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

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Smectite clay minerals are abundant in soils and sediments worldwide and are typically rich in Fe. While recent investigations have shown that the structural Fe(III) bound in clay minerals is reduced by microorganisms, previous studies have not tested growth with clay minerals as the sole electron acceptor. Here we have demonstrated that a pure culture of Shewanella oneidensis strain MR-1 as well as enrichment cultures of Fe(III)-reducing bacteria from rice paddy soil and subsurface sediments are capable of conserving energy for growth with the structural Fe(III) bound in smectite clay as the sole electron acceptor. Pure cultures of S. oneidensis were used for more detailed growth rate and yield experiments on various solid- and soluble-phase electron acceptors [smectite, Fe(III) oxyhydroxide FeOOH, Fe(III) citrate, and oxygen] in the same minimal medium. Growth was assessed as direct cell counts or as an increase in cell carbon (measured as particulate organic carbon). Cell counts showed that similar growth of S. oneidensis (108 cells ml−1) occurred with smectitic Fe(III) and on other Fe forms [amorphous Fe(III) oxyhydroxide, and Fe citrate] or oxygen as the electron acceptor. In contrast, cell yields of S. oneidensis measured as the increase in cell carbon were similar on all Fe forms tested while yields on oxygen were five times higher, in agreement with thermodynamic predictions. Over a range of particle loadings (0.5 to 4 g liter−1), the increase in cell number was highly correlated to the amount of structural Fe in smectite reduced. From phylogenetic analysis of the complete 16S rRNA gene sequences, a predominance of clones retrieved from the clay mineral-reducing enrichment cultures were most closely related to the low-G+C gram-positive members of the Bacteria (Clostridium and Desulfotobacterium) and the δ-Proteobacteria (members of the Geobacteraceae). Results indicate that growth with smectitic Fe(III) is similar in magnitude to that with Fe(III) oxide minerals and is dependent upon the mineral surface area available. Iron(III) bound in clay minerals should be considered an important electron acceptor supporting the growth of bacteria in soils or sedimentary environments.

Microbial Fe(III) reduction has been established as an important process catalyzing a large number of natural and contaminant biogeochemical cycles (21, 24, 29). However, no past studies have provided evidence for the respiration and growth on clay minerals as the sole electron acceptor. Further, it is unclear how respiration and growth on clay-bound Fe(III) compare to those on other Fe mineral forms.

In this study, we found that the respiration of structural Fe(III) bound in smectite clay minerals supports the growth of FeRB in pure culture and in enrichment cultures from two very different sedimentary environments. We also compared growth yields of a S. oneidensis strain MR-1 on a variety of Fe(III) forms with that on oxygen. Iron(III) oxide minerals are be-
lied to be more reactive or available for microbial reduction than clay minerals. In contrast, we show that the growth rate and yield of *S. oneidensis* on smectite appear to be similar to growth on amorphous Fe(III) oxyhydroxide. These discoveries have important implications for contaminated subsurface and surface aquatic environments, where Fe(III)-bearing clay minerals are abundant and at times comprise the predominant electron acceptor available to microorganisms.

**MATERIALS AND METHODS**

Bacterial cultures and cultivation methods. A pure culture of *Shewanella oneidensis* strain MR-1 was used which was isolated from the anoxic sediments of Lake Oneda, NY, and has been the subject of many physiological and genetic studies concerning the Shewanella (29). *S. oneidensis* is a facultative anaerobe and an obligately respiratory bacterium, incapable of fermentative growth (39).

Purified enrichment cultures consisted of FeRB consortia from two source inocula. Subsurface sediment samples were obtained from the saturated zone of unconsolidated alluvium (1 to 4 m below land surface) at the Field Research Center of the Department of Energy's Natural and Accelerated Bioremediation Research Program, Oak Ridge, Tenn. A second set of samples were collected at a 0.1-m depth in the surface soil of a rice paddy located in Nanjing, China. This rice field is flooded biannually, leading to prolonged anoxic conditions followed by alternating aerobic conditions between flood cycles. Inocula for enrichments were collected and transported chilled to the lab. Sample handling and cultivation procedures were carried out under anaerobic and strictly anaerobic conditions.

Enrichment cultures were purified by successive transfer using the culture medium and methods described below. The enrichments were selective for respiratory FeRB, as Fe(III) oxide was added as the sole electron acceptor and strictly anaerobic conditions were followed by alternating aerobic conditions between flood cycles. Inocula for enrichments were collected by taking cores or grab samples. All soil and sediment samples were collected and transported chilled to the lab. Sample handling and cultivation procedures were carried out under strict anaerobic conditions.

Preparation of oxidized Fe.

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

**Determination of reduction and growth.** The reduction of Fe(III) was measured as the production of reduced Fe in HCl extracts using the colorimetric reagent ferrozine under strictly anaerobic conditions (14, 23). This method was previously validated for use in clay cultures by comparison to HF extracts and Mossbauer spectroscopy (15, 16). Cell numbers were determined by direct counting using a Neubauer chamber and yield of *S. oneidensis* strain MR-1 over 3 days (Table 1). Nearly all of the Fe(III) citrate was reduced, compared to half of the Fe(III) oxyhydroxide (FeOOH) and approximately one-third of the structural Fe(III) bound in smectite. A white, ferrous carbonate precipitate was produced over time in Fe(III) citrate cultures, whereas a black magnetic

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

<table>
<thead>
<tr>
<th>Culture treatment</th>
<th>Conc of total cell carbon (μg liter⁻¹)</th>
<th>Conc of e-reduced (mM)</th>
<th>Growth (10⁷ cells ml⁻¹)</th>
<th>Growth per cell (10⁻¹⁰ g of C cell⁻¹)</th>
<th>Yield (g of C mol of e⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeOOH</td>
<td>1,600 ± 209</td>
<td>10.2</td>
<td>12.6 ± 0.3</td>
<td>1.3</td>
<td>0.169</td>
</tr>
<tr>
<td>FeOOH + AQDS</td>
<td>1,744 ± 244</td>
<td>24.0</td>
<td>14.7 ± 0.3</td>
<td>1.2</td>
<td>0.073</td>
</tr>
<tr>
<td>Smectite</td>
<td>2.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smectite + AQDS</td>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(III) citrate</td>
<td>10,885 ± 1,293</td>
<td>48.6</td>
<td>16.5 ± 0.7</td>
<td>6.6</td>
<td>0.198</td>
</tr>
<tr>
<td>O₂</td>
<td>25,490 ± 3,062</td>
<td>20.0</td>
<td>14.8 ± 0.2</td>
<td>17.2</td>
<td>1.29</td>
</tr>
</tbody>
</table>

* Each value is the mean ± 1 standard deviation or the average from the same triplicate cultures per treatment presented in Fig. 2.

**RESULTS**

The rapid reduction of soluble and solid Fe(III) forms was observed in cultures of *Shewanella oneidensis* strain MR-1 over 3 days (Table 1). Nearly all of the Fe(III) citrate was reduced, compared to half of the Fe(III) oxyhydroxide (FeOOH) and approximately one-third of the structural Fe(III) bound in smectite. A white, ferrous carbonate precipitate was produced over time in Fe(III) citrate cultures, whereas a black magnetic
precipitate, presumably magnetite, was formed in Fe(III) oxy-
hydroxide cultures. With smectite as the sole electron acceptor,
no solid form other than smectite was observed at the end of
the experiments.

Addition of the humic acid analog anthraquinone disulfon-
ate (AQDS) generally stimulated reduction of the FeOOH to
a larger extent than clay-bound Fe(III) (Table 1). Control
cultures (heat killed, exposed to HgCl2, or cultured aerobi-
cally) showed little or no reduction of clay-bound Fe(III) to
Fe(II). As has been demonstrated previously (16, 25), lactate
was depleted and carbon dioxide was produced according to
the 4:1 stoichiometry of Fe(III) reduced to carbon oxidized in
all cultures (data not shown).

Growth of *S. oneidensis*, measured by an increase in cell
number, paralleled the reduction of all Fe(III) forms tested
(Table 1; Fig. 1 and 2). Little or no growth was observed in
killed controls (with heat or HgCl2) or in control cultures to
which no electron acceptor had been added (Fig. 1). Interest-
ingly, the increase in cell number exhibited a similar range for
all electron acceptors tested (Table 1; Fig. 2). Within 3 days,
cell densities of *S. oneidensis* were observed to increase to a
range between $9.0 \times 10^7$ and $16.5 \times 10^7$ cells ml$^{-1}$. On the
third day, cultures were sacrificed for particulate organic car-
bon analysis and the yield within each culture was determined.
It was not possible to resolve the particulate organic carbon
yield in smectite cultures due to the presence of background
carbon impurities (data not shown). However, in contrast to
the increase in cell number, accumulation of *S. oneidensis*
biomass (measured as grams of C cell$^{-1}$) ranged over a factor
of 5 depending upon the electron acceptor utilized [O2, Fe
citrate, and Fe(III) oxyhydroxide] (Table 1).

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

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

![FIG. 1. Growth (A) and structural Fe(III) reduction (B) of *S. onei-
densis* strain MR-1 with lactate as the electron donor and smectite as
the electron acceptor. Results are expressed as means ± 1 standard
deviation from triplicate cultures. Symbols: open triangles, smectite
plus AQDS; solid circles, smectite; solid squares, no electron acceptor
added; open circles, heat-killed culture.](image1)

![FIG. 2. Growth of *S. oneidensis* strain MR-1 with lactate as the
electron donor in culture treatments which differ according to the
electron acceptor added. Results are expressed as the means ± 1
standard deviation from triplicate cultures. Symbols: open triangles,
Fe(III) citrate; open circles, O2; solid squares, FeOOH plus AQDS;
open squares, FeOOH; solid diamonds, smectite plus AQDS; open
diamonds, smectite.](image2)

![FIG. 3. Concentration of reduced structural Fe plotted against
growth (as cell number) for *S. oneidensis* strain MR-1 cultures with
lactate as the electron donor and where the particle load of smectite
was varied as the electron acceptor. Growth is expressed as means ±
1 standard deviation from triplicate cultures, while Fe(II) concentra-
tions are the averages from triplicate cultures.](image3)
DISCUSSION

Growth with smectite clay minerals as the sole electron acceptor. The growth of FeRB in culture has mostly been quantified by measuring the increase in cell number. Using the maximum cell number increase in comparison to control cultures, the range of growth measured for *S. oneidensis* coupled to smectite respiration reported here (1 x 10^8 to 2 x 10^8 cells ml^-1) is very similar to the range reported in previous studies for the growth of pure cultures of FeRB coupled to the respiration of synthetic Fe(III) oxide minerals (1 x 10^8 to 2 x 10^8 cells ml^-1) under similar culture conditions (7, 23, 25, 34). Our observation that microorganisms can gain energy for growth by catalyzing the respiration of smectite clay minerals is not surprising when the energy available from these reactions is considered (Table 2). The standard free energy at pH 7 that was
calculated to be available from lactate oxidation coupled to
smeectite reduction (Table 2, equation 1) is very similar to that
calculated to be available with Fe(III) oxyhydroxide or soluble
ferric citrate as the electron acceptor (Table 2, equations 2 and
3). Our findings suggest that a similar amount of energy for
growth is generated during Fe(III) reduction, regardless of the
form of Fe being utilized in cultures of S. oneidensis.

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

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

Growth with smectite as the sole electron acceptor was also
observed in Fe(III)-reducing consortia enriched from two very
different environments (contaminated subsurface sediment
and rice paddy soil) where Fe-rich clay minerals are abundant
and potentially important electron acceptors (41, 52). The 16S
rRNA gene sequences retrieved from these consortia were
dominated by those closely related to the δ-Proteobacteria
(Chloroflexiaceae) and low-G+C gram-positive members of the
Bacteria (Desulfitobacterium and Clostridium). Retrieval of
Geobacteraceae sequences is not unexpected, since these or-
organisms have been established as important members of FeRB
consortia in sediments (5, 37, 46). Detection of low-G+C
gram-positive members of FeRB consortia is more surprising
and intriguing. Members of the low-G+C Bacteria (Bacillus,
Desulfitobacterium, and Desulfovarcillum) been shown to be
able to reduce Fe(III) (6, 30, 31, 32, 45, 47). Gram-
positive organisms are thought to be more resistant to envi-
ronmental extremes such as desiccation in soils and sediments.

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

<table>
<thead>
<tr>
<th>Equation</th>
<th>Reactants</th>
<th>Products</th>
<th>Std G°a</th>
<th>kJ/reaction</th>
<th>kJ/mol of e⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>1.47[smectite]² + CH₃CHOHCOΟ⁻ + 2H₂O</td>
<td>1.47[smectite]²⁻ + CH₃CHOHCOΟ⁻ + HCO₃⁻ + 5H⁺</td>
<td>-436.09</td>
<td>-109.02</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12 Fe(OH)₃ + CH₃CHOHCOΟ⁻</td>
<td>4FeO₂⁻ + CH₃CHOHCOΟ⁻ + HCO₃⁻ + H⁺ + 18H₂O</td>
<td>-409.70</td>
<td>-102.42</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4Fe³⁺ + CH₃CHOHCOΟ⁻ + 2H₂O</td>
<td>4Fe²⁺ + CH₃CHOHCOΟ⁻ + HCO₃⁻ + 5H⁺</td>
<td>-460.34</td>
<td>-115.08</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>O₂ + CH₃CHOHCOΟ⁻</td>
<td>CH₃COO⁻ + HCO₃⁻ + H⁺</td>
<td>-478.28</td>
<td>-478.28</td>
<td></td>
</tr>
</tbody>
</table>

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

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

The chemical formula for smectite used in the calculations was Na₀.₁₁(Si₁.₇Al₀.₃)⁷[(Al₁₀⁻²₃Fe₀.₇₃Mg₀.²₃)O₂₀(OH)₆]⁷ (79).
suggested that the increase in biomass should be measured more often for these anaerobes. Given that previous perceptions on the growth of FeRB are heavily dependent upon direct counts in pure cultures, this observation could well revise our views on the energy obtained for growth via Fe(III) respiration. This conclusion is supported by the observation that both the predicted thermodynamic energy yield (Table 2) and the measured cell yield (Table 1) were higher by a factor of 5 when *S. oneidensis* cells were grown on oxygen than on various Fe(III) forms as the electron acceptor.

In agreement with the yield results (Table 1), growth rates of *S. oneidensis* calculated from the increase in cell number (Fig. 2) were higher for the soluble electron acceptors (0.489×10⁷ [O₂], and 0.520×10⁷ [Fe citrate] cells ml⁻¹ h⁻¹) in comparison to the solid electron acceptors (0.125×10⁷ [smectite] and 0.174 [FeOOH] cells ml⁻¹ h⁻¹) tested. Growth rates for smectite and FeOOH were similar to those observed in previous studies of *S. putrefaciens* growing on FeOOH (25). In contrast, our growth rates were 1 to 2 orders of magnitude higher than those observed in past studies of *Shewanella* strains growing on the crystalline Fe(III) oxide minerals goethite (36) and magnetite (13). It appears that the growth rate of *Shewanella* on smectite more closely resembles rates on poorly crystalline Fe(III) oxides (FeOOH) than those on crystalline Fe(III) oxides. We suggest that at least 20% of the Fe(III) bound in smectite is available as FeOOH for relatively rapid growth. However, the fact that the growth rate of *S. oneidensis* on smectite was 25% lower than that on FeOOH suggests that there is some metabolic cost associated with smectitic Fe(III) utilization.

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

Here we have demonstrated that FeRB, in a well-characterized pure culture and in purified enrichment cultures, can conserve energy for growth by coupling the reduction of structural Fe(III) bound in clay minerals to the oxidation of organic acids, lactate, or acetate. This is the first description of any organism capable of such metabolism. Given their abundance and ubiquity, Fe-containing clay minerals may be important and previously overlooked electron acceptors for the growth of bacteria in natural environments. Our results showing rapid bacterial growth via smectite respiration support past geochemical evidence (8, 9, 16, 17, 48) to indicate that a substantial portion (20 to 50%) of the Fe(III) bound in smectite is easily accessible to FeRB. Furthermore, our results show that the growth rate and yield on smectitic Fe(III) are comparable to those on poorly crystalline Fe(III) oxide minerals (FeOOH), which suggests that FeRB respire smectite in parallel with FeOOH. In other words, our data suggest that bacteria do not preferentially reduce Fe oxides over clay minerals. Microbial clay reduction may therefore be an important, but little-studied, process limiting natural and contaminant biogeochemical cycles.

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