

Growth of Iron(III)-Reducing Bacteria on Clay Minerals as the Sole Electron Acceptor and Comparison of Growth Yields on a Variety of Oxidized Iron Forms†

Joel E. Kostka,^{1*} Dava D. Dalton,¹ Hayley Skelton,¹ Sherry Dollhopf,¹ and Joseph W. Stucki²

Department of Oceanography, Florida State University, Tallahassee, Florida 32306,¹ and Natural Resources and Environmental Sciences Department, University of Illinois, Urbana, Illinois 61801²

Received 21 May 2002/Accepted 11 September 2002

Smectite clay minerals are abundant in soils and sediments worldwide and are typically rich in Fe. While recent investigations have shown that the structural Fe(III) bound in clay minerals is reduced by microorganisms, previous studies have not tested growth with clay minerals as the sole electron acceptor. Here we have demonstrated that a pure culture of *Shewanella oneidensis* strain MR-1 as well as enrichment cultures of Fe(III)-reducing bacteria from rice paddy soil and subsurface sediments are capable of conserving energy for growth with the structural Fe(III) bound in smectite clay as the sole electron acceptor. Pure cultures of *S. oneidensis* were used for more detailed growth rate and yield experiments on various solid- and soluble-phase electron acceptors [smectite, Fe(III) oxyhydroxide FeOOH, Fe(III) citrate, and oxygen] in the same minimal medium. Growth was assessed as direct cell counts or as an increase in cell carbon (measured as particulate organic carbon). Cell counts showed that similar growth of *S. oneidensis* (10^8 cells ml⁻¹) occurred with smectitic Fe(III) and on other Fe forms [amorphous Fe(III) oxyhydroxide, and Fe citrate] or oxygen as the electron acceptor. In contrast, cell yields of *S. oneidensis* measured as the increase in cell carbon were similar on all Fe forms tested while yields on oxygen were five times higher, in agreement with thermodynamic predictions. Over a range of particle loadings (0.5 to 4 g liter⁻¹), the increase in cell number was highly correlated to the amount of structural Fe in smectite reduced. From phylogenetic analysis of the complete 16S rRNA gene sequences, a predominance of clones retrieved from the clay mineral-reducing enrichment cultures were most closely related to the low-G+C gram-positive members of the *Bacteria* (*Clostridium* and *Desulfotobacterium*) and the δ -*Proteobacteria* (members of the *Geobacteraceae*). Results indicate that growth with smectitic Fe(III) is similar in magnitude to that with Fe(III) oxide minerals and is dependent upon the mineral surface area available. Iron(III) bound in clay minerals should be considered an important electron acceptor supporting the growth of bacteria in soils or sedimentary environments.

Microbial Fe(III) reduction has been established as an important process catalyzing a large number of natural and contaminant biogeochemical cycles (21, 24, 29). Iron(III) respiration is coupled to a substantial portion of organic matter remineralization in the surface sediments of marine and freshwater environments (18, 35, 48). The fate of organic and inorganic contaminants is intimately tied to the activities of Fe(III)-reducing bacteria (FeRB) in subsurface sediments (24). In addition to its importance in modern environments, Fe(III) respiration is suggested to have been one of the earliest forms of respiration on ancient Earth (21).

The majority of studies of FeRB carried out in pure culture have focused on soluble, complexed Fe forms (such as ferric citrate) or on Fe(III) oxyhydroxide minerals (ferrihydrite and goethite) as the Fe available for reduction. In contrast, most of the oxidized Fe by weight in natural sediments is associated with phyllosilicate clay minerals (48). Clay minerals are abundant and ubiquitous in soils and sediments (41). Along with microorganisms, clays provide some of the most catalytic surfaces in sedimentary environments, which are important to a

variety of biogeochemical cycles (41, 43). However, few studies have been carried out on the interactions between these two reactive components of porous media.

Of the organisms currently available in pure culture which are known to conserve energy for growth from the reduction of Fe(III) minerals, members of the shewanellae and of the *Geobacteraceae* have been characterized in the most detail (21, 29). A small but expanding database has been collected which shows that microorganisms from both of these families catalyze the reduction of clay-bound Fe(III) (16). Microbial clay reduction has been demonstrated at temperatures and pHs common to soils and sediments (15–17). As with Fe oxide minerals, organic chelates and electron transfer agents increased the bioavailability of clay bound Fe(III) for reduction (16, 26). However, no past studies have provided evidence for the growth of bacteria with clay minerals as the sole electron acceptor. Further, it is unclear how respiration and growth on clay-bound Fe(III) compare to those on other Fe mineral forms.

In this study, we found that the respiration of structural Fe(III) bound in smectite clay minerals supports the growth of FeRB in pure culture and in enrichment cultures from two very different sedimentary environments. We also compared growth yields of a *S. oneidensis* strain MR-1 on a variety of Fe(III) forms with that on oxygen. Iron(III) oxide minerals are be-

* Corresponding author. Mailing address: Department of Oceanography, Florida State University, Tallahassee, FL 32306-4320. Phone: (850) 645-3334. Fax: (850) 644-2581. E-mail: jkostka@ocean.fsu.edu.

† This paper is dedicated to the memory of Dava Dalton, friend and colleague, who passed away during review of the manuscript.

TABLE 1. Summary of growth yields for *S. oneidensis* strain MR-1 utilizing a variety of electron acceptors after 70 h in culture^a

Culture treatment	Concn of total cell carbon ($\mu\text{g liter}^{-1}$)	Concn of e-reduced (mM) ^b	Growth (10^7 cells ml^{-1})	Growth per cell (10^{-14} g of C cell ⁻¹)	Yield (g of C mol of e ⁻¹)
FeOOH	1,600 \pm 209	10.2	12.6 \pm 0.3	1.3	0.169
FeOOH + AQDS	1,744 \pm 244	24.0	14.7 \pm 0.3	1.2	0.073
Smectite ^c		2.8	9.0 \pm 0.4		
Smectite + AQDS ^c		4.0	12.0 \pm 0.3		
Fe(III) citrate	10,885 \pm 1,293	48.6	16.5 \pm 0.7	6.6	0.198
O ₂	25,490 \pm 3,062	20.0	14.8 \pm 0.2	17.2	1.29

^a Each value is the mean \pm 1 standard deviation or the average from the same triplicate cultures per treatment presented in Fig. 2.

^b Average concentration of electron acceptor reduced measured in triplicate cultures. Oxygen utilization was calculated from the stoichiometry presented in Table 2, assuming complete utilization of the lactate added.

^c It was not possible to resolve cell carbon or yield in smectite cultures due to the presence of background carbon impurities.

lieved to be more reactive or available for microbial reduction than clay minerals. In contrast, we show that the growth rate and yield of *S. oneidensis* on smectite appear to be similar to growth on amorphous Fe(III) oxyhydroxide. These discoveries have important implications for contaminated subsurface and surface aquatic environments, where Fe(III)-bearing clay minerals are abundant and at times comprise the predominant electron acceptor available to microorganisms.

MATERIALS AND METHODS

Bacterial cultures and cultivation methods. A pure culture of *Shewanella oneidensis* strain MR-1 was used which was isolated from the anoxic sediments of Lake Oneida, NY, and has been the subject of many physiological and genetic studies concerning the *Shewanellae* (29). *S. oneidensis* is a facultative anaerobe and an obligately respiratory bacterium, incapable of fermentative growth (39). Purified enrichment cultures consisted of FeRB consortia from two source inocula. Subsurface sediment samples were obtained from the saturated zone of unconsolidated alluvium (1 to 4 m below land surface) at the Field Research Center of the Department of Energy's Natural and Accelerated Bioremediation Research Program, Oak Ridge, Tenn. A second set of samples were collected at a 0.1-m depth in the surface soil of a rice paddy located in Nanjing, China. This rice field is flooded biannually, leading to prolonged anoxic conditions followed by alternating aerobic conditions between flood cycles. Inocula for enrichments were collected by taking cores or grab samples. All soil and sediment samples were collected and transported chilled to the lab. Sample handling and cultivation procedures were carried out under aseptic and strictly anoxic conditions.

Enrichment cultures were purified by successive transfer using the culture medium and methods described below. The enrichments were selective for respiratory FeRB, as Fe(III) oxide was added as the sole electron acceptor and acetate was added as the sole carbon source in a minimal medium throughout successive transfers. Dissimilatory Fe(III) reduction was shown repeatedly in the enrichments as the production of reduced Fe in acid extracts coupled to the depletion of acetate. Further, FeRB were observed to be abundant (10^4 to 10^6 cells g of wet sediment⁻¹) in the sediment used as an inoculum for the enrichment culture with a most probable number assay (Kostka et al., submitted for publication).

Standard methods for the culture of anaerobic bacteria were modified for clay reduction experiments as in the work of Kostka et al. (15). For *S. oneidensis*, a minimal culture medium was prepared as described by Kostka and Nealon (14). For the enrichment cultures, a carbonate-buffered minimal medium formulated to resemble groundwater was prepared and dispensed into Hungate tubes as described by Widdel and Bak (50). The freshwater version of the Widdel-Bak basal medium, originally designed for sulfate-reducing bacteria, was modified by replacing any sulfates with chloride salts and adding FeCl₂ instead of dissolved sulfide as a reductant (2 mM final concentration) (1).

Culture medium was prepared, the pH was adjusted to 7, and the medium was dispensed into serum bottles. Carbon substrates (lactate for *S. oneidensis*, acetate for enrichments) were added from sterile, anoxic stocks to a 10 mM final concentration for each. Unless otherwise specified, smectite clay SWa-1 was added to a particle concentration of 4 g liter⁻¹. Amorphous Fe(III) oxyhydroxide (FeOOH) or ferric citrate was added to a 50 mM final concentration. Culture bottles were sealed with butyl rubber stoppers and incubated at 30°C in the dark.

All manipulations of culture samples were carried out under strictly anoxic conditions within a Coy anaerobic chamber (90% N₂, 10% H₂). Inoculum cultures were grown anaerobically on Fe(III) to late log phase on the appropriate minimal medium. Heat-killed controls were heated by microwave radiation until boiling (11).

Preparation of oxidized Fe. Amorphous Fe(III) oxyhydroxide (surface area = 600 m² g⁻¹) was prepared as described by Schwertmann and Cornell (38). For all experiments with smectite clay, the 0.5- to 2- μm fraction of the ferruginous smectite SWa-1 from Grant County, Wash. (Source Clays Repository, The Clay Minerals Society), was used. The clay was Na⁺ saturated, fractionated, dialyzed, and freeze-dried prior to use (42). Lear and Stucki (20) reported the structural Fe content of the same dialyzed SWa-1 to be 3.549 mmol of Fe g⁻¹ (with less than 0.1 mmol of this Fe g⁻¹ present as Fe oxide impurities) and the surface area to be 720 m² g⁻¹. All solutions were made anoxic using an updated, commercially available version of the apparatus described by Stucki et al. (42). All Fe minerals were sterilized by heating via microwave radiation (11) before addition to the culture medium. Ferric citrate was prepared as described previously by Kostka and Nealon (14).

Determination of reduction and growth. The reduction of Fe(III) was measured as the production of Fe(II) in HCl extracts using the colorimetric reagent ferrozine under strictly anoxic conditions (14, 23). This method was previously validated for use in clay cultures by comparison to HF extracts and Mossbauer spectroscopy (15, 16). Cell numbers were determined by direct counting using acridine orange and epifluorescence microscopy as described previously (10, 23). Bacterial direct counting procedures were modified for solid-containing suspensions according to the work of Proctor and Souza (33). Biomass was determined by measuring the accumulation of particulate carbon using previously described methods (27).

Phylogenetic characterization of purified Fe(III)-reducing consortia. Genomic DNA was extracted with an ultraclean soil DNA kit (Mo Bio Laboratories, Inc, Solana Beach, Calif.). Extracted genomic DNA was used as a template for PCR amplification of nearly the entire 16S rRNA gene (~1,400 bp) with the bacterium-specific primers 8F and 1392R as previously described (3). The amplification products were subsequently cloned into *Escherichia coli* with the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.). The clones were screened by restriction analysis, and unique clones were sequenced with an automated sequencer (Applied Biosystems model 3100) using the terminator cycle sequencing method. The sequences were aligned to comparison strains in the Ribosomal Database Project in accordance with the secondary structure of the 16S rRNA molecule using the ARB software package (40). A phylogenetic distance matrix was calculated from the aligned sequences using distance and maximum likelihood methods.

RESULTS

The rapid reduction of soluble and solid Fe(III) forms was observed in cultures of *Shewanella oneidensis* strain MR-1 over 3 days (Table 1). Nearly all of the Fe(III) citrate was reduced, compared to half of the Fe(III) oxyhydroxide (FeOOH) and approximately one-third of the structural Fe(III) bound in smectite. A white, ferrous carbonate precipitate was produced over time in Fe(III) citrate cultures, whereas a black magnetic

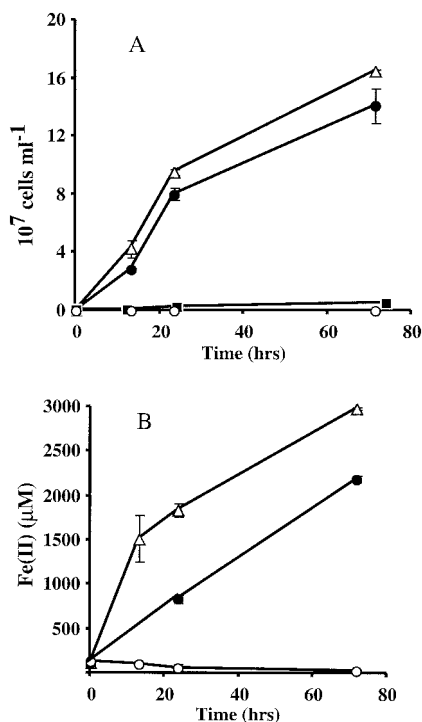


FIG. 1. Growth (A) and structural Fe(III) reduction (B) of *S. oneidensis* strain MR-1 with lactate as the electron donor and smectite as the electron acceptor. Results are expressed as means ± 1 standard deviation from triplicate cultures. Symbols: open triangles, smectite plus AQDS; solid circles, smectite; solid squares, no electron acceptor added; open circles, heat-killed culture.

precipitate, presumably magnetite, was formed in Fe(III) oxyhydroxide cultures. With smectite as the sole electron acceptor, no solid form other than smectite was observed at the end of the experiments.

Addition of the humic acid analog anthraquinone disulfonate (AQDS) generally stimulated reduction of the FeOOH to a larger extent than clay-bound Fe(III) (Table 1). Control cultures (heat killed, exposed to HgCl_2 , or cultured aerobically) showed little or no reduction of clay-bound Fe(III) to Fe(II). As has been demonstrated previously (16, 25), lactate was depleted and carbon dioxide was produced according to the 4:1 stoichiometry of Fe(III) reduced to carbon oxidized in all cultures (data not shown).

Growth of *S. oneidensis*, measured by an increase in cell number, paralleled the reduction of all Fe(III) forms tested (Table 1; Fig. 1 and 2). Little or no growth was observed in killed controls (with heat or HgCl_2) or in control cultures to which no electron acceptor had been added (Fig. 1). Interestingly, the increase in cell number exhibited a similar range for all electron acceptors tested (Table 1; Fig. 2). Within 3 days, cell densities of *S. oneidensis* were observed to increase to a range between 9.0×10^7 and 16.5×10^7 cells ml^{-1} . On the third day, cultures were sacrificed for particulate organic carbon analysis and the yield within each culture was determined. It was not possible to resolve the particulate organic carbon yield in smectite cultures due to the presence of background carbon impurities (data not shown). However, in contrast to the increase in cell number, accumulation of *S. oneidensis*

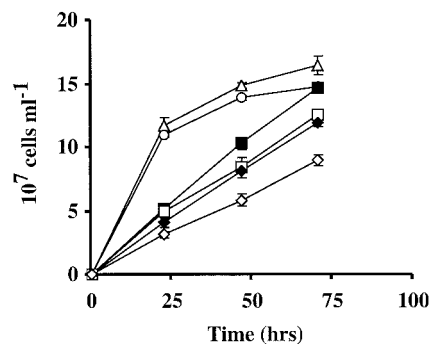


FIG. 2. Growth of *S. oneidensis* strain MR-1 with lactate as the electron donor in culture treatments which differ according to the electron acceptor added. Results are expressed as the means ± 1 standard deviation from triplicate cultures. Symbols: open triangles, Fe(III) citrate; open circles, O_2 ; solid squares, FeOOH plus AQDS; open squares, FeOOH; solid diamonds, smectite plus AQDS; open diamonds, smectite.

biomass (measured as grams of C cell^{-1}) ranged over a factor of 5 depending upon the electron acceptor utilized [O_2 , Fe citrate, and Fe(III) oxyhydroxide] (Table 1).

S. oneidensis conserved energy for growth by coupling the reduction of smectite to the oxidation of lactate (Fig. 1). *S. oneidensis* was then inoculated into minimal basal media supplemented with various particle concentrations of smectite as the sole electron acceptor. Not only was the degree of structural Fe(III) reduction proportional to the initial particle load, but a corresponding proportional increase in cell density was also observed (Fig. 3).

In enrichment cultures freshly purified from rice paddy soil and from contaminated subsurface sediment, Fe(III)-reducing consortia were shown to conserve energy for growth by coupling the reduction of structural Fe(III) in smectite to the oxidation of acetate. Growth paralleled the reduction of Fe(III) with smectite added as the sole electron acceptor, while little or no growth was observed in control cultures to which no electron acceptor had been added (Fig. 4). Representative growth curves are shown for parallel enrichment cultures enriched from the same subsurface sediment core (Fig. 4), and approximately twice the growth was observed for duplicate rice

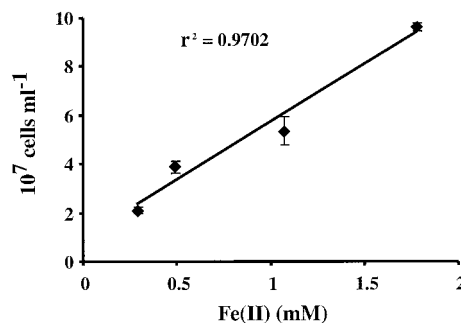


FIG. 3. Concentration of reduced structural Fe plotted against growth (as cell number) for *S. oneidensis* strain MR-1 cultures with lactate as the electron donor and where the particle load of smectite was varied as the electron acceptor. Growth is expressed as means ± 1 standard deviation from triplicate cultures, while Fe(II) concentrations are the averages from triplicate cultures.

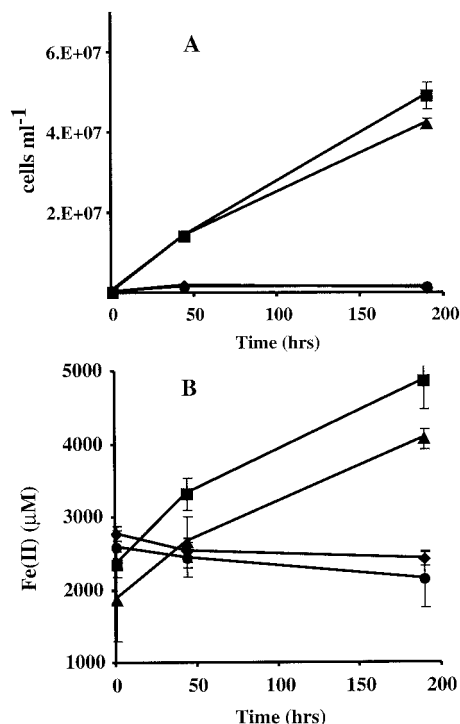


FIG. 4. Growth (A) and structural Fe(III) reduction (B) of an FeRB consortium purified from subsurface sediments with acetate as the electron donor and smectite as the electron acceptor. Results are expressed as means \pm 1 standard deviation for triplicate culture treatments from parallel enrichment cultures generated from the same sediment core. Symbols: squares and triangles, smectite added; diamonds and circles, no electron acceptor added. Squares and diamonds represent core sample A; triangles and circles represent core sample B.

paddy enrichments [13×10^7 cells ml⁻¹ and 2.5 mM smectite-bound Fe(III) reduced; data not shown]. Using a cloning and sequencing approach of the 16S rRNA genes in these enrichment cultures, the dominant members of the Fe(III)-reducing consortia were identified. In the subsurface enrichment (Ac032) (Fig. 5), five clones were obtained, and the dominant sequences retrieved showed the highest sequence similarity to “*Geobacter akaganeitrophicus*” (three clones, 90% similarity) and *Desulfotobacterium chlororespirans* (two clones, 90% similarity). In the rice paddy enrichment (LacRPS) (Fig. 5), six clones were obtained and the dominant sequences retrieved showed the highest sequence similarity to *Clostridium celerecrescens* (three clones, 96% similarity) and *Geobacter sulfurreducens* (two clones, 98% similarity).

DISCUSSION

Growth with smectite clay minerals as the sole electron acceptor. The growth of FeRB in culture has mostly been quantified by measuring the increase in cell number. Using the maximum cell number increase in comparison to control cultures, the range of growth measured for *S. oneidensis* coupled to smectite respiration reported here (1×10^8 to 2×10^8 cells ml⁻¹) is very similar to the range reported in previous studies for the growth of pure cultures of FeRB coupled to the respiration of synthetic Fe(III) oxide minerals (1×10^8 to 2×10^8 cells ml⁻¹) under similar culture conditions (7, 23, 25, 34). Our observation that microorganisms can gain energy for growth by catalyzing the respiration of smectite clay minerals is not surprising when the energy available from these reactions is considered (Table 2). The standard free energy at pH 7 that was

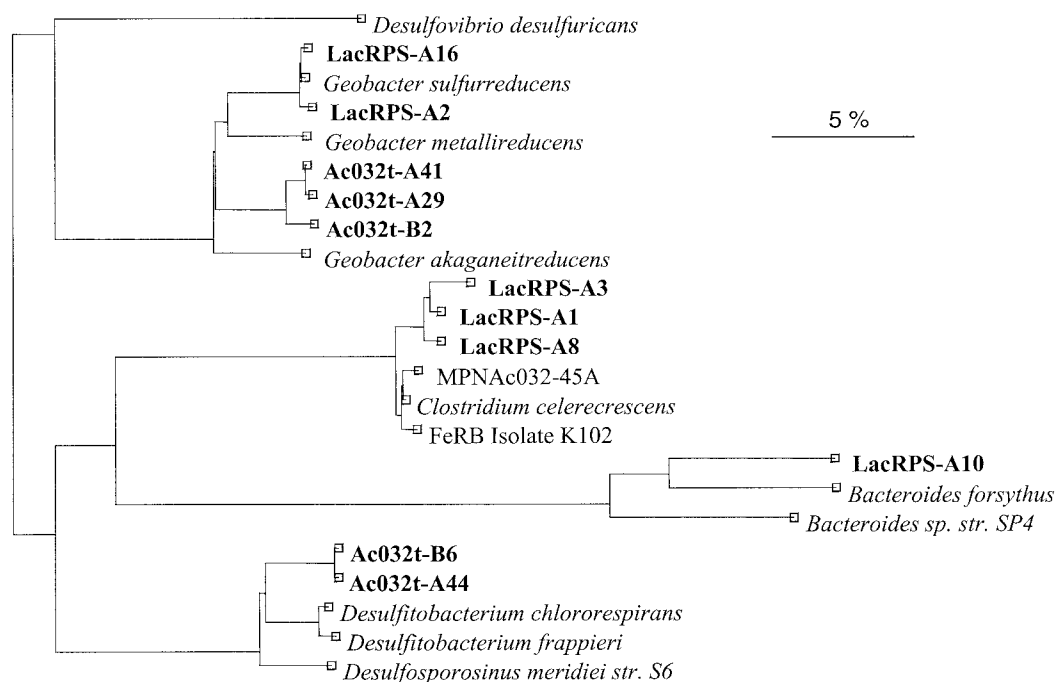


FIG. 5. Phylogenetic tree for enrichment cultures of Fe(III)-reducing consortia and selected strains of eubacteria, based on a distance matrix analysis (see Materials and Methods). Consortia purified from rice paddy soil and subsurface sediments are designated LacRPS and Ac032t, respectively. Scale bar, 5 substitutions per 100 bases. Parsimony and maximum likelihood analyses yielded equivalent results with respect to the phylogenetic positions of the Fe-reducing strains.

TABLE 2. Stoichiometry and free energy of reactions related to the metabolism of *S. oneidensis* strain MR-1 with various electron acceptors

Equation	Reactants	Products	Std G ^o	
			kJ/reaction	kJ/mol of e ⁻
1 ^b	1.47[smectite] ^c + CH ₃ CHOHCOO ⁻ + 2H ₂ O	1.47[smectite] ^{-2.73} + CH ₃ COO ⁻ + HCO ₃ ⁻ + 5H ⁺	-436.09	-109.02
2	12 Fe(OH) ₃ + CH ₃ CHOHCOO ⁻	4Fe ₃ O ₄ + CH ₃ COO ⁻ + HCO ₃ ⁻ + H ⁺ + 18H ₂ O	-409.70	-102.42
3	4Fe ³⁺ + CH ₃ CHOHCOO ⁻ + 2H ₂ O	4Fe ²⁺ + CH ₃ COO ⁻ + HCO ₃ ⁻ + 5H ⁺	-460.34	-115.08
4	O ₂ + CH ₃ CHOHCOO ⁻	CH ₃ COO ⁻ + HCO ₃ ⁻ + H ⁺	-478.28	-478.28

^a Free energy calculated from the standard free energies of formation of the products and reactants (44, 49) and by assuming standard conditions except for pH 7.

^b Free energy of formation for smectite was calculated from the standard reduction potential estimated by Amonette (2).

^c The chemical formula for smectite used in the calculations was Na_{0.81}(Si_{7.3}Al_{0.7})(Al_{1.06}Fe_{2.73}^{III}Mg_{0.26})O₂₀(OH)₄ (79).

calculated to be available from lactate oxidation coupled to smectite reduction (Table 2, equation 1) is very similar to that calculated to be available with Fe(III) oxyhydroxide or soluble ferric citrate as the electron acceptor (Table 2, equations 2 and 3). Our findings suggest that a similar amount of energy for growth is generated during Fe(III) reduction, regardless of the form of Fe being utilized in cultures of *S. oneidensis*.

Earlier culture studies indicated that the availability of Fe(III) minerals for reduction by microorganisms was determined by their crystallinity or mineral form (22). Amorphous Fe(III) oxyhydroxides were shown to be rapidly and extensively reduced by microbes, whereas crystalline Fe(III) minerals were reduced slowly and incompletely. More recent studies by Kostka and Neelson (13) and Roden and Zachara (36) revised this view by showing that FeRB were capable of growth on the crystalline Fe(III) oxide minerals magnetite and goethite, respectively. It was further suggested that the potential for cell growth and Fe(III) reduction was determined by the Fe(III) oxide surface area and not by crystallinity (36). Though Fe-containing clay minerals are operationally defined as crystalline Fe(III) minerals, rates of microbial clay reduction have been observed to be comparable to rates of reduction for poorly crystalline or amorphous Fe oxide minerals (16). In this study, we observed that growth was highly correlated to the particle concentration of smectite added to cultures of *S. oneidensis* (Fig. 3). The percentage of structural Fe(III) reduced in smectite remained at ~20% throughout the range of particle loading tested (Fig. 3). Thus, our results support previous observations which suggested that reduction and growth on crystalline Fe(III) minerals is determined by mineral surface area. We extend this concept to smectite clay minerals and suggest that their high surface area, comparable to that of amorphous Fe(III) oxyhydroxides (~700 m² g⁻¹) (38, 41), results in an increased availability for microbial reduction and growth.

Organic compounds such as humic acids are believed to facilitate the reduction of Fe(III) minerals by serving as an electron shuttle or by chelating and solubilizing Fe(III), thereby making the Fe more available for reduction. Our results concur with past studies to show that the reduction of smectite (26) or Fe(III) oxyhydroxide (21) is enhanced in the presence of the humic acid analog AQDS (Fig. 1; Table 1). However, we observed a minimal enhancement of cell growth in the presence of AQDS (Fig. 1 and 2).

Growth with smectite as the sole electron acceptor was also observed in Fe(III)-reducing consortia enriched from two very different environments (contaminated subsurface sediment

and rice paddy soil) where Fe-rich clay minerals are abundant and potentially important electron acceptors (41, 52). The 16S rRNA gene sequences retrieved from these consortia were dominated by those closely related to the δ -*Proteobacteria* (*Geobacteraceae*) and low-G+C gram-positive members of the *Bacteria* (*Desulfitobacterium* and *Clostridium*). Retrieval of *Geobacteraceae* sequences is not unexpected, since these organisms have been established as important members of FeRB consortia in sediments (5, 37, 46). Detection of low-G+C gram-positive members of FeRB consortia is more surprising and intriguing. Members of the low-G+C *Bacteria* (*Bacillus*, *Desulfitobacterium*, and *Desulfotomaculum*) been shown to be capable of Fe(III) respiration (6, 30, 31, 32, 45, 47). Gram-positive organisms are thought to be more resistant to environmental extremes such as desiccation in soils and sediments.

Growth rate and yield of *S. oneidensis* strain MR-1 on various electron acceptors. *S. oneidensis* is a facultative anaerobe which grows well on oxygen and is incapable of fermentative growth (29, 39). Thermodynamic calculations of free energy are often used as the basis for comparing the energetics of Fe(III) respiration to aerobic respiration by FeRB of the *Shewanellae* family, though few quantitative growth data are available to support this comparison. Soluble Fe(III) forms such as ferric citrate are also believed to be more available for reduction and therefore may provide more energy for growth than solid Fe(III) minerals. To our knowledge, growth rates or yields of FeRB on solid versus soluble Fe forms have not been compared, and no studies have compared growth coupled to Fe(III) respiration versus aerobic respiration.

We observed growth yields of *S. oneidensis* as the increase in total cell carbon in the following order: O₂ > ferric citrate >> Fe(III) oxyhydroxide. When normalized per mole of electron acceptor (e⁻) utilized, growth yields were nearly identical for cells growing on Fe(III) oxyhydroxide (0.17 g of C mol of e⁻⁻¹) compared to ferric citrate (0.20 g of C mol of e⁻⁻¹), while the growth yield on oxygen was larger by a factor of 5 (Table 1). By measuring an increase in cell protein, Bazylinski et al. (4) observed a growth yield, similar to that in our study, of 0.40 g of cell C mol of Fe(III) reduced⁻¹ for *Geobacter* cells with ferric citrate as the electron acceptor.

In support of the growth yield data, cell sizes visualized under epifluorescence microscopy appeared to be substantially larger in cultures grown on soluble electron acceptors (oxygen or ferric citrate) than in those grown with solid electron acceptors [Fe(III) oxyhydroxide or smectite]. Therefore, we conclude that cell counts are not always directly proportional to growth yield in cultures of *S. oneidensis*. Such an observation

suggests that the increase in biomass should be measured more often for these anaerobes. Given that previous perceptions on the growth of FeRB are heavily dependent upon direct counts in pure cultures, this observation could well revise our views on the energy obtained for growth via Fe(III) respiration. This conclusion is supported by the observation that both the predicted thermodynamic energy yield (Table 2) and the measured cell yield (Table 1) were higher by a factor of 5 when *S. oneidensis* cells were grown on oxygen than on various Fe(III) forms as the electron acceptor.

In agreement with the yield results (Table 1), growth rates of *S. oneidensis* calculated from the increase in cell number (Fig. 2) were higher for the soluble electron acceptors (0.489×10^7 [O₂] and 0.520×10^7 [Fe citrate] cells ml⁻¹ h⁻¹) in comparison to the solid electron acceptors (0.125×10^7 [smectite] and 0.174 [FeOOH] cells ml⁻¹ h⁻¹) tested. Growth rates for smectite and FeOOH were similar to those observed in previous studies of *S. putrefaciens* growing on FeOOH (25). In contrast, our growth rates were 1 to 2 orders of magnitude higher than those observed in past studies of *Shewanella* strains growing on the crystalline Fe(III) oxide minerals goethite (36) and magnetite (13). It appears that the growth rate of *Shewanella* on smectite more closely resembles rates on poorly crystalline Fe(III) oxides (FeOOH) than those on crystalline Fe(III) oxides. We suggest that at least 20% of the Fe(III) bound in smectite is as available as FeOOH for relatively rapid growth. However, the fact that the growth rate of *S. oneidensis* on smectite was 25% lower than that on FeOOH suggests that there is some metabolic cost associated with smectitic Fe(III) utilization.

Biogeochemical significance. The form and concentration of reactive Fe(III) minerals are of paramount importance to the environmental significance of microbial Fe(III) reduction in sedimentary environments. Clay minerals are particularly important because they are highly reactive and account for a large fraction of Fe-containing minerals in nature (16, 41, 43, 48). In aquatic and marine sediments, silicates or clay minerals comprise 65% of all Fe minerals (48). Geochemical evidence has shown that Fe(III) in clay minerals is rapidly reduced and may constitute a significant fraction of the redox-active Fe from terrestrial environments to the deep sea (43, 52). Evidence also suggests that Fe-rich smectites comprise an important electron acceptor available for dissimilatory metal reducing metabolism in some surface (marine and aquatic) (12) and terrestrial subsurface sediments (52). In some contaminated subsurface sediments within the Department of Energy complex, iron-rich clay minerals are the primary electron acceptor available for microbial Fe(III) reduction (52). Thus, the microbial reduction of Fe-rich clay minerals is thought to have a significant impact on nutrient cycles, agricultural productivity, and the environmental fate of contaminants (8, 9, 41, 43, 51). However, the role of microbial clay reduction in natural environments has not been extensively determined.

Here we have demonstrated that FeRB, in a well-characterized pure culture and in purified enrichment cultures, can conserve energy for growth by coupling the reduction of structural Fe(III) bound in clay minerals to the oxidation of organic acids, lactate, or acetate. This is the first description of any organism capable of such metabolism. Given their abundance and ubiquity, Fe-containing clay minerals may be important

and previously overlooked electron acceptors for the growth of bacteria in natural environments. Our results showing rapid bacterial growth via smectite respiration support past geochemical evidence (8, 9, 16, 17, 48) to indicate that a substantial portion (20 to 50%) of the Fe(III) bound in smectite is easily accessible to FeRB. Furthermore, our results show that the growth rate and yield on smectitic Fe(III) are comparable to those on poorly crystalline Fe(III) oxide minerals (FeOOH), which suggests that FeRB respire smectite in parallel with FeOOH. In other words, our data suggest that bacteria do not preferentially reduce Fe oxides over clay minerals. Microbial clay reduction may therefore be an important, but little-studied, process limiting natural and contaminant biogeochemical cycles.

ACKNOWLEDGMENTS

This research was funded by the Natural and Accelerated Bioremediation Research (NABIR) program, Biological and Environmental Research (BER), the U.S. Department of Energy (grant DE-FG02-00ER62986), and by the U.S. Department of Agriculture (grant 98-35107-6313).

We thank Jun Wu for preparing the smectite clay and for assistance with the iron extractions. We acknowledge Lita Proctor and her lab personnel for their assistance with the epifluorescence microscopy as well as the technical support provided by the FSU Biological Science Imaging Resource Center. We also thank Jean Cowan of the Dauphin Island Sea Lab in Mobile, Ala., for help with the total organic carbon analyses.

REFERENCES

- Achtlich, C., A. Schuhmann, T. Wind, and R. Conrad. 1995. Role of interspecies H₂ transfer to sulfate and ferric iron-reducing bacteria in acetate consumption in anoxic paddy soil. *FEMS Microbiol. Ecol.* **16**:61-70.
- Amonette, J. E. Iron redox chemistry of clays and oxides: environmental applications. In A. Fitch (ed.), *Electrochemistry of clays*, in press. Clay Minerals Society, Aurora, Colo.
- Balkwill, D. L., G. R. Drake, R. H. Reeves, J. K. Fredrickson, D. C. White, D. B. Ringelberg, D. P. Chandler, M. F. Romine, D. W. Kennedy, and C. M. Spadoni. 1997. Taxonomic study of aromatic-degrading bacteria from deep-terrestrial-subsurface sediments and description of *Sphingomonas aromaticivorans* sp. nov., *Sphingomonas subterranea* sp. nov., and *Sphingomonas stygia* sp. nov. *Int. J. Syst. Bacteriol.* **47**:191-201.
- Bazylinski, D. A., A. J. Dean, D. Schuler, E. J. P. Phillips, and D. R. Lovley. 2000. N₂-dependent growth and nitrogenase activity in the metal-metabolizing bacteria, *Geobacter* and *Magnetospirillum* species. *Environ. Microbiol.* **2**:266-273.
- Bond, D. R., D. E. Holmes, L. M. Tender, and D. R. Lovley. 2002. Electrode-reducing microorganisms that harvest energy from marine sediments. *Science* **295**:483-485.
- Boone, D. R., Y. Liu, Z. Zhao, D. L. Balkwill, G. Drake, T. O. Stevens, and H. C. Aldrich. 1995. *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. *Int. J. Syst. Bacteriol.* **45**:441-448.
- Coates, J. D., D. J. Lonergan, E. J. P. Phillips, H. Jenter, and D. R. Lovley. 1995. *Desulfuromonas palmitatis* sp. nov., a marine dissimilatory Fe(III) reductor that can oxidize long-chain fatty acids. *Arch. Microbiol.* **164**:406-413.
- Gates, W. P., H. T. Wilkinson, and J. W. Stucki. 1993. Swelling properties of microbially reduced ferruginous smectite. *Clays Clay Miner.* **41**:360-364.
- Gates, W. P., J. W. Stucki, and R. J. Kirkpatrick. 1996. Structural properties of reduced Upton montmorillonite. *Phys. Chem. Miner.* **23**:535-541.
- Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33**:1225-1228.
- Keller, M. D., W. K. Bellows, and R. R. L. Guillard. 1988. Microwave treatment for sterilization of phytoplankton culture media. *J. Exp. Mar. Biol. Ecol.* **117**:279-283.
- Kostka, J. E., and G. W. Luther III. 1994. Partitioning and speciation of solid phase Fe in saltmarsh sediments. *Geochim. Cosmochim. Acta* **58**:1701-1710.
- Kostka, J. E., and K. H. Nealson. 1995. Dissolution and reduction of magnetite by bacteria. *Environ. Sci. Technol.* **29**:2535-2540.
- Kostka, J. E., and K. H. Nealson. 1998. Isolation, cultivation, and characterization of iron- and manganese-reducing bacteria, p. 58-78. In R. S. Burlage et al. (ed.), *Techniques in microbial ecology*. Oxford University Press, New York, N.Y.

15. Kostka, J. E., J. W. Stucki, K. H. Neelson, and J. Wu. 1996. Reduction of structural Fe(III) in smectite by a pure culture of *Shewanella putrefaciens* strain MR-1. *Clays Clay Miner.* **44**:522–529.
16. Kostka, J. E., E. Haeefe, R. Viehweger, and J. W. Stucki. 1999. Respiration and dissolution of Fe(III)-containing clay minerals by bacteria. *Environ. Sci. Technol.* **33**:3127–3133.
17. Kostka, J. E., J. Wu, K. H. Neelson, and J. W. Stucki. 1999. The impact of structural Fe(III) reduction by bacteria on the surface chemistry of clay minerals. *Geochim. Cosmochim. Acta* **63**:3705–3713.
18. Kostka, J. E., B. Gribsholt, E. Petrie, D. Dalton, H. Skelton, and E. Kristensen. 2002. The rates and pathways of carbon oxidation in bioturbated saltmarsh sediments. *Limnol. Oceanogr.* **47**:230–240.
19. Lear, P. R., and J. W. Stucki. 1985. Role of structural hydrogen in the reduction and reoxidation of iron in nontronite. *Clays Clay Miner.* **33**:539–545.
20. Lear, P. R., and J. W. Stucki. 1989. Effects of iron oxidation state on the specific surface area of nontronite. *Clays Clay Miner.* **37**:547–552.
21. Lovley, D. R. 2000. Fe(III) and Mn(IV) reduction, p. 3–30. *In* D. R. Lovley (ed.), *Environmental microbe-metal interactions*. ASM Press, Washington, D.C.
22. Lovley, D. R., and E. J. P. Phillips. 1986. Availability of ferric iron for microbial reduction in bottom sediments of the freshwater tidal Potomac River. *Appl. Environ. Microbiol.* **52**:751–757.
23. Lovley, D. R., and E. J. P. Phillips. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl. Environ. Microbiol.* **54**:1472–1480.
24. Lovley, D. R., and R. T. Anderson. 2000. Influence of dissimilatory metal reduction on fate of organic and metal contaminants in the subsurface. *Hydrogeol. J.* **8**:77–88.
25. Lovley, D. R., E. J. P. Phillips, and D. J. Lonergan. 1989. Hydrogen and formate oxidation coupled to dissimilatory reduction of iron or manganese by *Aeromonas putrefaciens*. *Appl. Environ. Microbiol.* **55**:700–706.
26. Lovley, D. R., J. L. Fraga, E. L. Blunt-Harris, L. A. Hayes, E. J. P. Phillips, and J. D. Coates. 1998. Humic substances as a mediator from microbially catalyzed metal reduction. *Acta Hydrochim. Hydrobiol.* **26**:152–157.
27. Moser, D. P., J. R. Brozowski, and K. H. Neelson. 1996. Elemental analysis for biomass determination in the presence of insoluble substrates. *J. Microbiol. Methods* **26**:271–278.
28. Myers, C. R., and J. M. Myers. 1994. Ferric iron reduction-linked growth yields of *Shewanella putrefaciens* MR-1. *J. Appl. Bacteriol.* **76**:253–258.
29. Neelson, K. H., and D. Saffarini. 1994. Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. *Annu. Rev. Microbiol.* **48**:311–343.
30. Niggemeyer, A., S. Spring, E. Stackebrandt, and R. F. Rosenzweig. 2001. Isolation and characterization of a novel As(V)-reducing bacterium: implications for arsenic mobilization and the genus *Desulfotobacterium*. *Appl. Environ. Microbiol.* **67**:5568–5580.
31. Ottow, J. C. G. 1970. Selection, characterization, and iron-reducing capacity of nitrate-reductase-less (Nit⁻) mutants or iron-reducing bacteria. *Z. Allg. Mikrobiol.* **10**:55–62.
32. Ottow, J. C. G., and H. Glathe. 1971. Isolation and identification of iron-reducing bacteria from gley soil. *Soil Biol. Biochem.* **3**:43–55.
33. Proctor, L. M., and A. Souza. 2001. Method for enumeration of 5-cyano-2,3-dithiolytetrazolium chloride (CTC)-active cells and cell-specific activity of benthic bacteria in riverine, estuarine, and coastal sediments. *J. Microbiol. Methods* **43**:213–222.
34. Roden, E. E., and D. R. Lovley. 1993. Dissimilatory Fe(III) reduction by the marine microorganism *Desulfuromonas acetoxidans*. *Appl. Environ. Microbiol.* **59**:734–742.
35. Roden, E. E., and R. G. Wetzel. 1996. Organic carbon oxidation and suppression of methane production by microbial Fe(III) oxide reduction in vegetated and unvegetated freshwater wetland sediments. *Limnol. Oceanogr.* **41**:1733–1748.
36. Roden, E. E., and J. M. Zachara. 1996. Microbial reduction of crystalline Fe(III) oxides: influence of oxide surface area and potential for cell growth. *Environ. Sci. Technol.* **30**:1618–1628.
37. Rooney-Varga, J. N., R. T. Anderson, J. L. Fraga, D. Ringelberg, and D. L. Lovley. 1999. Microbial communities associated with anaerobic benzene mineralization in a petroleum-contaminated aquifer. *Appl. Environ. Microbiol.* **65**:3056–3063.
38. Schwertmann, U., and R. M. Cornell. 1991. *Iron oxides in the laboratory*. VCH, Weinheim, Germany.
39. Scott, J. H., and K. H. Neelson. 1994. A biochemical study of the intermediary carbon metabolism of *Shewanella putrefaciens*. *J. Bacteriol.* **176**:3408–3411.
40. Strunk, O., and W. Ludwig. 1997. ARB: software for phylogenetic analysis. Technical University of Munich, Munich, Germany.
41. Stucki, J. W. 1988. Structural iron in smectites, p. 625–675. *In* J. W. Stucki, B. A. Goodman, and U. Schwertmann (ed.), *Iron in soils and clay minerals*. D. Reidel, Dordrecht, The Netherlands.
42. Stucki, J. W., D. C. Golden, and C. B. Roth. 1984. Preparation and handling of dithionite reduced smectite suspensions. *Clays Clay Miner.* **32**:191–197.
43. Stucki, J. W., G. W. Bailey, and H. Gan. 1996. Oxidation-reduction mechanisms in iron-bearing phyllosilicates. *Appl. Clay Sci.* **10**:417–430.
44. Stumm, W., and J. J. Morgan. 1996. *Aquatic chemistry*, 3rd ed. John Wiley & Sons, New York, N.Y.
45. Switzer Blum, J., A. B. Burns Bindi, J. Buzzelli, J. F. Stolz, and R. S. Oremland. 1998. *Bacillus arsenicoselenatis*, sp. nov., and *Bacillus selenitireducens*, sp. nov.: two haloalkaliphiles from Mono Lake, California that respire oxyanions of selenium and arsenic. *Arch. Microbiol.* **171**:19–30.
46. Synoeybos-West, O. L., K. P. Nevin, R. T. Anderson, and D. R. Lovley. 2000. Enrichment of *Geobacter* species in response to stimulation of Fe(III) reduction in sandy aquifer sediments. *Microb. Ecol.* **39**:153–167.
47. Tebo, B. M., and A. Y. Obraztsova. 1998. Sulfate-reducing bacterium grows with Cr(IV), U(VI), Mn(IV), and Fe(III) as electron acceptors. *FEMS Microbiol. Lett.* **162**:193–198.
48. Thamdrup, B. 2000. Bacterial manganese and iron reduction in aquatic sediments. *Adv. Microb. Ecol.* **16**:41–84.
49. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41**:100–180.
50. Widdel, F., and F. Bak. 1992. Gram negative mesophilic sulfate reducing bacteria, p. 3352–3378. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, and applications*, 2nd ed., vol. 2. Springer-Verlag, New York, N.Y.
51. Xu, J. C., J. W. Stucki, J. Wu, J. E. Kostka, and G. K. Sims. 2001. Fate of atrazine and alachlor in redox-treated ferruginous smectite. *Environ. Toxicol. Chem.* **20**:2717–2724.
52. Zachara, J. M., P. L. Gassman, S. C. Smith, and D. Taylor. 1995. Oxidation and adsorption of Co(II) EDTA₂⁻ complexes in subsurface materials with iron and manganese oxides. *Geochim. Cosmochim. Acta* **59**:4449–4463.