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Biomineralization associated with microbial reduction of Fe^{3+} and oxidation of Fe^{2+} in solid minerals

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ABSTRACT

Iron-reducing and oxidizing microorganisms gain energy through reduction or oxidation of iron, and by doing so play an important role in the geochemical cycling of iron. This study was undertaken to investigate mineral transformations associated with microbial reduction of Fe^{3+} and oxidation of Fe^{2+} in solid minerals. A fluid sample from the 2450 m depth of the Chinese Continental Scientific Drilling project was collected, and Fe^{3+} -reducing and Fe^{2+} -oxidizing microorganisms were enriched. The enrichment cultures displayed reduction of Fe^{3+} in nontronite and ferric citrate, and oxidation of Fe^{2+} in vivianite, siderite, and monosulfide (FeS). Additional experiments verified that the iron reduction and oxidation was biological. Oxidation of FeS resulted in the formation of goethite, lepidocrocite, and ferrihydrite as products. Although our molecular microbiological analyses detected *Thermoanaerobacter ethanolicus* as a predominant organism in the enrichment culture, Fe^{3+} reduction and Fe^{2+} oxidation may be accomplished by a consortia of organisms. Our results have important environmental and ecological implications for iron redox cycling in solid minerals in natural environments, where iron mineral transformations may be related to the mobility and solubility of inorganic and organic contaminants.

Keywords: CCSD, iron redox cycling, nontronite, subsurface, *Thermoanaerobacter ethanolicus*

INTRODUCTION

Iron is the fourth most abundant element and one of the most dominant redox active metals in the Earth's crust. Depending on environmental conditions, iron can form stable minerals in both the divalent and trivalent state. Iron cycling depends on redox reactions that are driven by both abiotic and biotic factors, which often result in precipitation and dissolution of Fe-bearing minerals. Coupled with the carbon, nitrogen, phosphorous, and sulfur cycles (Petsch and Berner 1998; Berner et al. 2003), microbial activity can mediate the iron cycle (Fortin and Langley 2005; Kappler and Straub 2005; Lovley 2000a).

Microorganisms can gain energy by reducing Fe^{3+} with either organic acids or H_2 as electron donors (Lovley 1993; Nealson and Saffarini 1994) or oxidizing Fe^{2+} with either O_2 (Kappler and Newman 2004; Kappler and Straub 2005; Neubauer et al. 2002) or nitrate (Benz et al. 1998; Kappler and Straub 2005) as electron acceptors. Numerous Fe^{3+} -reducing microorganisms have been

isolated and characterized (Lovley 1993, 2000a). Whereas it is now well established that diverse microorganisms are capable of reducing both aqueous and solid Fe^{3+} in near-surface and subsurface environments (Liu et al. 1997; Lovley and Chapelle 1995; Lovley et al. 1996; Roh et al. 2002; Zhang et al. 2007a, 2007b), the microbial role in Fe^{2+} oxidation requires further investigation. Although aerobic oxidation of Fe^{2+} -bearing minerals by acidophiles has been recognized for many years (Ghiorse 1984; Harrison 1984; Straub et al. 2001), nitrate-dependent, phototrophic Fe^{2+} oxidation was discovered only recently (Kappler and Newman 2004; Kappler and Straub 2005). In those studies, certain phototrophic and nitrate-reducing bacteria have been demonstrated to be capable of utilizing Fe^{2+} as an electron donor coupled with reduction of CO_2 and nitrate, respectively. The role of anaerobic Fe^{2+} -oxidizing bacteria in iron cycling has recently become a subject of intense investigation (Fortin and Langley 2005; Kappler and Straub 2005; Roden et al. 2004; Straub et al. 2001, 2004; Weber et al. 2006). A recent study reported that a single bacterial species (*Desulfitobacterium frappieri*) was capable of both reducing Fe^{3+} with H_2 as an electron donor and oxidizing Fe^{2+} with nitrate as an electron acceptor (Shelobolina et al. 2003). Clearly, such microbially mediated iron cycling is

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very important to several environmental processes. For example, by regenerating Fe^{3+} , anaerobic microbial Fe^{2+} oxidation may enhance mineralization of organic materials in sediments by Fe^{3+} -reducing bacteria. However, mineral transformations as a result of iron redox cycling need further study.

The purpose of this study was to investigate microbially mediated mineral reactions under controlled laboratory conditions. Microbial enrichment cultures obtained from a deep drilling project, the Chinese Continental Scientific Drilling (CCSD) project, were used to study Fe^{3+} reduction and Fe^{2+} oxidation in solid minerals. Biogenic products were characterized with X-ray diffraction, scanning and transmission electron microscopy, and Mössbauer spectroscopy. Our results indicated that microbial communities enriched from the deep subsurface may collectively perform biogeochemical cycling of iron.

MATERIALS AND EXPERIMENTAL METHODS

Site description

The CCSD drilling site is located in Donghai County, Lianyungang City, Jiangsu Province. Geologically, the site lies within the eastern part of the Dabie-Sulu ultrahigh-pressure metamorphic (UHPM) belt. The Dabie-Sulu UHPM belt was formed by collision between the Sino-Korean Plate and the Yangtze Plate about 240 Ma (Zheng et al. 2003). The occurrence of diamond and coesite in the rocks reveals temperature-pressure conditions of 700–850 °C and 2.8 GPa for the UHP metamorphism. The temperature gradient at the site was 19–26 °C/km (Wang et al. 2001).

Sample collection and preservation

Drilling fluid is often regarded as a source of contamination during the investigation of deep subsurface microbiology, but it is also a means for sampling geological fluids at depth and studying microbial communities from deep subsurface environments. Our previous studies of the drilling fluids that circulated within the CCSD borehole (down to 3350 m) suggest the presence of unique microbes from the deep subsurface (Zhang et al. 2006). In this case, subsurface media (rocks and fluids) that the circulating drilling fluid incorporated, not the drilling fluid itself, were sources of the detected unique microbes. These detected microbes were different from those that may have been present in the components of the drilling fluid (such as bentonite, recycled rocks from shallower depths, and other additives). Therefore, drilling fluid samples were collected every 50 m at the CCSD drilling site, along with rock cores, using sterile tools. Upon collection, drilling fluid samples were immediately frozen at –80 °C (Zhang et al. 2005, 2006). An unfrozen drilling fluid sample, from 2450 m below the ground surface, was shipped to the United States in ice. This sample was used in this study because our previous molecular-based survey suggested the presence of thermophilic, metal-reducing bacteria.

Geochemical analyses

The supernatant of the drilling fluid sample (2450 m), separated by high-speed centrifuge, was analyzed for F^- , Cl^- , NO_3^- , and SO_4^{2-} concentrations by high performance liquid chromatography (HPLC). The pH of the sample was measured with an YSI pH probe and salinity by an YSI salinity probe. In addition, the total Fe^{3+} and Fe^{2+} contents in the drilling fluid sample were measured by direct current plasma (DCP) emission spectroscopy and titration (Andrade et al. 2002), respectively.

Direct microscopic counts

One aliquot was used for direct count to estimate the total number of microbial cells in the sample. Microbial cells were first detached from solids by strong agitation in a 0.7% NaCl solution for 10 min (Bottomley 1994) followed by acridine orange staining and counting with an epifluorescence microscope.

Enrichment of Fe^{3+} -reducing bacteria

Nontronite (NAu-2), an iron-rich smectite clay mineral, was purchased from the Source Clays Repository of the Clay Minerals Society. The bulk sample was size-fractionated and the fraction of 0.5–2 μm was used for the experiment. The total Fe content was 23.4%, and 0.5% of that was Fe^{2+} (Jaisi et al. 2005). Synthetic

ferrihydrite (HFO) (Schwertmann and Cornell 2000) and ferric iron citrate were used, along with NAu-2, to test for Fe^{3+} reduction.

M1 medium (Table 1) (Kostka and Nealson 1998) was prepared to target iron-reducing bacteria. Enrichments were set up in the M1 medium under strictly anaerobic conditions ($\text{N}_2:\text{CO}_2$, 80:20) with 2–5% inoculum at an incubation temperature of 65 °C (the in situ temperature at the sampling depth). Fe^{3+} in either NAu-2 or iron citrate was provided as the sole electron acceptor, and acetate (5 mM) and lactate (5 mM) as the electron donors. Cell growth was monitored by acridine orange direct count (AODC). Production of Fe^{2+} was measured by Ferrozine assay (Dong et al. 2003b; Stookey 1970). This procedure has been shown to be effective for extracting microbially produced Fe^{2+} from reduction of iron oxides including adsorbed form and Fe^{2+} in biogenic solids except for highly crystalline magnetite (Fredrickson et al. 1998). Aqueous Fe^{2+} concentration was measured by the Ferrozine assay after passing 0.3 mL of cell-mineral suspension through a 0.22 μm filter.

Fe^{2+} oxidation experiment by the same enrichment culture

Our results from the Fe^{3+} reduction experiments suggest that the enrichment culture may have contained some Fe^{2+} -oxidizing microorganisms. Thus, separate experiments were performed to examine microbial iron oxidation using Fe^{2+} in synthetic FeS (mackinawite) and siderite (FeCO_3) as electron donors. Mackinawite and siderite were synthesized according to published methods (Ehrenreich and Widdel 1994; Emerson and Moyer 1997). Either FeS or siderite (FeCO_3) was used as the sole electron donor in AG medium (Table 1), a medium for *Anaerobranca gottschalkii*, a thermoalkaliphilic bacterium that grows anaerobically at high pH (9.5) and high temperature (55 °C) (Prowe and Antranikian 2001). The Fe^{2+} oxidation experiments were set up in the AG medium under strictly anaerobic conditions (N_2) with 2–5% inoculum at an incubation temperature of 65 °C. The extent of FeS or FeCO_3 oxidation was determined by measuring Fe^{2+} disappearance with the Ferrozine assay. Bio-oxidized FeS products were characterized by X-ray diffraction (XRD), scanning and transmission electron microscopy (SEM and TEM). Details of the TEM sample preparation, instrumentation, and data collection are described elsewhere (Kim et al. 2003).

XRD and electron microscopy

Solid samples from both abiotic control and reduced NAu-2 were studied by XRD at the U.S. Geological Survey in Boulder, Colorado, to identify mineralogical changes as a result of NAu-2 interaction with the enrichment culture. Solid samples were dispersed in 2 mL distilled water using an ultrasonic water bath, and then pipetted and dried onto glass slides for XRD analyses. Samples were X-rayed with a Siemens D5000 X-ray diffraction system using Cu radiation and a monochromator, and were scanned in 0.02 °2 θ steps with a count time of 2 s per step. To identify the possible presence of mixed layer smectite-illite phases as a result of reduction of Fe^{3+} , bio-reduced NAu-2 was treated with ethylene glycol overnight.

For the reduction experiment with ferric iron citrate as the electron acceptor, the solid products were identified by XRD and SEM. XRD samples were prepared and air dried in a glove box ($\text{N}_2:\text{H}_2$, 95:5). XRD patterns were obtained at Miami University with a Scintag X1 powder diffractometer system using $\text{CuK}\alpha$ radiation with a variable divergent slit and a solid-state detector. The routine power was 1400 W (40 kV, 35 mA). Low-background quartz XRD slides (Gem Dugout, Inc., Pittsburgh, Pennsylvania) were used. Mineral identification was made using a combination of the search-match software JADE, 6.5 and manual search. For SEM observations, the samples were prepared following previously published procedures (Dong et al. 2003a; Zhang et al. 2007a, 2007b).

TABLE 1. Composition of M1 and AG medium

Compounds	M1 Medium	AG Medium
$(\text{NH}_4)_2\text{SO}_4$	9.0 mM	11.4 mM
K_2HPO_4	5.7 mM	2.7 mM
KH_2PO_4	3.3 mM	~
MgSO_4	1.0 mM	0.4 mM
CaCl_2	0.5 mM	0.3 mM
NaCl	~	51.3 mM
NaHCO_3	2.0 mM	11.2 mM
Na_2CO_3	~	26.2 mM
Yeast extract	0.1 g/L	0.1 g/L
Lactate	5 mM	5 mM
Acetate	5 mM	5 mM
pH	6.2	9.2

Mössbauer spectroscopy

^{57}Fe transmission Mössbauer spectroscopy was employed to characterize the Fe mineralogy of the ferric citrate reduction products. Room-temperature, 20, and 12 K spectra were acquired. The sample was analyzed under anaerobic conditions in an environmental chamber. Details of the instrumentation, data collection, calibration, and folding of the data were described elsewhere (Kukkadapu et al. 2001, 2004).

DNA isolation, amplification, cloning, sequencing, and phylogenetic analysis

To determine the microbial community structure in the drilling fluid and the enrichment, DNA was extracted, and the 16S rRNA gene was amplified by polymerase chain reaction followed by cloning and sequencing. Phylogenetic analyses were performed and trees were constructed. These procedures were the same as those described in our previous publications (Jiang et al. 2006; Zhang et al. 2005, 2006).

RESULTS

Geochemistry and biomass of the drilling fluid sample

The drilling fluid sample (2450 m) was alkaline (pH 9.4) with a salinity of 2.5% and various levels of cations and anions (mM): F, 0.3; Cl, 9.9; NO_3^- , 0.2; SO_4^{2-} , 1.2; Na, 73.0; K, 1.1; Mg, 0.2; Ca, 0.6; NH_4^+ , 0.4. The in situ temperature was calculated to be 66–83 °C based on the measured geothermal gradient (Wang et al. 2001). The total iron concentration in the fluid sample was 22.7 mM and the ratio of $\text{Fe}^{2+}/\text{Fe}^{3+}$ was 35.5%. The AODC data showed that the total number of cells was 4.3×10^8 cells/mL.

Microbial reduction of Fe^{3+} in nontronite and iron citrate

The enrichment culture was capable of reducing Fe^{3+} in both solid nontronite and soluble citrate (Fig. 1). The extent of Fe^{3+} reduction in nontronite was similar to that by pure culture of *Shewanella putrefaciens* (Zhang et al. 2007a). When ferric iron citrate was used as the sole electron acceptor, Fe^{3+} was steadily reduced to Fe^{2+} , reaching an Fe^{2+} concentration of 19 mM (95% extent of reduction), followed by a decrease to ~14 mM. The saturation index of iron minerals was calculated using Visual MINTEQ (Gustafsson 2008). At the Fe^{2+} concentrations of 19 and 14 mM, vivianite was the only iron mineral supersaturated (saturation index of 13.4 and 12.0, respectively). Because 0.5 N HCl is able to fully extract Fe^{2+} from vivianite (Fredrickson et al. 1998), this decrease of Fe^{2+} concentration from 19 to 14 mM may be an indication of Fe^{2+} oxidation, and therefore iron oxidation experiments were conducted.

Microbial FeS oxidation experiments

FeS was oxidized at pH 9.5 with an obvious color change from black to brown (Fig. 2a). Ferrozine measurement indicated a decrease in Fe^{2+} concentration from 2.4 to 0.8 mM and then a slight increase to 1.0 mM (Fig. 2b), confirming FeS oxidation. The synthetic siderite was similarly oxidized (Fig. 2b).

X-ray diffraction analysis of iron-redox products

XRD revealed that vivianite was the dominant Fe^{2+} mineral when ferric iron citrate was used as the sole electron acceptor (data not shown). XRD detected the presence of an amorphous phase, possibly ferrihydrite, as a result of microbial oxidation of Fe^{2+} in FeS (Fig. 3).

FIGURE 1. Microbial reduction of Fe^{3+} in nontronite and ferric iron citrate by an enrichment culture from a 2450 m fluid sample of the CCSD project. (a) Change in 0.5 M HCl-extractable Fe^{2+} with time as the enrichment culture reduced Fe^{3+} in nontronite in M1 medium. A = inoculated samples; C = abiotic control. Fe^{3+} (20.8 mM) in NAu-2 was used as the sole electron acceptor and acetate and lactate (5 mM each) as electron donors. (b) Change in 0.5 M HCl-extractable Fe^{2+} with time in AG medium. A = inoculated samples; C = abiotic control. In this treatment, ferric iron citrate was used as the sole electron acceptor. The donors were the same as above. The amount of Fe^{2+} at time zero came from abiotic reduction of Fe^{3+} to Fe^{2+} by cysteine, a chemical reductant used to create a fully anoxic atmosphere in the treatment tubes. The values presented in the graph are single measurements, and good experimental reproducibility can be seen from duplicate or triplicate curves overlapping with one another.

Electron microscopy

SEM observations revealed that vivianite was observed as a reduction product of ferric iron citrate (Fig. 4). The EDS spectrum revealed the presence of P, O, and Fe (see inset EDS spectrum in Fig. 4b). With a prolonged time, vivianite dissolved, forming dissolution pits in association with some encrusted cells (Fig. 4b). Amorphous-looking precipitates formed, which consisted of only iron and oxygen (EDS spectrum obtained from spot B on Fig. 4b). Calculations of mineral saturation indices suggest that ferrihydrite and goethite were possible products of vivianite oxidation. Thus, we infer that the amorphous-looking precipitates were possibly ferrihydrite. With further development of dissolution, etched pits connected to form long cracks or channels and certain cells were associated with these channels (Fig. 4c). Some cells formed a biofilm in close association with the amorphous-looking precipitates (Fig. 4d).

The FeS oxidation products were also studied with SEM and TEM. SEM micrographs of the oxidized FeS showed aggregates of several micrometers in size in association with biofilms (Fig. 5). The synthetic FeS appeared as layered aggregates (Fig. 5a), which were distinct from microbially oxidized minerals (Fig. 5b). These oxidized aggregates were composed of aggregates

FIGURE 2. (a) A photograph of roll tubes showing oxidized FeS (apparently by Fe^{2+} oxidizing bacteria) and an FeS control. (b) A photograph of the culture tubes showing bio-oxidized FeS and abiotic controls. Oxidation of FeS changed color from black to brown. (c) Decrease of Fe^{2+} with time as measured by the 0.5 N HCl extraction in the microbially oxidized FeS and abiotic control (no cells added). Slight increase of Fe^{2+} after 4 days may indicate Fe^{3+} reduction.

FIGURE 3. XRD patterns of bio-oxidized FeS, abiotic control, and reference ferrihydrite from XRD database. The ferrihydrite reference shows two broad peaks corresponding to two-line ferrihydrite.

of hundreds of nanometers in size with various shapes such as needles. Generally, the precipitates appeared to be fine grained (Fig. 5c) and with time, these precipitates formed micrometer-sized crystals (Fig. 5d). The corresponding EDS spectrum (inset A) revealed that the amorphous-looking precipitates consisted of iron and oxygen (Point A on Fig. 5d). In addition to this iron oxide precipitate, there was formation of pyrite. The pyrite possessed a framboidal texture (a spherical or sub-spherical structure composed of numerous microcrystals that are often equal-dimensional) (Fig. 5e) and EDS analysis revealed Fe and S as major elements. The framboidal texture was not present in abiotic FeS control. Pyrite has previously been observed as a product of FeS oxidation (Butler and Rickard 2000).

Transmission electron microscopy images clearly showed crystalline and poorly crystalline iron oxides in the bio-oxidized FeS sample (Fig. 6). High-magnification (up to 400,000 times) TEM was employed to capture the structure of the iron oxide minerals. Aggregates of Fe-precipitates with 3 Å spacing were predominant (Fig. 6a). The upper inset showed 0.3 nm lattice fringes, and selected area electron diffraction (SAED) of the Fe-precipitates displayed ring patterns with 2.1, 2.5, and 3.2 Å spacings (Fig. 6a). These layer spacings were consistent with ferrihydrite (Janney et al. 2000). Needle-like aggregates of the Fe-precipitates with 10 Å spacings were dominant crystalline minerals (Fig. 6b) and its corresponding SAED pattern revealed

ring patterns with 3.2, 4.2, and 10 Å spacings, which is consistent with goethite (Schwertmann and Taylor 1989). Crystalline lepidocrocite was also observed as a product of FeS oxidation.

Mössbauer spectroscopy

Variable-temperature Mössbauer spectroscopy was used to characterize the Fe mineralogy of the bio-reduced ferric iron citrate. The room-temperature (RT) Mössbauer spectrum showed two doublets representing Fe^{2+} associated with vivianite site A (56%) and vivianite site B (35%), and one doublet representing Fe^{3+} (9%) (Fig. 7a). Fe^{3+} exhibited a doublet (central doublet) at room temperature. Mössbauer spectroscopy of 20 and 12 K were employed to further study Fe^{2+} and Fe^{3+} species (Fig. 7b). The 12 K spectrum, however, was virtually identical to the 20 K spectrum. The central Fe^{3+} doublet did not split and did not exhibit a well-defined sextet feature at 12 K, which revealed that the Fe^{3+} (9%) was from the oxidized vivianite (McCammon and Burns 1980). These data confirm the SEM observations that vivianite formed from reduction of ferric iron citrate, which was subsequently microbially oxidized.

Bacterial clone library

Approximately 118 bacterial clones were sequenced for the original drilling fluid sample and the enrichment culture. The bacterial diversity was the highest in the original drilling fluid sample, and the rarefaction curve for the bacterial clone library was not fully saturated. The bacterial diversity for the enrichment culture was much lower, and the rarefaction curve was fully saturated. However, even in the clone library for the drilling fluid sample, multiple sequences were highly similar to one another (>98–99% similarity), implying that the dominant organisms were not missed.

The 16S rRNA gene sequences for the drilling fluid sample (2450 m) clustered into three lineages of bacteria: *Firmicutes*, *Gammaproteobacteria*, and *Deltaproteobacteria* (Fig. 8). In the lineage of *Firmicutes*, the most abundant group of clone sequences clustered with (95–99% similarity) an anaerobic, mesophilic, fermentative, and benzaldehyde-converting bac-

FIGURE 4. SEM images showing that ferric iron citrate was reduced to form vivianite. Vivianite was then partially oxidized in AG medium. (a) Overview of vivianite and amorphous-looking precipitate; (b) crystalline vivianite with dissolution features (pits and channels). Amorphous precipitates and encrusted cells are visible. The insets are SEM-EDS spectra showing the composition of vivianite and oxidized vivianite (i.e., possibly Fe oxides); (c) vivianite with dissolution pits and associated cells; (d) oxidized products of vivianite in association with cells and biofilms.

FIGURE 5. SEM images showing oxidation products of FeS: (a) abiotic control of FeS; (b) overview of minerals in microbially oxidized FeS; (c) amorphous-looking iron oxides; (d) crystalline iron oxides (goethite); (e) framboidal pyrite (enlarged image of the lower-right corner of b). The insets are SEM-EDS spectra of iron oxides in d and pyrite in e.

terium *Soehngenia saccharolytica*. The second most abundant group of clones was closely or moderately (92–99%) related to *Alkalibacterium*. Four sequences were closely related to *Acetobacterium psammolithicum*. It is an acetogen isolated from a subsurface sandstone (Krumholz et al. 1999) and can produce acetate by addition of autoclaved shales (Krumholz et al. 2002).

One sequence was closely related to *Thermoanaerobacter ethanolicus* (Fig. 8). Some strains of this species are capable of using acetate, lactate, and H_2 as electron donors and Fe^{3+} , Co^{3+} , Mn^{4+} , and U^{6+} as electron acceptors (Roh et al. 2002). Growth occurs between 37 and 78 °C and at pH 4.4 to 9.8 (Wiegel and Ljungdahl 1981). These conditions were similar to the measured pH

FIGURE 6. TEM micrographs of (a) bio-oxidized FeS showing Fe-precipitates with 0.3 nm lattice fringes (shown in the inset) and the SAED pattern showing 2.1, 2.5, and 3.2 Å spacings typical of ferrihydrite; (b) bio-oxidized FeS with a needle shape and 1 nm lattice fringe. The inset SAED shows typical goethite with 3.2, 4.2, and 10 Å spacings.

of 9.4 and the in situ temperature of 63–83 °C at the depth from which the drilling fluid sample was collected. In the lineage of *Gammaproteobacteria*, two clones were remotely or moderately related to *Halomonas desiderata*, an alkaliphilic, halotolerant, and denitrifying bacterium. In the lineage of *Deltaproteobacteria*, one sequence was closely related to *Desulfomicrobium norvegi-*

FIGURE 7. Mössbauer spectra of the bio-reduced ferric iron citrate at: (a) room temperature (RT) showing the relative percentage of Fe^{2+} and Fe^{3+} associated with vivianite; (b) 20 K Mössbauer spectra showing the Fe^{3+} is from oxidized vivianite.

cum, a sulfate-reducing bacterium that is able to reduce Co^{3+} and U^{6+} (Genthner et al. 1997; Michel et al. 2001).

DNA was also extracted from the enrichment. Eighty-two (out of 84) clone sequences were closely related (>98–99%) to *Thermoanaerobacter ethanolicus*, suggesting that the enrichment process enriched this thermophilic metal-reducing organism. The other two clone sequences were remotely or moderately related to *Halomonas desiderata*.

DISCUSSION

Biological Fe^{2+} oxidation

Our initial goal was to enrich Fe^{3+} -reducing bacteria in the fluid sample. However, the enrichment culture also displayed an Fe^{2+} oxidation capacity (Fig. 1b). Because of the possibility of chemical oxidation of Fe^{2+} , it was thus important to verify that this oxidation was truly biological. In our experiments, strictly anaerobic techniques were used and it was unlikely that any O_2 leaked into the tubes. Resazurin, a sensitive O_2 indicator, should have turned pink if there was any exposure to oxygen, but a pink color was not observed. The control experiments further indicated that oxygen penetration through butyl rubber stoppers of the culture tubes was not responsible for the observed Fe^{2+} oxidation. We were not aware of any other possible chemical oxidants in the enrichment medium (M1 or AG) that could oxidize Fe^{2+} . Abiotic control did not show any Fe^{2+} oxidation by

FIGURE 8. Phylogenetic relationships of representative phylotypes of bacterial 16S rRNA gene sequences as determined by the neighbor-joining method. Scale bar = 0.02 nucleotide substitution per site. Phyla were determined by using classification in the Bergey's Manual of Systematic Bacteriology (Garrity 2001). *Aquifex pyrophilus* was used as an outer group. For a group of sequences with high similarity to each other, only one representative sequence is shown. CCSD_DF2450_B is from the drilling fluid sample; CCSD_DF2450_Enrichment_B is from enriched culture.

any components of the media. Speciation of biogenic Fe^{2+} into acid-insoluble solids such as magnetite was not likely either, because such solids were not detected by XRD, SEM, TEM, and Mössbauer spectroscopy. Furthermore, addition of certain antibiotics, i.e., chloramphenicol (200 $\mu\text{g}/\text{mL}$) and carbonyl cyanide *m*-chlorophenyl-hydrazone or CCCP (41 $\mu\text{g}/\text{mL}$) at the time of maximal Fe^{2+} concentration, stopped Fe^{2+} oxidation (data not shown). In addition, brown-colored materials surrounding FeS

oxidizing colonies were unevenly distributed on the interior wall of the roll-tubes (Fig. 2a), further indicating biological oxidation of FeS. Based on these data, we conclude that anaerobic Fe^{2+} oxidation was a biologically catalyzed reaction.

Based on the current state of knowledge, only two pathways are known for anaerobic oxidation of Fe^{2+} . Oxygen-independent biological oxidation of Fe^{2+} was first recognized in cultures of anoxygenic phototrophic bacteria (Widdel et al. 1993), show-

ing that Fe^{2+} can be oxidized in anoxic environments (Straub et al. 2001). Fe^{2+} -oxidizing photoautotrophic bacteria use light for CO_2 fixation coupled with oxidation of Fe^{2+} to Fe^{3+} . The reaction is $4\text{Fe}^{2+} + \text{CO}_2 + 11\text{H}_2\text{O} (\text{light}) \rightarrow 4\text{Fe}(\text{OH})_3 + (\text{CH}_2\text{O}) + 8\text{H}^+$ (Ehrenreich and Widdel 1994). The second pathway involves nitrate-dependent oxidation of Fe^{2+} as follows: $10\text{Fe}^{2+} + 2\text{NO}_3^- + 24\text{H}_2\text{O} \rightarrow 10\text{Fe}(\text{OH})_3 + \text{N}_2 + 12\text{H}^+$ or $2\text{Fe}^{2+} + \text{NO}_3^- \rightarrow 2\text{Fe}^{3+} + \text{NO}_2^-$ (Shelobolina et al. 2003; Straub et al. 1996; Straub and Buchholz-Cleven 1998). Because all of our experiments were performed in the dark without nitrate, iron oxidation in our enrichment culture should not have depended on either photosynthesis or nitrate.

Microbial reduction of phosphate to phosphine can be carried out by several fermentors and sulfate-reducing bacteria (Roels and Verstraete 2001). It is conceivable that Fe^{2+} oxidation may be coupled with phosphate reduction. Phosphate is abundant in AG medium. This speculation would be consistent with the fact that phosphate reduction is often linked to metal corrosion (Roels and Verstraete 2001). Unfortunately, phosphine concentration was not measured in our study. In addition, bacteria could possibly reduce SO_4^{2-} to SO_3^{2-} by oxidizing Fe^{2+} to Fe^{3+} . Our thermodynamic calculations suggest this possibility (data not shown), but more data were needed to confirm this speculation.

Identity of Fe^{3+} -reducing and Fe^{2+} -oxidizing bacteria

The drilling fluid sample was dominated by bacterial sequences belonging to the lineage of *Firmicutes*, and only several sequences were clustered into *Proteobacteria*. Many clone sequences were related to either alkaliphilic bacteria (such as *Alkalibacterium olivoapovlenticus*), fermentors, or those sequences previously found in alkaline and saline environments. After the enrichment process, the bacterial diversity decreased dramatically. The bacterial community in the enrichment culture was dominated by strains of *Thermoanaerobacter ethanolicus*. Although the enrichment culture (and subsequent transfers) was predominated by *T. ethanolicus*, a known iron-reducer (Roh et al. 2001), Fe^{2+} -oxidizing bacteria may be present that was not detected by our sequencing method. Although unlikely, *T. ethanolicus* might be capable of reducing Fe^{3+} and oxidizing Fe^{2+} under different conditions (such as pH and medium composition). Indeed, a wide diversity of acidophilic bacteria (*Acidithiobacillus ferrooxidans*, *Acidimicrobium ferrooxidans*, and *Ferrimicrobium acidophilus*), mesophilic and neutrophilic *Geobacter metallireducens*, and *Desulfitobacterium frappieri* have been reported to have the ability to conserve energy to support growth from both Fe^{3+} reduction and anaerobic Fe^{2+} oxidation (Blake and Johnson 2000; Finneran et al. 2002; Shelobolina et al. 2003; Weber et al. 2006).

Mineral transformations associated with Fe^{3+} reduction and oxidation

With nearly two decades of research on microbial iron reduction, it is now clear that various microorganisms can gain energy from reduction of Fe^{3+} in solid minerals (iron oxides and silicates) to support growth (Dong et al. 2000; Lovley 2000b, 2004; Stucki 2006). Iron-reducing microorganisms reduce solid Fe^{3+} via different mechanisms including direct contact, via naturally present or microbially produced electron shuttles (Nevin and Lovley 2002),

and others (such as dissolution and reduction). However, it is not yet well understood if microbes can oxidize Fe^{2+} in solids. Past studies have shown that Fe^{2+} -oxidizing bacteria largely oxidize aqueous Fe^{2+} , and few studies have demonstrated microbial oxidation of Fe^{2+} in solids, except by acidophilic bacteria (Pisapia et al. 2007). For example, phototrophic bacteria can grow with relatively soluble Fe^{2+} minerals such as siderite or ferrous monosulfide (Kappler and Newman 2004). However, nitrate-dependent Fe^{2+} oxidizers have not been demonstrated to be capable of conserving energy from oxidation of solid Fe^{2+} (Kappler and Straub 2005).

In this study, we have demonstrated that iron-oxidizing microorganisms can oxidize Fe^{2+} in solid mineral siderite, vivianite, and ferrous monosulfides. These results are important considering that dissolved Fe^{2+} is insignificant relative to solid Fe^{2+} at neutral pH environments (Kappler and Straub 2005). Regardless of which organisms are involved in the oxidation, our mineralogical evidence revealed mineral transformations as a result of Fe^{2+} oxidation. Vivianite first formed as a result of microbial reduction of soluble Fe^{3+} , but subsequently partially dissolved by iron-oxidizing bacteria to precipitate amorphous iron oxides. Likewise, iron monosulfide (FeS) was oxidized to form amorphous iron oxides as verified by SEM and TEM observations. Siderite, vivianite, and monosulfides are common products of microbial Fe^{3+} reduction, and the products of microbial Fe^{2+} oxidation, iron oxides, can in turn feed iron-reducing bacteria to support their growth. Effectively, our results demonstrate for the first time that there is an iron cycle in an anoxic environment, where Fe^{2+} and Fe^{3+} can cycle back and forth in the solid state.

In contrast to our knowledge of the mechanisms of iron reduction, the mechanisms of microbial Fe^{2+} oxidation in solid minerals have remained poorly understood. By analogy to Fe^{3+} reducers, it may be reasonable to speculate that Fe^{2+} oxidizers may require direct contact or electron shuttles to facilitate electron transport. However, it is premature to propose any possible mechanisms even in a tentative sense.

Environmental and ecological implication of iron redox cycling

The observed iron redox cycling in the solid state has at least three important environmental implications. First, dissimilatory microbial reduction of Fe^{3+} is coupled to oxidation of H_2 or other fermentation products in pristine environments or to organic compounds in contaminated environments (Lovley 2000a). Thus, the extent of iron redox cycling may be linked to the amount of degradation of organic compounds. This linkage is important for accelerated and natural remediation. Second, because reduced and oxidized iron minerals have dramatically different surface area, grain size, and surface adsorption properties for heavy metals and organic compounds, it is likely that every cycle of iron redox state would affect the mobility and ultimate fate of adsorbed organic and inorganic contaminants (Martinez and Ferris 2005; Zachara et al. 2000). The high metal uptake affinity of poorly ordered iron oxides may be important in the global cycling of trace elements throughout the world's oceans (Kennedy et al. 2003), because iron oxides can play an important role in heavy metal and nutrition sorption (Kreller et al. 2003). In contrast,

reduced iron minerals do not exhibit similar properties. Third, many biogenic minerals are nano-particles and possess high reactivity toward several environmental constituents (Wigginton et al. 2007). For example, studies have shown that Fe-oxide and As-sulfides synthesized abiotically and biotically show different electrical and photoconductive properties (Lee et al. 2007) and rates of heterogeneous catalytic efficiency (Jung et al. 2007).

In addition to these environmental implications, microbially catalyzed iron redox cycling has important ecological implications. In the deep subsurface, the availability of electron acceptors usually constrains biomass and microbial activity (Pedersen 2000). The ability of organisms to recycle electrons between Fe^{2+} and Fe^{3+} minerals has a tremendous impact not only on the redox potential of the environment, but also on the energetics of the microorganisms involved. For example, *Desulfotobacterium frapperi* not only reduces Fe^{3+} , but also anaerobically oxidizes Fe^{2+} when nitrate is available. This ability provides a competitive advantage for the organism (Shelobolina et al. 2003). Thus, the enrichment culture used in this study, in which *Thermoanaerobacter ethanolicus* was a predominant organism, may be able to use this type of advantage. Microorganisms need a constant supply of energy to survive, grow, and reproduce. Hydrogen gas is continuously generated in the interior of our planet via various mechanisms (Dong and Yu 2007) and probably constitutes sustainable sources of energy for deep terrestrial biosphere ecosystems (Chapelle et al. 2002; Stevens 1997; Stevens and McKinley 1995, 2000). The lack of energy substrates often forces an organism to switch to a different type of metabolism, or may even cause a shift in the composition of a microbial community (Brune et al. 2000), but the recycling of electrons between Fe^{2+} and Fe^{3+} may provide a renewable energy source for the microorganisms so that it may not be necessary for them to switch metabolic pathways even under stressed conditions.

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