG. 2. Kinetics of adhesion of *S. alga* BrY (●) and *S. alga* RAD20 (■) to amorphous Fe(III) oxide. Error bars represent the standard deviations from the means ($n = 3$).

TABLE 1. Comparison of carbohydrate concentrations of *S. alga* cells

Strain	Protein (mg)	Total hexose (μg)	Specific hexose (μg mg of protein ⁻¹)	Total uronic acid (μg)	Specific uronic acid (μg mg of protein ⁻¹)
BrY	60	3,600	60.4	4,670	78.3
RAD20	50	4,200	84.7	5,620	113.4

Bulk cell chemistry. The results of bulk cell chemical analyses are shown in Table 1. *S. alga* RAD20 contained 16% more total carbohydrate as neutral hexose, 40% more specific carbohydrate (normalized to protein) as neutral hexose, 20% more total carbohydrate as uronic acids, and 45% more specific carbohydrate (normalized to protein) as uronic acids than *S. alga* BrY.

Physicochemical cell surface characteristics. A comparison of the physicochemical cell surface characteristics of both *S. alga* strains is shown in Table 2. The cell surface charges, as measured by zeta potential at pH 7, were similar for both *S. alga* BrY and *S. alga* RAD20. The zeta potentials of amorphous Fe(III) oxide and SiO_2 were 33.6 and 38.7, respectively. The pI was lower for *S. alga* RAD20 (pH 2.2) than it was for *S. alga* BrY (pH 4.4) (Fig. 3). The cell surface hydrophobicity of *S. alga* BrY, as measured by contact angle, was significantly greater than that of *S. alga* RAD20 (Table 2). Analytical limitations precluded contact-angle measurements of amorphous Fe(III) oxide and SiO_2 .

Chemistry specific to the cell surface. The positive ion TOF-SIMS spectra obtained from both *S. alga* strains are shown in Fig. 4A and B. Ion peaks at 110, 120, and 136 m/z were present in the spectra of both strains. These peaks are typical of those arising from protein molecules (Fig. 4C). An ion peak at 165 m/z that was significantly above background was present only in the *S. alga* RAD20 spectrum. This peak is associated with protonated sugars (Fig. 4D).

Table 3 contains the ESCA atomic concentrations for the cell surfaces of both *S. alga* strains. *S. alga* RAD20 contained a higher atomic concentration of oxygen and lower atomic concentrations of carbon and nitrogen than *S. alga* BrY. The salt ions present on the surfaces of cells of both strains were most likely due to small amounts of contamination from the growth medium. Carbon, oxygen, and nitrogen were not observed in controls containing only growth medium. The silica present on the cell surfaces was most likely from silicone contamination, as ion fragments indicative of silicone were observed in TOF-SIMS spectra of both *S. alga* strains.

The percent distribution of the state of carbon on the surfaces of cells of both *S. alga* strains is presented in Table 4. The C—O or C—N band intensity was greater for *S. alga* RAD20 than for *S. alga* BrY. The C=O content was the same for both *S. alga* strains. The O—C=O or N—C=O bands were of equal intensities for both *S. alga* strains.

TABLE 2. Physicochemical characteristics of *S. alga* strains^a

<i>S. alga</i> strain	Cell length (μm)	pI	Zeta potential (mV)	Contact angle (°)
BrY	1.13 ± 0.01	4.4	-37.7 ± 0.70	116 ± 3
RAD20	1.31 ± 0.03	2.2	-32.0 ± 0.90	51 ± 2

^a Values for cell length ($n = 5$), zeta potential ($n = 10$), and contact angle ($n = 3$) are means ± standard deviations.

FIG. 3. Relationships between zeta potential and pH for cells of *S. alga* BrY (●) and *S. alga* RAD20 (■). Error bars represent the standard deviations from the means ($n = 10$).

Relationship between cell adhesion and amorphous Fe(III) oxide reduction. *S. alga* RAD20 reduced amorphous Fe(III) oxide at a rate identical to that of *S. alga* BrY (Fig. 5). However, the percentage of *S. alga* RAD20 cells that adhered to the amorphous Fe(III) oxide decreased over the duration of the experiment from approximately 90% at the beginning of the experiment to less than 50% at the end of the experiment. Approximately 100% of the *S. alga* BrY cells were adhered to the amorphous Fe(III) oxide over the duration of the experiment. The specific Fe(III) reduction activities of both cell suspensions with soluble ferric pyrophosphate as an electron acceptor were similar [13.3 ± 0.58 nmol of Fe(II) min^{-1} cell^{-1} for BrY and 17.1 ± 4.16 nmol of Fe(II) min^{-1} cell^{-1} for RAD20]. The rates of soluble ferric pyrophosphate reduction by both *S. alga* strains were also identical (data not shown).

DISCUSSION

This study is the first quantitative analysis of the adhesion of a DIRB to amorphous Fe(III) oxide. Previous studies have found the use of adhesion-deficient mutants an invaluable approach for identifying surface components involved in bacterial adhesion (11, 12, 31, 40, 43, 44). The adhesion assay developed in this study provided rapid acquisition of reproducible adhesion data, which allowed us to obtain an adhesion-deficient strain of *S. alga* BrY and thus distinguish between strains of the same species which differed only in their abilities to adhere to amorphous Fe(III) oxide.

A broadly based approach was used to determine the important factors which govern *S. alga* adhesion to amorphous Fe(III) oxide. The physicochemical analyses demonstrated that the surface charges of both *S. alga* strains are similar, suggesting that electrostatic interactions are not important factors in *S. alga* adhesion to amorphous Fe(III) oxide. Although the surface charges of Fe(III) oxide and SiO_2 were similar, *S. alga* adhered only weakly to SiO_2 . These results also suggest that factors other than surface charge dictate *S. alga* adhesion to Fe(III) oxide.

The results of the contact-angle measurements demonstrated that *S. alga* BrY cells are significantly more hydrophobic than *S. alga* RAD20 cells. Rijnaarts et al. (33) found that a contact angle greater than 50° is indicative of a hydrophobic cell surface and that a contact angle between 20 and 50° is

indicative of an intermediately hydrophobic cell surface. The methods used to measure contact angle in this study were slightly different from those used by Rijnaarts et al., which may affect the exact values of the measured contact angles. In spite of this, the contact-angle measurements suggest that *S. alga* BrY is more hydrophobic than *S. alga* RAD20. The results of the hydrophobic interaction assay also suggest that *S. alga* BrY is hydrophobic.

TOF-SIMS and ESCA were used to examine the surfaces of *S. alga* cells for chemical components that may affect adhesion to amorphous Fe(III) oxide. TOF-SIMS probes the outermost 20 Å of a surface, using a primary ion beam in the keV energy range to evolve secondary ions from the sample surface. The secondary ions obtained are unique to the molecules from which the ion originated. Based on the observed ions and ion

FIG. 4. Positive ion TOF-SIMS spectra obtained from *S. alga* BrY cell surfaces (A), *S. alga* RAD20 cell surfaces (B), bovine serum albumin (C), and glucose (D).

TABLE 3. ESCA atomic concentrations for *S. alga* strains

Strain	Atomic concn (%) ^a							
	C	O	N	S	P	Na	Cl	Si
BrY	63.1	22.2	12.6	0.4	0.5	0.9	0.3	0.0
RAD20	60.2	24.2	11.8	0.3	0.2	1.0	1.0	1.3

^a Values are the averages of measurements from duplicate samples. The difference between duplicates was within the relative experimental error associated with each measurement (0.5 atomic percent).

patterns, information about the molecular conformation of the surface is obtained. TOF-SIMS spectra of the surfaces of cells of *S. alga* BrY and *S. alga* RAD20 showed ion peaks at 110, 120, and 135 *m/z*, which are typical of proteins, as inferred from the TOF-SIMS spectrum of bovine serum albumin. TOF-SIMS spectra also indicated an ion peak at 165 *m/z* on the *S. alga* RAD20 cell surface which was absent from the *S. alga* BrY cell surface. This peak was indicative of protonated sugar molecules, as determined from the glucose spectrum. These results suggest that *S. alga* RAD20 cells have more sugar (exopolysaccharide) on their surfaces than *S. alga* BrY cells. These results support those of the bulk chemical analysis of both *S. alga* strains, which showed that *S. alga* RAD20 contained significantly more carbohydrate, both as neutral hexose and uronic acids, than *S. alga* BrY. This is the first reported study using TOF-SIMS to examine the molecular chemistries of bacterial cell surfaces.

ESCA provides the elemental compositions for all elements in the periodic table (except nonhydride H₂) for the outermost 20 to 100 Å of a surface (23, 42). The technique utilizes an X-ray source to generate photoelectrons. The binding energy of each electron is unique to each atomic element, allowing quantitative identification of the elements present on a cell surface. Additionally, shifts in the core electron energy are a function of valence electron bonding environment. These energy shifts can therefore be used to quantitatively determine the distribution of oxidation states as well as the atomic concentrations of the elements present in the outermost layers of a surface.

A number of previous studies have analyzed the surface chemistries of bacterial cells by ESCA (1, 3, 22, 38, 39, 41). ESCA analysis of *S. alga* cell surfaces showed a higher atomic concentration of oxygen and lower atomic concentrations of carbon and nitrogen on the surfaces of *S. alga* RAD20 cells than on the surfaces of *S. alga* BrY cells. These results suggest that oxygen-rich molecules, such as polysaccharides, predominate on the *S. alga* RAD20 cell surface but that nitrogen-rich molecules, such as proteins, predominate on the *S. alga* BrY cell surface. While the differences in atomic concentrations are not large, they support the results found in the studies of bulk cell chemistry and TOF-SIMS analysis. This conclusion was

TABLE 4. Percent distributions of the states of carbon for *S. alga* strains as determined by ESCA analysis

Strain	% Distribution ^a			
	CH _x	C—O or C—N	C=O	O—C=O or N—C=O
BrY	56.3	21.0	19.0	2.0
RAD20	54.2	24.8	19.0	2.0

^a Values are the averages of curve fits for data obtained from duplicate samples. The difference between duplicates was within the relative error associated with each curve fit as follows: ± 2.0 for CH_x, ± 1.0 for C—O or C—N, ± 1.0 for C=O, and ± 0.5 for O—C=O or N—C=O.

FIG. 5. Relationships between cell adhesion and Fe(III) reduction by *S. alga* strains. The concentrations of Fe(II) produced by *S. alga* BrY (●) and *S. alga* RAD20 (■) and the percentages of *S. alga* BrY (▲) and *S. alga* RAD20 (▼) cells adhered to amorphous Fe(III) oxide were measured over time. Error bars represent the standard deviations from the means (*n* = 3).

also supported by analysis of the percent distributions of carbon oxidation states for both *S. alga* strains. The increased C—O or C—N band intensity for the *S. alga* RAD20 cell surface relative to that for the *S. alga* BrY cell surface suggests that *S. alga* RAD20 cells contain more polysaccharide on their surfaces.

Taken collectively, the bulk chemical analysis data, the TOF-SIMS data, and the ESCA data suggest that the predominant chemical difference between the cell surfaces of the *S. alga* strains is an abundance of exopolysaccharide produced by *S. alga* RAD20 relative to that produced by *S. alga* BrY. This conclusion is also supported by the colony morphology of each strain. *S. alga* BrY colonies are flat, opaque, and often crenated, while *S. alga* RAD20 colonies are round, glossy, and mucoid.

Exopolysaccharide overproduction may explain the difference in the abilities of these strains to adhere to amorphous Fe(III) oxide. Previous studies have used bacterial pI measurements to determine the tendency of bacterial surface polymers to inhibit cell adhesion (33). The pI of a bacterium reflects a balance between anionic and cationic acid-base groups on the bacterial cell surface and specific ion adsorption (33). A pI between 2.0 and 2.8 results from a cell surface predominated by glucuronic acids or other polysaccharide-associated carboxyl groups (33). These polymers can sterically inhibit cell adhesion (33, 38). Bacteria with a pI greater than or equal to 3.2 have cell walls of predominately protein rather than polysaccharide (6, 12, 26, 33). The pIs of *S. alga* BrY (4.4) and *S. alga* RAD20 (2.2) therefore reflect the results of the TOF-SIMS, ESCA, and bulk chemical analysis and support the conclusion that the surfaces of cells of *S. alga* BrY are predominately protein while the surfaces of cells of *S. alga* RAD20 are predominately polysaccharide. Collectively, the results of this study support the hypothesis that *S. alga* adhesion to amorphous Fe(III) oxide is dictated primarily by cell surface hydrophobicity, which may be promoted by hydrophobic surface protein(s). Overproduction of exopolysaccharide may sterically hinder the interaction of this protein(s) with the Fe(III) oxide, resulting in the weak adhesion (desorption) of *S. alga* RAD20 observed in Fig. 2 and Fig. 5.

Previous studies have suggested that exopolysaccharides me-

diate the adhesion of DIRB to Fe(III) minerals (2, 27). Arnold et al. (2) assumed that adhered cells were tethered to Fe(III) minerals by exopolysaccharides, but this conclusion was never chemically substantiated. Obuekwe et al. (27) demonstrated, through ruthenium red staining, that Fe(III)-reducing biofilms were attached to steel surfaces via exopolysaccharides. However, this determination was made from scanning electron micrographs of fully colonized steel coupons and the role of exopolysaccharides in the initial adhesion of these cells was not determined. The results of this study suggest that proteins, in addition to exopolysaccharides, may be important for DIRB adhesion to Fe(III) minerals.

A number of previous studies have suggested that DIRB cell contact with insoluble Fe(III) minerals is a necessary prerequisite for Fe(III) mineral reduction (2, 9, 10, 21, 37). The results of the present study suggest that, for *S. alga*, this evaluation may be an oversimplification. Approximately 50% of the *S. alga* RAD20 cells and 100% of the *S. alga* BrY cells adhered to amorphous Fe(III) oxide during Fe(III) oxide reduction. The specific Fe(III) reduction activities of soluble ferric pyrophosphate were identical for both strains. If cell contact were necessary for Fe(III) reduction, one would expect that the rate of Fe(III) reduction by *S. alga* RAD20 would be half of that by *S. alga* BrY. However, *S. alga* RAD20 reduced amorphous Fe(III) oxide at a rate identical to that of *S. alga* BrY. These results suggest that irreversible bacterial adhesion is not necessary for amorphous Fe(III) oxide reduction. We are currently investigating the mechanisms by which DIRB can reduce amorphous Fe(III) oxide through transient contact with the mineral. Preliminary studies suggest that *S. alga* produces a cell surface protein that can efficiently chelate ferric iron (8a). The physical and chemical interactions between DIRB and amorphous Fe(III) oxide appear to be more specific and complex than previously believed.

ACKNOWLEDGMENTS

We thank Thomas Ernst Dueholm for assistance with the physicochemical analyses and Ace Baty for assistance with the bulk chemical analyses.

P.C.S. was supported by grant N00014-96-1-371 from the Office of Naval Research, Biological and Biomedical Science and Technology Division.

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