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Respiration and Dissolution of Iron(III)-Containing Clay Minerals by Bacteria

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A quantitative study of microbial clay mineral reduction coupled to the oxidation of organic carbon was carried out using an Fe(III)-reducing bacterium (*Shewanella putrefaciens* strain MR-1). Total CO_2 production, organic acid depletion, and Fe(III) reduction were measured in the same cultures with formate or lactate as the carbon source and clay as the sole electron acceptor. Mean ratios of 1.6:1 and 4.9:1 were observed for structural Fe(III) reduction coupled to formate oxidation and lactate oxidation, respectively. When organic ligands were added under similar culture conditions, the extent of clay reduction was enhanced up to 2-fold in the order of nitrilotriacetic acid (NTA) > oxalate > citrate > malate. Further, dissolution of the clay mineral structure was inferred as dissolved Fe(II) comprised up to 50% of the total clay-bound Fe reduced in cultures to which organic ligand was added. Here we provide the first direct measurements which show that (1) bacteria may couple the respiration of Fe(III) bound in smectite clay minerals to carbon cycling, (2) organic ligands increase the bioavailability of Fe(III) bound in clay minerals, and (3) bacterial Fe(III) reduction in the presence of organic ligands may lead to clay mineral dissolution. These discoveries have important implications for the biogeochemistry of soils where Fe(III)-bearing clay minerals are abundant.

Introduction

Clay minerals are among the most important structural materials of the earth's surface playing a significant role in many aspects of life, ranging from their domination of soil/sediment physical properties to their ubiquitous commercial use in many products and industries (*1*−*3*). Clays have large surface areas, balanced by exchangeable cations, which bind a large number of inorganic and organic molecules including contaminants in soils and sediments (*4*). Interactions between microbes and clay minerals result in maintenance of soil structure, soil fertility, and soil chemistry (cation exchange, pH buffering).

Virtually all soil clay minerals contain some Fe in their crystal structures, and studies over the past few decades have discovered that the oxidation state of this structural Fe profoundly alters many physicochemical properties of the clay, which has important industrial and environmental implications (*3, 5*). Studies of the chemical reduction of Fe(III) bound in clay minerals have shown effects on swelling (*6*), cation exchange and fixation capacity (*7*−*9*), specific surface area (*8*), color (*10*), and magnetic exchange interactions (*11*). During reduction, the clay structure collapses in response to the reduction of structural Fe, thereby trapping cations (such as K^+ and NH_4^+) in their mineral matrix which may be detrimental to agricultural use of a soil because valuable nutrients could become unavailable for plant growth. In addition, the fate and transport of redox active contaminants in soil, heavy metals, and organic pollutants, are likely to be highly dependent on transformations at the clay surface.

Though reduction of structural Fe in clays within soils and sediments is thought to be mediated primarily by the enzymatically catalyzed activity of indigenous microorganisms (*12, 13*), most research has focused on chemical mechanisms of structural Fe reduction in clays. Chemical reduction studies have employed potent chemical reductants such as dithionite or hydrazine, which are not likely to play a significant role in clay reduction in natural environments (*4, 5*). These inorganic chemical reductants are likely to be minor components of soils; whereas, microorganisms capable of Fe(III) reduction have been observed at up to 10^5 cells per gram dry weight of agricultural soil (*14*).

Biogeochemical evidence exists to support the potential importance of crystalline Fe minerals as an electron acceptor for Fe-reducing bacteria in soils and subsurface sediments. Several studies have shown that Fe-reducers are capable of coupling the reduction of crystalline Fe minerals, including oxides goethite (*15*) and magnetite (*16*), and the silicate, smectite (*17*), to energy generation. Studies of soils and subsurface aquifer sediments have shown that the crystalline Fe fraction, containing a mixture of Fe(III) oxides and layered silicates, is available for reduction to seeded cultures and natural populations of Fe-reducing bacteria (*15, 18, 19*).

The form of Fe(III) available for bacterial reduction has been shown to be paramount to its reactivity. The rate of Fe mineral reduction by bacteria has been shown to be directly proportional to particle surface area (*15*), and the crystallinity of Fe(III) minerals has also been positively correlated with their availability for bacterial reduction (*18, 19*). Consistent with these hypotheses, amorphous Fe(III) oxyhydroxide, which has a high surface area and is marginally crystalline in character, is most often implicated as the solid Fe(III) form reduced by bacteria in natural environments (*20*−*22*). Although not easily detected in the environment, soluble Fe(III) chelated to organic ligands has been shown to be an effective electron acceptor for Fe-reducing bacteria in laboratory studies, and the organic ligand nitrilotriacetic acid (NTA) was observed to increase the availability of solid Fe(III), presumably Fe(III) oxyhydroxides, in sediments (*23, 24*).

Until recently, Fe(III) bound in clays was considered unreactive or unavailable for use by bacteria. Recent evidence from our combined laboratories has shown that a known dissimilatory Fe(III)-reducing bacterium, *Shewanella putrefaciens*, was capable of rapidly reducing the structural Fe(III) in smectite (29−41%) in less than 24 h (*17*). Clay reduction occurred at temperatures and pHs common to soils/sediments, and it was linked to energy generation and carbon metabolism in this Fe(III)-reducer. In this study, we elucidate the mechanism of clay reduction as anaerobic respiration coupled to carbon metabolism in *Shewanella* through determination of reactant/product stoichiometry. Further, we show that, in the presence of organic ligands (some of which are commonly found in soils), bacterial reduction of

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TABLE 1. Summary of Studies to Date Which Have Quantified the Rate and Extent of Crystalline Fe(III) Mineral Reduction by *Shewanella* sp. in Characterized Mineral Suspensions

	iron mineral	surf. area ($m^2\ g^{-1}$)	load ($g\ L^{-1}$)	red. extent (%)	red. rate[a] (10^{-8} mol $m^{-2}\ h^{-1}$)
Arnold et al. (*39*)	goethite	23	0.2	ND	52.7
	hematite	11.2	0.6	ND	22.5
Kostka and Nealson (*16*)	magnetite	4	3.7	17−42	2450
Roden and Zachara (*15*)	amorph. Fe(III)	600	0.1−1.1	44	1.36
	goethite	55.2	0.4−8.9	3	1.42
this study	smectite (SWa-1)	600	1.0	30−46	20−30
			7.5	9−14	1.25−1.53
	montmorillonite	800	1.0	74−90	ND[b]

[b] ND = not determined [a] Average derived from at least triplicate culture experiments.

structural Fe(III) can lead to dissolution of a substantial portion of the clay mineral.

Materials and Methods

Bacterial Cultures. *Shewanella putrefaciens* strain MR-1 was used. MR-1 was isolated from the anoxic sediments of Lake Oneida, NY (*25*), and has been the subject of many physiological and genetic studies concerning *S. putrefaciens* (*26*). MR-1 is a facultative anaerobe and an obligate-respiratory bacterium, incapable of fermentative growth (*27*).

Standard methods for the culture of anaerobic bacteria were used, which were modified for clay reduction experiments as in Kostka et al. (*17*). A minimal culture medium was prepared according to Kostka and Nealson (*28*). The minimal medium had the following base composition: 9.0 mM $(NH_4)_2SO_4$, 5.7 mM K_2HPO_4, 3.3 mM KH_2PO_4, 2.0 mM $NaHCO_3$, 1.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 67 μM Na_2EDTA, 57 μM H_3BO_3, 10 μM NaCl, 5.4 μM $FeSO_4$, 5.0 μM $CoSO_4$, 5.0 μM $Ni(NH_4)_2(SO_4)_2$, 3.9 μM Na_2MoO_4, 1.5 μM Na_2SeO_4, 1.3 μM $MnSO_4$, 1.0 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 20 mg L^{-1} arginine, 20 mg L^{-1} glutamate, and 20 mg L^{-1} serine. For the stoichiometry experiments, $NaHCO_3$ was deleted from the minimal medium. Carbon substrates were added at 2−8 mM, and organic ligands were added at 2−50 mM final concentration from sterile, anoxic stock solutions. Clay minerals (see next paragraph) were sterilized and added as the sole electron acceptor in the culture medium at 1.0−7.5 g L^{-1} final concentration. Culture medium was prepared and dispensed into serum bottles under strict anoxic conditions. 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 strict anoxic conditions within a Coy anaerobic chamber (90% N_2, 10% H_2). Inoculum cultures were grown aerobically to late log phase on the above medium. The initial cell density was $\sim 2 \times 10^8$ mL^{-1}, as estimated by the relationship between optical density (absorbance a 500 nm) and direct cell counts.

Heat-killed controls were heated by microwave radiation until boiling (*29*). For control cultures to test the reduction capacity of the supernatant, MR-1 was grown for 1 week with 1 g L^{-1} clay (SWa-1; see below) as the sole electron acceptor and 8 mM lactate as the carbon source. The culture medium was centrifuged (at 10000*g* for 10 min), and the supernatant was filtered successively through sterile 0.2 μm and 0.02 μm syringe filters. Sterile clay (SWa-1) was then reintroduced into the filter-sterilized supernatant, and the medium was incubated as above. After the 2 week period, an average of only 1.4% of the Fe present in smectite was reduced in duplicate treatments as compared to the 30−46% reduction observed in the presence of MR-1 cells under similar culture conditions (Table 1). Sterility of the supernatant was tested periodically using aerobic viable counts on agar plates in a nutrient-rich, Luria Broth medium. The pH of the medium (pH 7) was monitored and did not change upon reduction of Fe(III) clay minerals.

Clay Mineral Preparation. For all experiments with smectite clay, the 0.5−2 μm size fraction of the ferruginous smectite SWa-1 from Grant County, WA (Source Clays Repository, The Clay Minerals Society) was used. The clay was fractionated, dialyzed, and freeze-dried prior to use (*7*). Lear and Stucki (*8*) reported the structural Fe content of the same dialyzed SWa-1 to be 3.549 mmol Fe g^{-1} (with less than 0.1 mmol g^{-1} of this Fe present as Fe oxide impurities). Further, less than 0.2 mmol of Fe g^{-1} was liberated by a dilute HCl extraction from SWa-1. Upton montmorillonite (API no. 25, Ward's Natural Science Establishment) was prepared using identical methods to those described for SWa-1, and its Fe content (0.522 mmol Fe g^{-1}) was determined previously by Komadel and Stucki (*30*). Clay minerals were sterilized by heating via microwave radiation (*29*) before addition to the culture medium. Particle surface area was measured using the ethylene glycol-monoethyl ether (EGME) method of Carter et al. (*31*) as modified by Odom and Low (*32*).

Reactant/Product Measurements. The reduction of Fe in clay minerals was measured as the production of Fe(II) in HCl extracts using the colorimetric reagent, ferrozine, according to Lovley and Phillips (*19*) under strict anoxic conditions. This method was previously validated for use in clay cultures by comparison to HF extracts and Mossbauer spectroscopy (*17*). For fractionation experiments (Figure 4), the total amount of extractable, reduced Fe was measured as above. Dissolved Fe was measured as above after centrifuging culture samples and filtering the supernatant through a 0.2 μm syringe filter. Adsorbed Fe was extracted from centrifuged pellets of culture samples by resuspending in ferrozine buffer without acid extraction. Solid Fe bound in the clay structure was then calculated by difference from the total extractable Fe and the exchangeable Fe (dissolved + adsorbed) pools. Organic acids were measured by ion exclusion chromatography on a Dionex 500 ion chromatography system. Total dissolved inorganic carbon was measured by conductivity detection using flow injection analysis as in Hall and Aller (*33*).

Results

The extent of structural Fe reduction by MR-1 was observed to reach up to 46% in the smectite, SWa-1, and up to 90% in Upton montmorillonite after 14 days in anaerobic cultures (Table 1). More extensive experiments with SWa-1 showed that both the rate and extent of smectite reduction decreased at higher particle densities (Table 1). Physiological and stoichiometric experiments were carried out with SWa-1 because this clay mineral provided a higher Fe(III) concentration for use as an electron acceptor.

Stoichiometry of Clay-bound Fe(III) Respiration. Our hypothesis has been that *S. putrefaciens* strain MR-1 couples the dissimilatory reduction of structural Fe(III) in smectite to the oxidation of organic acids, formate and lactate, according to the following reactions:

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

$$0.73[Na_{0.81}(Si_{7.3}Al_{0.7})(Al_{1.06}Fe_{2.73}^{III}Mg_{0.26})O_{20}(OH)_4] + HCOO^- + H_2O \rightarrow 0.73[Na_{0.81}(Si_{7.3}Al_{0.7})(Al_{1.06}Fe_{2.73}^{II}Mg_{0.26})O_{20}(OH)_4]^{-2.73} + HCO_3^- + 2H^+ \quad (1)$$

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

$$1.47[Na_{0.81}(Si_{7.3}Al_{0.7})(Al_{1.06}Fe_{2.73}^{III}Mg_{0.26})O_{20}(OH)_4] + CH_3CHOHCOO^- + 2H_2O \rightarrow 1.47[Na_{0.81}(Si_{7.3}Al_{0.7})(Al_{1.06}Fe_{2.73}^{II}Mg_{0.26})O_{20}(OH)_4]^{-2.73} + CH_3COO^- + HCO_3^- + 5H^+ \quad (2)$$

According to eq 1, 2 mol of structural Fe(III) is reduced and 1 mol of HCO_3^- is produced for every 1 mol of formate oxidized. According to eq 2, 4 mol of structural Fe(III) is reduced for every 1 mol of lactate oxidized while 1 mol each of acetate and HCO_3^- is produced. This would be consistent with previous studies of *S. putrefaciens* under similar culture conditions in which the stoichiometry of dissimilatory Fe(III) reduction was documented (*34*).

In this study, we have measured structural Fe(III) reduction (Figures 1A and 2), total carbon dioxide production (Figures 1B and 2), and organic acid depletion (Table 2) in duplicate cultures with formate or lactate as the carbon source and clay as the sole electron acceptor. In four experiments, each containing duplicate treatments (for a total of eight cultures), a mean ratio (±1 standard deviation) of (1.6 ± 0.2):1 was observed for structural Fe(III) reduction coupled to formate oxidation, measured as the production of carbon dioxide. For the same number of cultures, a mean ratio of (4.9 ± 1.8):1 was observed for lactate oxidation, measured as carbon dioxide production, coupled to structural Fe(III) reduction. These ratios are near those predicted by the above balanced reactions. Neither C substrate was depleted, and little or no carbon dioxide production (and structural FeIII reduction) was observed in the absence of electron acceptor or in sterile controls (Figure 1).

In three of the above-mentioned culture experiments, organic acid measurements were made at the beginning and end of the incubation (Table 2). In duplicate cultures to which formate was added as the sole carbon source, it was shown that the amount of formate taken up by MR-1 cells was about twice that oxidized to carbon dioxide. In two separate experiments with lactate added as a carbon substrate, different results were obtained. With 1 mM lactate added as the sole carbon source, nearly equimolar amounts of lactate uptake and carbon dioxide production were observed [normalized to the Fe(III) reduced] while less acetate accumulated in the cultures relative to the amount of lactate oxidized. With 2 mM lactate and a small amount of yeast extract (0.02%) added, larger amounts of lactate utilization and acetate accumulation [normalized to the Fe(III) reduced] were found relative to carbon dioxide production. In the cultures with formate or lactate added as the "sole" carbon source (Table 2), the larger amounts of organic acid uptake observed (relative to the amount of carbon oxidized to CO_2) can be explained by significant incorporation of carbon into cell biomass. Similar to the ratio of Fe(III) reduced to lactate consumed observed in this study (2.5; Table 2), Roden and Zachara (*15*) observed a ratio of 2.8 for a closely related *Shewanella* strain with the crystalline Fe(III) oxide mineral, goethite, as the sole electron acceptor and lactate as the sole carbon source. In our cultures with a small amount of yeast extract added in addition to lactate, we suggest that fermentative metabolism, as shown to be coupled to Fe(III) reduction in *S. putrefaciens* by Lovley et al. (*34*), was stimulated by the extra carbon source, and this could explain the higher ratio of Fe reduction to lactate oxidation measured

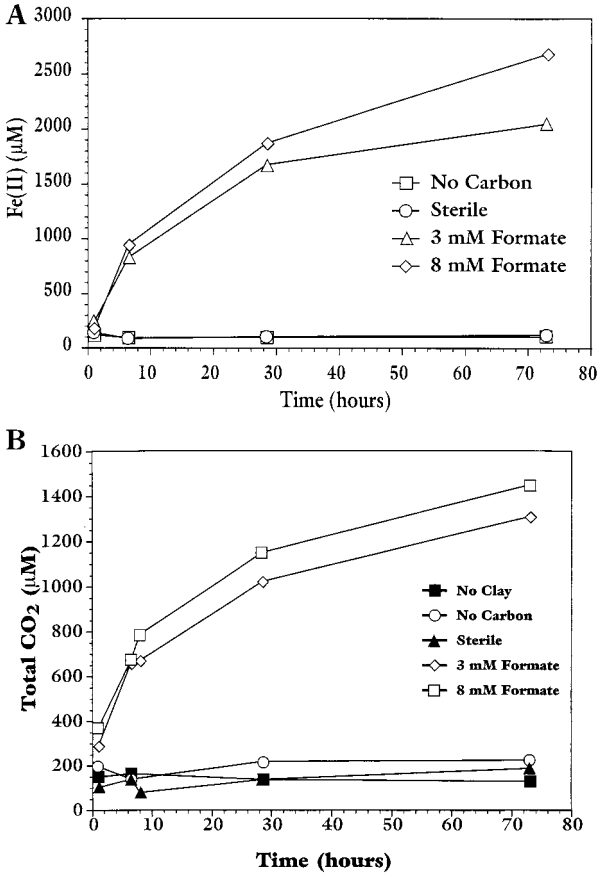

FIGURE 1. (A) Respiration of structural Fe(III) in smectite by MR-1 in a minimal medium with clay added as the sole electron acceptor and formate added as the sole electron donor. (B) Production of $\sum CO_2$ in the same duplicate MR-1 cultures. Smectite was added to 7.5 g L^{-1}. Each value is the average calculated from duplicate cultures and measurements ranged less than 5% from the average of duplicates.

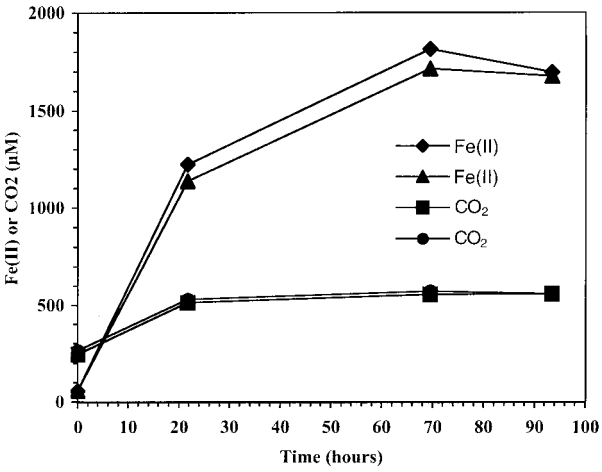

FIGURE 2. Respiration of structural Fe(III) and production of $\sum CO_2$ in the same duplicate MR-1 cultures in a minimal medium with clay added as the sole electron acceptor and lactate added as the sole electron donor. Smectite was added to 7.5 g L^{-1}. Each value is the average calculated from duplicate cultures and measurements ranged less than 5% from the average of duplicates. Control cultures showed similar results to those found in Figure 1.

TABLE 2. Stoichiometry of Carbon Oxidation Coupled to Structural Fe(III) Respiration in Smectite Clay by MR-1[a]

sole carbon source	reactant/product (μM)		Fe(III)/C ratio
2 mM formate	formate utilized	1986	1.0
	CO_2 produced	1309	1.6
	Fe(III) reduced	2047	
1 mM lactate	lactate utilized	877	2.5
	acetate produced	594	3.7
	CO_2 produced	863	2.6
	Fe(III) reduced	2204	
2 mM lactate	lactate utilized	2236	1.7
	acetate produced	2793	1.4
	CO_2 produced	592	6.4
	Fe(III) reduced	3792	

[a] Each value represents the difference between initial and terminal values and is the average of at least duplicate cultures. Culture measurements [organic acids, CO_2, Fe(II)] ranged less than 5% from the average as shown for Fe(II) and CO_2 in Figures 1 and 2.

TABLE 3. Reduction of Clay-Bound Fe(III) by MR-1 in a Minimal Culture Medium With and Without the Addition of Organic Ligands (to 2 mM final concentration)[a]

culture treatment	Fe(III) reduced (μM)
no ligand	1144
NTA + MR-1	2359
NTA, sterile	48
oxalic + MR-1	1557
oxalic, sterile	63
malate + MR-1	1309
malate, sterile	38

[a] Smectite was added to 2.0 g L^{-1}. Each value is the average from duplicate treatments incubated at 30 °C for 50 days. Culture measurements ranged less than 5% from the average of duplicates.

compared to that predicted from the balanced reaction. This suggestion is further supported by the higher amounts of acetate produced relative to lactate utilized in the cultures to which yeast extract was added as compared to cultures without yeast extract (Table 2).

Effect of Organic Ligands on Bacterial Clay Reduction. Organic ligands, some of which are commonly found in soils, were tested to determine their potential for increasing the availability of clay-bound Fe(III) as an electron acceptor for MR-1. In initial long-term cultures, oxalate and malate additions resulted in only minor increases in the extent of clay reduction relative to MR-1 cultures with no ligand added, and sterile controls showed little or no clay reduction (Table 3). In contrast, greater than twice the amount of clay-bound Fe(III) was reduced in cultures amended with nitrilotriacetic acid (NTA) (Table 3).

To further explore the effect of organic ligands on clay reduction, a range of organic ligand concentrations was tested and clay reduction was monitored over a shorter time period (Figure 3). Again, NTA treatments showed the largest extent of reduction (up to 50% greater than MR-1 cultures without ligand added) with smaller effects observed in citrate and malate treatments (14% over no ligand). Further evidence for the effects of organic ligands on clay reduction by MR-1 was observed when the Fe extracted from long-term cultures (Table 3) was fractionated into dissolved, adsorbed, and solid phases (Figure 4). With no ligand present, though, substantial Fe(III) reduction occurred, only 8% of the Fe reduced was observed in the dissolved and adsorbed fractions combined (Figure 4). With organic ligand present, 2359 μM of structural Fe in smectite (=37% of Fe in clay) was reduced and >50% of the total Fe extracted (1201 μM = 17% of structural Fe in clay) was found to be present in the dissolved/adsorbed phase, with nearly all of this Fe in the dissolved fraction. This

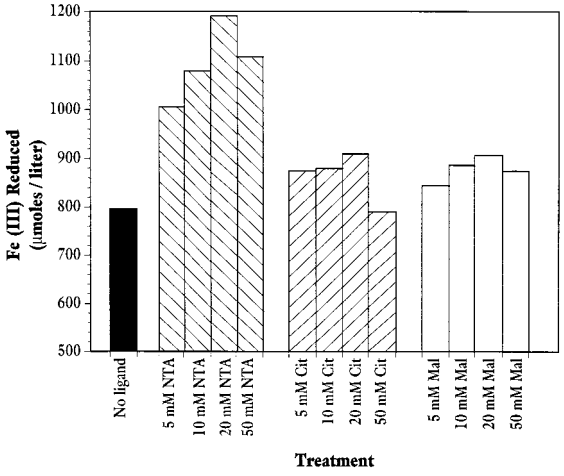

FIGURE 3. Reduction of clay-bound Fe(III) after 1 week in duplicate MR-1 cultures containing a range of organic ligand concentrations. Smectite was added to 2.0 g L^{-1}. Each value is the average calculated from at least duplicate cultures and measurements ranged less than 5% from the average of duplicates.

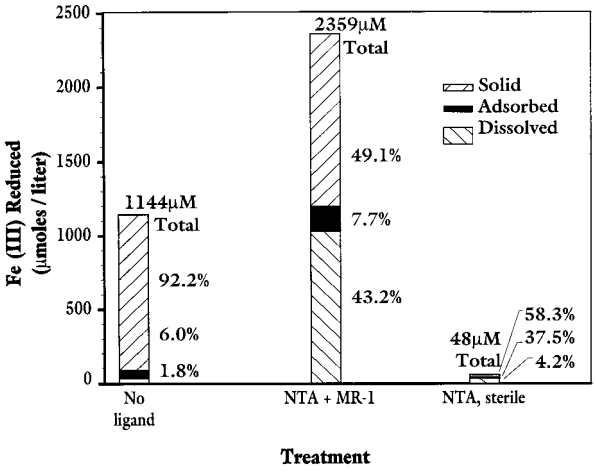

FIGURE 4. Partitioning of reduced Fe into dissolved, adsorbed, and solid-phase fractions in same duplicate MR-1 cultures represented in Table 3. Dissolved Fe is operationally defined as that which passes through a 0.2 μm filter; adsorbed Fe is that extracted with ferrozine buffer alone; solid-phase Fe is calculated by difference from the total Fe extracted in HCl. Each value is the average calculated from at least duplicate cultures and measurements ranged less than 5% from the average of duplicates.

dissolved Fe fraction is 17 times larger than the highest possible concentration of any Fe oxide impurities present in the clay suspension (see Materials and Methods). A similar result was found when fractionating extracted Fe from cultures amended with citrate or malate. In abiotic, sterile controls with the same concentrations of organic ligand added, little or no clay-bound Fe was reduced or dissolved relative to sterile no-ligand controls (Table 3; Figure 4).

Quantitative measurements of reductive dissolution were supported by absorbance measurements and visual observations. Clay suspensions were observed to clear upon reduction when organic ligand was added to the medium and the centrifuged pellets of clay were considerably smaller in ligand-amended treatments. Treatments to which no ligand was added turned green, indicating reduction but did not clear; whereas sterile controls showed no change in color or transparency of the clay suspension. Absorbance measurements of MR-1 cultures (same cultures as those in Figure 4) were >50% lower in treatments to which NTA was added ($A_{550nm} = 0.161 \pm 0.015$) as compared to killed controls (A_{550nm}

$= 0.410 + 0.016$) and MR-1 cultures with no ligand added ($A_{550nm} = 0.366 \pm 0.015$). Reduction by MR-1 occurred in the presence or absence of organic ligand, but dissolution only occurred when ligand was present.

The growth of MR-1 with smectite clay as the sole electron acceptor was tested in several experiments. Routine microbiological tests for growth proved difficult due to methodological problems. Using a simple Lowry extraction to measure protein biomass proved impossible because the smectite dissolved in 1 N NaOH rendering colorimetric methods such as Bradford (*35*) assay unreliable. Direct counts of bacterial cells using epiflourescence with acridine orange or DAPI stains were also inconclusive due to the clay particles (which are similar in size to the bacteria) obscuring the counts.

No direct evidence was observed for growth with smectite clay as the sole electron acceptor as yet. However, indirect evidence indicates respiration is coupled to growth. MR-1 cultures maintained viability (assessed by reduction of millimolar amounts of structural Fe in smectite) after more than five successive transfers with clay as the only electron acceptor added and lactate as a carbon source in a minimal medium. Since *S. putrefaciens* has been demonstrated to be incapable of fermentative growth (*27*), continued respiration of clay after succesive transfer implies growth.

Discussion

Bacterial Clay Mineral Reduction Coupled to Carbon Cycling. Many bacterial groups have been shown to utilize amorphous Fe(III) oxyhydroxide minerals either as a sole electron acceptor during anaerobic respiration (*34, 36*) or as a minor pathway for electron disposal during carbon metabolism (*18, 37, 38*). Two strains of the *Shewanella* group have been shown to couple respiration of crystalline Fe(III) oxides to energy generation and growth (*15, 16*). Here we provide the first and only example of studies which show that bacteria couple the rapid respiration of Fe(III) in clay minerals to organic carbon cycling. *S. putrefaciens* strain MR-1 was shown to reduce clay-bound Fe(III) and concomitantly oxidize millimolar amounts of organic acids in less than 1 day.

Our study has extended the list of crystalline Fe minerals that may be used by bacteria as an electron acceptor during carbon metabolism to include one of the most abundant mineral forms on earth, layered silicates. This discovery has important implications for Fe-rich agricultural soils and contaminated aquifers where crystalline Fe(III) has been shown to be an important oxidant.

Reduction of Clay Minerals vs Fe(III) Oxides. As mentioned above, the rate and extent of bacterial Fe mineral reduction is thought to depend heavily upon direct contact between the cell and mineral surfaces, mineral surface area, and crystallinity. We compare the reduction rates we observed in *Shewanella* cultures with clay minerals to other studies of Fe(III) oxide minerals which range in surface area and crystallinity (Table 1). The layered silicates studied contained surface areas similar to that of amorphous Fe(III) oxide, so it is not surprising that the extent of reduction as measured by the percent of Fe reduced was also similar. Goethite, a crystalline Fe(III) oxide with an order of magnitude smaller surface area, displayed a comparably lower extent of reduction by *Shewanella*. Since Fe(III) minerals of drastically different crystallinities (clay minerals and amorphous Fe oxide) gave such similar extents of reduction, mineral surface area appears to be much more important than crystallinity determining the amount of each Fe mineral which is available for reduction. The importance of mineral surface area in regulating bacterial clay reduction was further evident in the rates of reduction by *Shewanella*. When normalized to the surface area of Fe mineral present, the range of clay reduction rates overlapped with those observed for oxides, regardless of their crystal structure (Table 1).

As for Fe oxides, the bacterial reduction of clay minerals is clearly limited by particle surface area. However, other factors, such as particle aggregation or morphology are also important. The extent of bacterial clay reduction decreased at higher particle loading (Table 1), and normalized clay reduction rates were over an order of magnitude higher when nearly 10 times less clay was added (Table 1). Roden and Zachara (*15*) observed no rate change for goethite reduction over a large range of particle densities ($4-40$ g L^{-1}). At a comparable particle surface area, Arnold et al. (*39*) observed 50 times higher rates of goethite reduction at generally lower particle densities as compared to Roden and Zachara (*15*) (Table 1). Changes to particle aggregation brought on by particle loading may affect reduction rates as much or more than particle surface area or crystallinity. This same phenomenon, which may not be reflected in direct surface area measurements, was implicated as the cause for lower bacterial reduction rates observed previously with two-line ferrihydrite at high particle densities (*15*). In support of this interpretation, we observed that the culture medium became highly viscous in response to increased loading of smectite clays, resembling a semisolid agar at 7.5 g of smectite L^{-1}. It is suggested that increased viscosity represents a change in clay particle aggregation that somehow inhibits MR-1 cells from making optimum contact with the clay mineral surface. Particle morphology and uniformity in Fe mineral suspensions may also be an important factor regulating the kinetics of bacterial reduction. Alterations to particle aggregation in response to increased loading may not be an important factor in natural environments since clays will be present at lower concentrations.

Results for bacterial magnetite reduction did not follow with the interpretations given above for clay minerals and the remaining crystalline Fe oxides. Magnetite, a crystalline Fe oxide mineral with a mixed oxidation state and a low particle surface area, was reduced by *Shewanella* strains at a rate which was several orders of magnitude higher than all other Fe minerals studied to date (Table 1; ref *16*).

Clay Bioavailability and Reductive Dissolution in the Presence of Organic Ligands. Organic ligands were observed to increase the extent of microbial clay reduction (Table 3, Figure 3), whereas no significant reduction or dissolution was observed in control experiments with organic ligands alone. These data strongly suggest that organic ligands are capable of increasing the bioavailability of clay-bound Fe(III) for utilization as an electron acceptor. Further, organic ligands, especially strong chelators such as NTA, not only increase the amount of clay-bound Fe reduced, they also appear to catalyze the reductive dissolution of clay by bacteria (Figure 4). Our results concur with those of Lovley et al. (*40*), which observed that smectite reduction by the Fe-reducing bacterium, *Geobacter metallireducens*, was enhanced by a factor of $2-4$ in the presence of humic acid or a humic acid analogue.

Bacterial clay reduction in our model system appears to be stimulated by a different mechanism compared to that observed for Fe(III) oxide minerals. Previous pure culture studies observed enhanced bacterial reduction rates of Fe oxides in the presence of NTA (*24, 39*). Since NTA was shown to solubilize Fe(III) in soils and in cultures with synthetic oxides in the absence of seeded bacteria (*24*), it was concluded that solubilization of Fe(III) from the oxide surface was responsible for the enhanced Fe reduction rates. In contrast, we observed no solubilization of structural Fe(III) in smectite in the absence of bacteria (Figure 4), and the addition of organic ligand such as NTA to our cultures enhanced bacterial clay reduction rates to a smaller extent over the short term (Figure 3).

Several mechanisms other than the dissolution of Fe(III) could stimulate the extent of Fe(III) reduction in the presence of organic ligands. For example, organic chelates may remove Fe(II) from reactive sites in the mineral structure, thus making structural Fe(III) more available for reduction. Organic ligands could also provide a bridge or a shuttle for the transport of electrons to bound Fe(III) (*39*). We hypothesize that the former is operative during the anaerobic respiration of smectite coupled to carbon metabolism. It has been shown in studies with chemical reductants that as Fe(III) is reduced in the octahedral layer of smectites, dehydroxylation occurs rendering the layer structure less stable (*5*). Dissolution then may be abiotic and catalyzed after bacterial reduction by ligand enhanced leaching of Fe(II) from dehydroxylated sites in the octahedral sheet of smectite.

Many of the organic ligands employed in this study were chosen because of their common occurrence in soils. Oxalate, citrate, and malate are all important constituents of root exudates which have been detected routinely in a range of soil evironments at concentrations of up to 1 mM (*41*, *42*). These organic ligands have been shown to react with Fe oxide minerals and to affect metal solubility/speciation in soil (*41*, *43*). Further, Urrutia et al. (*44*) recently demonstrated that high concentrations of malate led to enhanced bacterial reduction of the crystalline Fe(III) oxide goethite through a mechanism analogous to that described above for clay mineral reduction. We have extended the database to show that naturally occurring organic compounds may make crystalline Fe(III) minerals such as layered silicates more available for dissimilatory reduction.

Potential Significance of Layered Silicates as an Electron Acceptor in the Environment. Crystalline Fe minerals have been shown to be abundant in agricultural soils (*18, 45*) and contaminated aquifers (*46, 47*), but the contribution of silicate minerals to soil biogeochemistry has so far not been characterized in detail. Crystalline Fe minerals have been operationally partitioned in soils and sediments as that fraction which is not easily extractable in HCl or oxalate, but may be extracted in dithionite-citrate buffer (*48*, *49*). In this study, we observed that only a small fraction of Fe ($4.5 \pm 1\%$) bound in the model layered silicate, SWa-1, was easily extractable in triplicate HCl treatments prior to exposure to bacteria, while $50.0 \pm 2.5\%$ of Fe bound in SWa-1 was extracted in triplicate dithionite treatments, close to the largest percentage of structural Fe(III) reduced in SWa-1 by *Shewanella* (46%; Table 1). Therefore, smectite, though operationally defined as part of the more recalcitrant crystalline Fe fraction, still may be rapidly reduced by bacteria. Further, the reactivity of smectite, as defined by its chemical extractability, is correlated to its availability for microbial reduction.

This research provides new, basic scientific data on a widespread biogeochemical process in soils, Fe(III) reduction, and on the microorganisms believed to mediate the process, the Fe(III)-reducing bacteria. Clay minerals (layered silicates) must be added to the list of Fe(III) forms which may be respired by Fe-reducing bacteria and coupled to the oxidation of organic carbon. Organic ligands were shown to increase the bioavailability of clay-bound Fe(III) and catalyze the reductive dissolution of clays in the presence of Fe-reducing bacteria. Together with environmental data, our observations of rapid bacterial clay reduction suggest that layered silicates comprise a significant fraction of crystalline Fe(III) minerals that may be used as an oxidant by bacteria in soils and sediments. These laboratory results should be utilized to direct a search for the significance of microbial clay reduction in the field with the goal of controlling the Fe oxidation state of clay minerals in situ for the benefit of environmental, agricultural, and engineering concerns.

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