

REDUCTION OF STRUCTURAL Fe(III) IN SMECTITE BY A PURE CULTURE OF *SHEWANELLA PUTREFACIENS* STRAIN MR-1

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Abstract—*Shewanella putrefaciens* is a species of metal-reducing bacteria with a versatile respiratory metabolism. This study reports that *S. putrefaciens* strain MR-1 rapidly reduces Fe(III) within smectite clay minerals. Up to 15% of the structural Fe within ferruginous smectite (sample SWa-1, Source Clays Repository of the Clay Minerals Society) was reduced by MR-1 in 4 h, and a range of 25% to 41% of structural Fe was reduced after 6 to 12 d during culture. Conditions for which smectite reduction was optimal, that is, pH 5 to 6, at 25 to 37 °C, are consistent with an enzymatic process and not with simple chemical reduction. Smectite reduction required viable cells, and was coupled to energy generation and carbon metabolism for MR-1 cultures with smectite added as the sole electron acceptor. Iron(III) reduction catalyzed by MR-1 was inhibited under aerobic conditions, and under anaerobic conditions it was inhibited by the addition of nitrate as an alternate electron acceptor or by the metabolic inhibitors tetrachlorosalicylanilide (TCS) or quinacrine hydrochloride. Genetic mutants of MR-1 deficient in anaerobic respiration reduced significantly less structural Fe than wild-type cells. In a minimal medium with formate or lactate as the electron donor, more than three times the amount of smectite was reduced over no-carbon controls. These data point to at least one mechanism that may be responsible for the microbial reduction of clay minerals within soils, namely, anaerobic respiration, and indicate that pure cultures of MR-1 provide an effective model system for soil scientists and mineralogists interested in clay reduction. Given the ubiquitous distribution and versatile metabolism of MR-1, these studies may have further implications for bioremediation and water quality in soils and sediments.

Key Words—Clay, Fe(III) reduction, Metal-reducing bacteria, Sediments, Smectite, Soils

INTRODUCTION

The reduction of clays and associated swellability changes have been related to important processes in soils and sediments such as nutrient cycling (Chen et al. 1987; Shen and Stucki 1994), sediment structure (Stucki 1988), permeability (Gates et al. 1993) and plant growth (Lamb and Grady 1963). Reduced clays have a more blocky morphology (Stucki and Tessier 1991) and expose less surface area (Lear and Stucki 1989); whereas, the oxidized clay particles are thinner, have larger lateral extent and greater surface area. Chemical reduction studies have shown that the clay structure collapses in response to the reduction of structural Fe, causing a swellability decrease measured by swelling pressure (Stucki et al. 1984b; Lear and Stucki 1989), a decrease in surface area (Stucki and Lear 1990) and a cation exchange capacity (CEC) increase (Stucki 1988).

Though clay reduction within soils and sediments is thought to occur primarily as a result of the activity of indigenous microorganisms (Stucki et al. 1987; Gates et al. 1993), most research has focused on chemical mechanisms of Fe reduction within clays. Chemical reduction studies have employed potent inorganic

reductants such as dithionite or hydrazine, which are not likely to play a significant role in clay reduction for natural environments (Stucki 1988). The fact is that these inorganic chemical reductants are likely to be minor components of soils and sediments; whereas, metal-reducing bacteria have been reported at 10^3 to 10^5 cells per cm^3 for aquatic sediments (Nealson et al. 1991; Nealson and Myers 1992).

Within the past decade, a variety of bacteria within soils and sediments have been found to couple the oxidation of organic carbon to the reduction of oxidized metals [Fe(III) or Mn(IV)] (Myers and Nealson 1988; Lovley 1991; Nealson and Myers 1992; Nealson and Saffarini 1994). Through the catalysis of such reactions, metal-reducing bacteria can form an effective link between the cycles of oxidized metals and organic carbon. While the turnover or recycling of the metals for most cases is undetermined, for some sedimentary environments Fe and/or Mn respiration can account for a large portion of the organic carbon oxidation that occurs (Chapelle and Lovley 1992; Burdige 1993; Canfield et al. 1993).

Reduction of amorphous Fe(III) oxide minerals and of the crystalline Fe(III) minerals, goethite and hematite, has been demonstrated using pure cultures of metal-reducing bacteria (Arnold et al. 1988; Lovley 1991). However, though clay minerals often dominate the solid phase of sediments where metal-reducing

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bacteria have been isolated, few studies of the microbial reduction of Fe-rich clay minerals have been carried out (Stucki et al. 1987; Wu et al. 1988; Gates et al. 1993). None of these studies has explored the biological mechanism by which microbes catalyze clay reduction nor have they specified the conditions under which microbial clay reduction would be favored for soils and sediments. No previous clay reduction studies have been carried out using pure cultures of metal-reducing bacteria, organisms which are likely to mediate clay reduction *in situ*.

The possible importance of the process and the paucity of knowledge for the area warranted a study of structural Fe reduction within smectites using a pure culture of the dissimilatory Fe(III)-reducing bacterium, *Shewanella putrefaciens* strain MR-1.

MATERIALS AND METHODS

Clay Mineral Preparation

The 0.5 to 2 μm size fraction of the ferruginous smectite SWa-1 (Source Clays Repository of the Clay Minerals Society) from Grant County, WA, was used. The elemental composition of SWa-1 was reported by Goodman et al. (1976). The clay was fractionated, dialyzed and freeze-dried prior to use (Stucki et al. 1984a). The total Fe content of SWa-1 (total Fe = 3.549 mmol Fe/g SWa-1) was reported previously by Lear and Stucki (1989). Clays were sterilized by heating via microwave radiation (Keller et al. 1988) before addition to the culture medium.

Bacterial Cultures

Shewanella putrefaciens strain MR-1 was used. MR-1 was isolated from the anoxic sediments of Lake Oneida, NY (Myers and Nealson 1988), and has been the subject of many physiological and genetic studies concerning *S. putrefaciens* (Nealson and Saffarini 1994). MR-1 is an obligate-respiratory bacterium, incapable of fermentation (Scott and Nealson 1994).

Unless otherwise stated, the minimal (M1) medium described by Myers and Nealson (1988) was used. The M1 medium contained 20 mM formate or lactate as the electron donor in a base medium of the following composition: 9.0 mM $(\text{NH}_4)_2\text{SO}_4$, 5.7 mM K_2HPO_4 , 3.3 mM KH_2PO_4 , 2.0 mM NaHCO_3 , 1.01 mM MgSO_4 , 0.485 mM CaCl_2 , 67.2 μM Na_2EDTA , 56.6 μM H_3BO_3 , 10.0 μM NaCl , 5.4 μM FeSO_4 , 5.0 μM CoSO_4 , 5.0 μM $\text{Ni}(\text{NH}_4)_2(\text{SO}_4)_2$, 3.87 μM Na_2MoO_4 , 1.5 μM Na_2SeO_4 , 1.26 μM MnSO_4 , 1.04 μM ZnSO_4 , 0.2 μM CuSO_4 , 20 mg/L arginine, 20 mg/L glutamate, and 20 mg/L serine. Cultures were grown within this medium with 80 mM Fe(III) citrate as the electron acceptor and then transferred to fresh medium with 1.25 mg/mL smectite added as the sole electron acceptor. Cultures were shaken at approximately 50 rpm and incubated at 22 °C unless otherwise indicated. All

manipulations and incubations were carried out under strict anoxic conditions within a Coy anaerobic chamber (90% N_2 , 10% H_2). Heat-killed controls were heated by microwave radiation until boiling (Keller et al. 1988) and aerobic cultures were shaken at 50 rpm in air. Metabolic inhibitors were used as in previous studies (Kostka et al. 1995; Kostka and Nealson 1995). Transposon (Tn5) and chemical mutagenesis procedures were used to produce genetic mutants of MR-1, which were deficient in the respiration of Fe (Saffarini et al. 1994).

Fe Measurements

The oxidation state of structural Fe within smectite was measured using a variety of techniques. Production of Fe(II) was monitored in culture by HCl extraction according to Kostka and Nealson (1995) under strict anoxic conditions. Culture samples were extracted using 0.5 M HCl for 30 min and then subsamples of the extract were pipetted into ferrozine buffered in 50 mM Hepes (pH 7). After 10 to 15 min the ferrozine mixture was filtered and the absorbance was measured at 562 nm (Stookey 1970). Iron concentration was determined via comparison to a standard curve generated with ferrous ammonium sulfate. The percentage of structural Fe reduced was determined mathematically by comparison to the total Fe concentration for SWa-1 (3.549 mmol Fe/g) reported by Lear and Stucki (1989).

The oxidation state of the clay was further determined as described by Stucki (1981) and modified by Komadel and Stucki (1988). Briefly, the clay sample was digested with HF and H_2SO_4 , in the presence of 1,10-phenanthroline. The digestate was diluted with sodium citrate and the absorbance measured at 510 nm before and after photochemical reduction using a mercury vapor lamp. Mössbauer spectroscopy was used to measure the amount of structural Fe reduced within the smectite (Amonette et al. 1994).

RESULTS AND DISCUSSION

Iron(III) reduction catalyzed by MR-1 had an apparent large effect upon clay mineral properties. The oxidation state of structural Fe within smectite determines much of the mineral's color (Stucki 1988). Color changes occurred within a few hours during incubation of all treatments and were indicative of the Fe(II) contents measured. Oxidized smectite appeared as fine yellow particles that remained in suspension. After microbial reduction, the clay became a dark green to blue color, the particles appeared to flocculate and sank to the bottom of the serum bottle. This change occurred within 6 to 12 h from a 1.25 mg/mL smectite suspension with an initial cell number of 10^6 to 10^8 cells/mL of MR-1. These observations are consistent with previous studies of microbial enrichments from clay minerals (Stucki et al. 1987), enrichments

Table 1. Comparison of measurements for the extent of structural Fe reduction in smectite after 72 h exposure to *Shewanella putrefaciens* strain MR-1 under anaerobic conditions. Results are the average from duplicate cultures and the same bottles were sampled for all 3 measurements.

Method	Fe(II) (% of total Fe)
HCl/Ferrozine	29.8
HF/H ₂ SO ₄ /1,10-phenanthroline (Stucki 1981)	31.5
Mössbauer spectroscopy	29.8

from paddy soils (Wu et al. 1988) and in *Pseudomonas* cultures isolated from the wheat rhizosphere of soils from the State of Washington (Gates et al. 1993).

The Fe chemistry of smectite reduced by MR-1 was studied (Table 1). Wet chemical methods were used to measure the oxidation state of Fe within culture samples. MR-1 reduced a substantial percentage of the structural Fe within smectite in a few hours. The HCl extraction used for this study was not expected to dissolve away the entire clay structure (Kostka and Luther 1994). As expected, no Fe was extracted at time zero (Figure 1) and little or no Fe was extracted from the bacteria controls. From parallel measurements using duplicate samples taken from the same culture after 72 h exposure to MR-1, the HCl extraction gave the same Fe(II) values within 1 to 2% of the Fe(II) measured by the HF dissolution method of Stucki (1981) (Table 1). Wet chemistry was supported by close agreement with Mössbauer spectroscopy that confirmed the change of oxidation state following microbial reduction (Table 1).

Approximately half of the structural Fe reduction by MR-1, that is, 0.51 mmol Fe/g or 15% of total Fe in SWa-1, occurred within the first 4 h after inoculation (Figure 1) and the amount of Fe reduced increased exponentially during the first 10 h (Figure 1). From these same cultures after 2 weeks, 1.167 mmol Fe(II)/g or 33% of the total Fe in SWa-1 was reduced (data not shown). Therefore, structural Fe reduction occurred at an exponential rate for the first day and then a linear rate was observed afterward.

To explore the physiological mechanisms associated with microbial clay reduction, smectite reduction was monitored over time using a variety of culture treatments. Figure 2a shows the effect of inoculum size (cell number) on the rate and extent of Fe reduction in smectite. A clear relationship exists between the inoculum size and the rate of Fe reduction, but it is a complex relationship that is undoubtedly related to contact between the clay and cell surfaces. This suggestion was supported by experiments which showed that when the clay suspension was separated from MR-1 cells with dialysis tubing no reduction was observed.

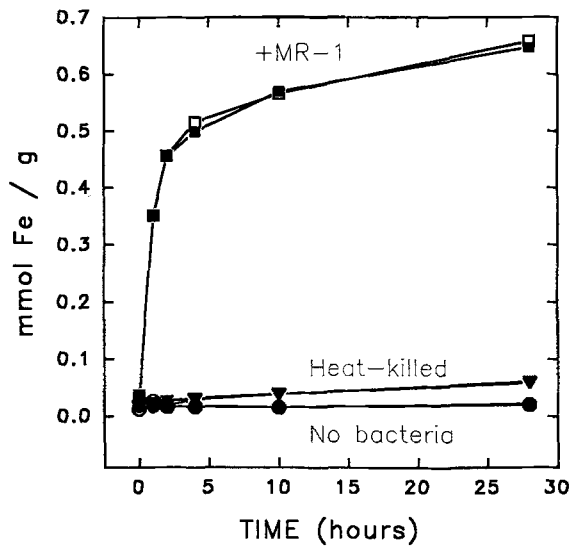


Figure 1. Reduction of structural Fe(III) to Fe(II) in smectite (1.25 mg/mL) under anoxic conditions for the minimal medium (with no bacteria added), with heat-killed cells, and within live cultures of MR-1.

Figure 2b shows that alternate electron acceptors such as oxygen or nitrate inhibit the microbial reduction of clay structural Fe, which is consistent with reports involving other Fe minerals. Molecular oxygen has been shown to inhibit strongly the respiration of Fe(III) by metal-reducing bacteria, while nitrate or a product of nitrate reduction has been observed to partially inhibit metal respiration (Nealson and Saffarini 1994). Table 2 shows a summary of the results of experiments involving metabolic inhibitors. Neither cyanide nor azide, inhibitors of aerobic metabolism, showed significant inhibition of Fe reduction (Table 2), while quinacrine, an inhibitor of electron transport, inhibited Fe(III) reduction by 41%. Heat-killed cells and cells treated with the anaerobic protonophore salicylanilide (TCS) showed almost no ability to reduce the clay structural Fe (Table 2).

The extent of Fe reduction for various genetic mutants of MR-1 is shown in Figure 2c. A rough mutant, with an altered morphology, that was normal for Fe reduction showed similar clay reduction as did the wild type, while 3 mutants isolated as deficient in Fe reduction, namely, A1, A2 and 13, all showed decreased levels of reduction of clay structural Fe. While the genetics of Fe respiration for *S. putrefaciens* are not yet completely elucidated, the lower rates of Fe reduction by the mutants suggest that either regulatory or structural genes needed for Fe reduction have been altered.

For the minimal medium used in these studies, more than 3 times the amount of smectite was reduced upon addition of carbon substrate than was observed for the controls to which no carbon was added in duplicate

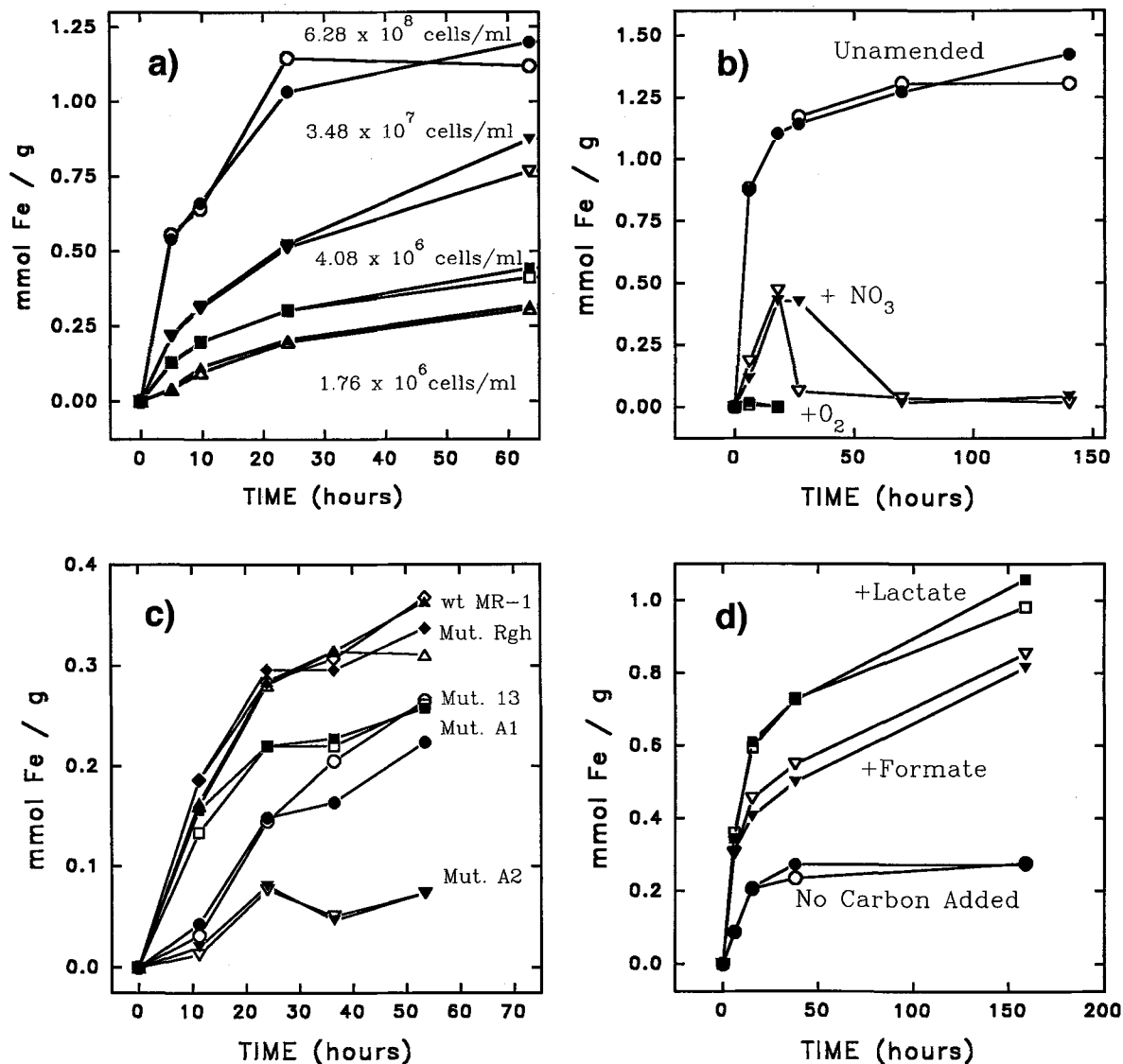


Figure 2. Structural Fe(III) reduction in smectite (1.25 mg/mL) over time in various treatments of MR-1: a) at a range of different initial cell numbers; b) with electron acceptors added; c) within cultures of genetic mutants of MR-1 compared to the wild type; and d) within a minimal medium with/without electron donors added. Each point is the average of duplicate cultures. Oxygen treatments were shaken in air and nitrate was added to 5 mM [for cultures shown in b)].

Table 2. Effect of metabolic inhibitors on the reduction of structural Fe(III) within smectite (SWa-1) by *Shewanella putrefaciens* strain MR-1.

Inhibitor	Degree of inhibition (%)†
Quinacrine hydrochloride (1 mM)	41
Tetrachlorosalicylanilide (0.2 mM)	83
Cyanide (0.5 mM)	5
Azide (1 mM)	10
Heat-killed (100 °C)	91

† Calculated from the amount of structural Fe(III) within SWa-1 reduced (as % of total Fe) after 24 h in duplicate MR-1 cultures.

culture treatments at a constant cell number (Figure 2d). The initial reduction observed for the nocarbon controls could be due to residual carbon within the cells upon dilution into the minimal medium or to the low levels of amino acids used to supplement this medium. Regardless of the source, after 1 to 2 d, clearly no more Fe reduction occurred unless an exogenous source of organic carbon, for example, lactate or formate, was added. These results indicate that clay reduction is coupled to carbon metabolism and electron flow in MR-1. With formate added as the sole electron donor (Figure 2d), the data provide strong evidence that the mechanism of clay reduction is anaerobic res-

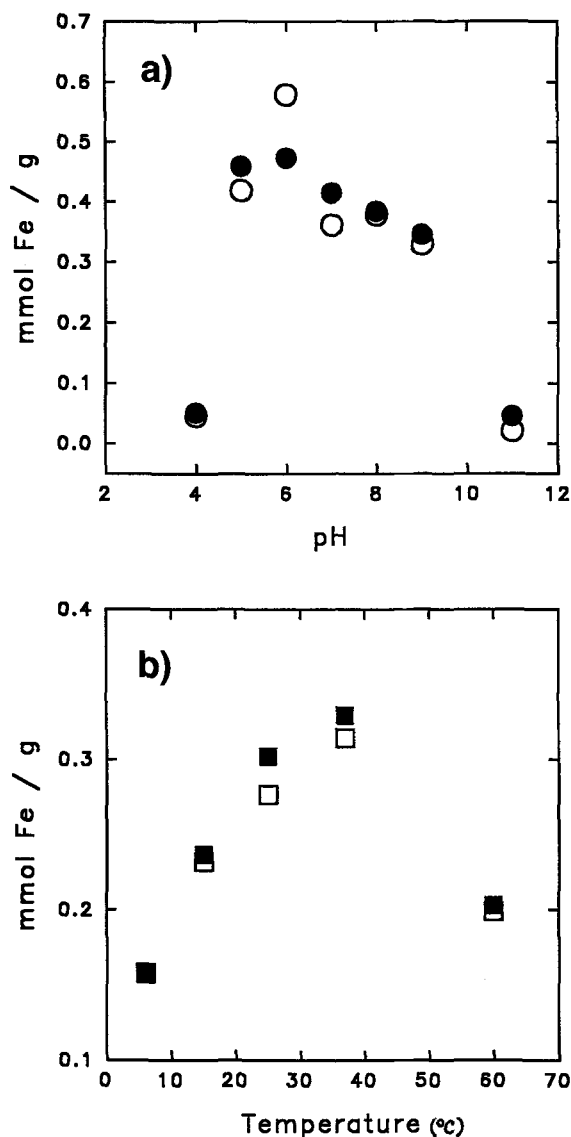


Figure 3. a) Optimum pH of smectite reduction after 2.5 h exposure to MR-1. Each point is the average of duplicate cultures which contained an initial cell number of 10^8 cells/mL; b) Temperature optimum of smectite reduction after 2.5 h exposure to MR-1.

piration through dissimilatory reduction of structural Fe(III) coupled to organic carbon oxidation.

To explore conditions under which microbial clay reduction is likely to occur within soils and sediments, smectite reduction by MR-1 cultures was measured at a range of temperatures and pH values. Once the initial pH of cultures was raised to a value at which MR-1 is physiologically active ($> \text{pH } 4$), the optimum range was 5 to 6 as the rate of smectite reduction decreased with increasing pH above 6 (Figure 3a). However, clay reduction rates were rapid and remained within a factor of 2 from pH 5 to pH 9. Similarly, reduction rates

Table 3. Comparison of the extent of structural Fe(III) reduction by MR-1 for the present study to previous studies of microbial clay reduction.

Study	Time in culture (d)	Cell count (cells/ml)	Maximum extent of reduction	
			(mmol/g)	(% of Total)
Stucki et al. (1987)	14	10^8 to 10^{12}	0.31	8.7
Wu et al. (1988)	14	ND†	1.73	39
Gates et al. (1993)	14	10^7	1.24	35
	60	10^7	NA†	NA†
This Study	7	10^6 (n = 4)	1.46	41
	7	10^7 (n = 2)	NA†	NA†
	6 to 12	10^8 (n = 12)	NA†	NA†

† Key: ND = not determined; NA = not available.

remained within a factor of 2 at temperatures from 6 to 60 °C (Figure 3b). Substantial microbial Fe reduction was observed, as a green color, for treatments from 6 to 37 °C while little or no microbial reduction was observed at 60 °C or for no-bacteria controls, which remained golden brown in color. Optimum rates were observed for the middle of the range of both pH and temperature, indicative of an enzymatically catalyzed reduction reaction (Figures 3a and 3b).

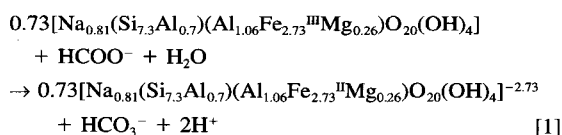
Smectite reduction remained rapid over a larger pH range than for previous studies of magnetite reduction (Kostka and Nealson 1995) or for studies of Mn(IV) reduction by MR-1 (Myers and Nealson 1988). Rates of clay reduction also appeared to remain rapid over a wider range of temperature than observed from previous studies of the reduction of crystalline Fe(III) oxide by MR-1 (Figure 3b) (Kostka and Nealson 1995). Though further study is needed to better define the kinetics of structural Fe(III) reduction by MR-1, rapid microbial clay reduction appears to be possible over a wide range of temperature and pH found within soils and sediments.

Comparison of the rate and extent of Fe(III) reduction by MR-1 to other studies of microbial clay reduction is difficult because few studies have been conducted. Moreover, previous work emphasized the effects of microbial reduction on clay structure. The existing data are summarized in Table 3. The comparison reveals that though the extent of reduction observed for the present study is within the range reported from previous studies, the reduction rate observed for this study is much more rapid, occurring on the scale of hours rather than days (Table 3). Stucki et al. (1987) reported that 3 to 8% of the total Fe in SWa-1 was reduced after 14 d from cultures of soil enrichments containing 10^8 to 10^{12} cells/mL. No cell counts were carried out by Wu et al. (1988), but the extent of nontronite reduction after 14 d (39%) fell within the range reported from the present study for smectite at 6 to 12 d (29–41%). Gates et al. (1993) found that pure cul-

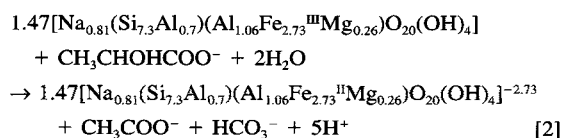
tures of *Pseudomonas sp.* reduced up to 21% of the structural Fe within SWa-1 after 14 d, and mixed cultures reduced 35% after 60 d. At comparable cell numbers and smectite concentrations, one-half of the structural Fe(III) reduction observed within MR-1 cultures occurred during the first 4 h. Results from the present study conclusively show the dependence of structural Fe(III) reduction upon cell number as well as upon the physiology of metal-reducing bacteria.

Similar to studies of Fe(III) oxide minerals (Arnold et al. 1988), the present study also shows that cell contact is necessary for the reduction of structural Fe(III) within smectite. The requirement for cell contact is consistent with an anaerobic respiration mechanism involving transfer of an electron from the cell surface to the clay causing Fe(III) reduction. This observation, coupled with experiments on carbon metabolism (Figure 2d), provide strong evidence that clay reduction occurs through an enzymatic, dissimilatory mechanism. We propose that *Shewanella putrefaciens* couples the dissimilatory reduction of structural Fe(III) within smectite to the oxidation of organic acids according to the following reactions:

Dissimilatory Fe(III) smectite reduction coupled to formate oxidation:



Dissimilatory Fe(III) smectite reduction coupled to lactate oxidation:



This is consistent with previous studies of *S. putrefaciens* under similar culture conditions in which the stoichiometry of dissimilatory metal reduction was documented (Kostka et al. 1995).

The mechanisms for electron transport to insoluble Fe(III) and Mn(IV) minerals by dissimilatory metal-reducing bacteria are largely unknown (Lovley 1993a). One study by Myers and Myers (1992) found that *S. putrefaciens* produces cytochromes, which are located on the outer membrane, suggesting a possible mechanism of electron transport to external electron acceptors. Reduction at the outer membrane seems more plausible at this time for smectite reduction by MR-1 in that the Fe is likely to remain bound within the clay structure as it is reduced. Further studies are in progress to elucidate the electron transfer mechanism between MR-1 and clays in the laboratory.

Environmental Implications of Microbial Clay Reduction

Iron(III) minerals are often the most abundant oxidant available within soils and sediments (Ponnamperuma et al. 1967; Lovley 1991), and changes in the oxidation state of Fe within clay minerals have a large effect on the physical-chemical characteristics of these environments (Stucki 1988). Recent studies have found that a large percentage of Fe reduction in sediments is due to bacterial metabolism (Lovley 1993b). Bacterial Fe reduction is thought to be intimately involved in the cycling of organic C and other nutrients. This process may be manipulated to facilitate the remediation of sediments contaminated with metals and organics.

Several strains of bacteria have been isolated from sediments, which are capable of coupling the reduction of Fe(III) to the oxidation of organic C (Nealson and Saffarini 1994). *Shewanella putrefaciens* is one of the better studied organisms and has been isolated from oil field samples (Obuekwe and Westlake 1982), freshwater sediments (Myers and Nealson 1988) and marine environments where they are found in high numbers at redox interfaces (Nealson et al. 1991; Brettar and Hoefle 1993). *S. putrefaciens* has also been studied for its potential to remediate organic contaminants (Petrovskis et al. 1994; Nealson et al. 1995). Though only a fraction of the presumed population of bacteria capable of metal reduction has been cultivated, the abundance and physiological versatility observed to date suggests that these organisms may contribute significantly to *in situ* bioremediation and water quality within soils and sediments.

Metal-reducing bacteria like *S. putrefaciens* may catalyze a number of important reactions associated with clay mineral surfaces within soils and sediments by one or more of the following ways:

1) Through reduction of structural Fe(III), metal-reducers may decrease the swelling of clays leading to decreased surface area and increased CEC (Stucki 1988). Therefore, structural Fe reduction by microbes could lead to changes in soil structure (Gates et al. 1993) and fertility (Chen et al. 1987).

2) The bacterial reduction of metal oxyhydroxides associated with clay-rich soils and sediments may release trace metals and nutrients that co-precipitate with these oxides, thereby affecting the flow of these important components within soils (Jenne 1977).

3) Metal-reducing bacteria oxidize organic carbon compounds, which are likely to be found in soil organic matter (Lovley 1991), and interactions of metal-reducers with soil organic matter may subsequently affect the sorption capacity of solid surfaces in soils (Schlautman and Morgan 1994).

The wealth of physicochemical changes caused by structural Fe(III) reduction (Stucki 1988) coupled with

the other processes mentioned above suggest that synergies between metal-reducing bacteria and clay minerals have the potential to play a substantial role for the biogeochemistry of soils and sediments.

SUMMARY AND CONCLUSIONS

Smectites are best known for their large swellability, surface area and cation exchange capacity. This group of phyllosilicates exists extensively within aquatic soils and sediments (Isphording 1975; Stucki 1988). The smectite bentonite has been used as a sorbent for the containment of hydrophobic organic pollutants. Therefore, results from the present study may be applied to natural soils and sediments, as well as to waste systems and clay-rich aquifer sediments where metal-reducing bacteria have been isolated and Fe(III) reduction has been identified as an important process (Lovley et al. 1990; Chapelle and Lovley 1992). Inorganic reducing agents such as dithionite are unlikely to be present in sufficient quantities within soils or sediments to significantly alter Fe oxidation state *in situ*. The use of such chemical reducing agents for controlling the oxidation state of sediments is both economically and environmentally infeasible (Gates et al. 1993). However, indigenous microorganisms or seeded microorganisms may prove useful for controlling the oxidation state of these environments.

A substantial proportion of the structural Fe(III) within smectite may be reduced by sedimentary bacteria in 6 to 12 h. This reduction occurs at temperatures and pHs common to soils and sediments, and the process appears to be coupled to energy generation and carbon metabolism for strain MR-1. Anaerobic respiration is apparently one mechanism by which microbes may reduce clays within soils and sediments. Changes in Fe chemistry indicate that the process has a large influence upon clay mineral structure, and therefore, may be important to the biogeochemistry and remediation of pollutants within the environment. Hopefully these studies will stimulate and direct a search for microbial clay reduction in the field.

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