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Lack of Production of Electron-Shuttling Compounds or Solubilization of Fe(III) during Reduction of Insoluble Fe(III) Oxide by Geobacter metallireducens

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Studies with the dissimilatory Fe(III)-reducing microorganism Geobacter metallireducens demonstrated that the common technique of separating Fe(III)-reducing microorganisms and Fe(III) oxides with semipermeable membranes in order to determine whether the Fe(III) reducers release electron-shuttling compounds and/or Fe(III) chelators is invalid. This raised doubts about the mechanisms for Fe(III) oxide reduction by this organism. However, several experimental approaches indicated that G. metallireducens does not release electron-shuttling compounds and does not significantly solubilize Fe(III) during Fe(III) oxide reduction. These results suggest that G. metallireducens directly reduces insoluble Fe(III) oxide.

The mechanisms by which pure cultures of dissimilatory Fe(III)-reducing microorganisms reduce insoluble Fe(III) oxides is of interest because this may provide insights into how Fe(III) oxides are microbially reduced in soils and sediments. It is generally considered that if Fe(III) chelators or electron-shuttling compounds are not added to the culture medium, then Fe(III)-reducing microorganisms must establish direct contact with Fe(III) oxides to reduce them (11). Circumstantial evidence consistent with the need for direct contact with Fe(III) oxide is the visual observation that Fe(III)-reducing microorganisms often seem to be attached to, or at least closely associated with, Fe(III) oxides (1, 14, 22). The only experimental evidence that has appeared to directly support the need for direct contact between Fe(III)-reducing microorganisms and Fe(III) oxides has come from studies in which the Fe(III) reducers are physically separated from the Fe(III) oxides by semipermeable membranes (1, 3, 14, 15, 20, 22). Fe(III) reduction is invariably inhibited. This result has been interpreted as an indication that pure cultures of Fe(III) reducers do not release compounds that can solubilize Fe(III) or act as electron shuttles between Fe(III) reducers and Fe(III) oxides. The assumption has been that if Fe(III) reducers did release such compounds, then the compounds would diffuse through the membranes, permitting Fe(III) reduction without direct contact. However, this basic assumption does not appear to have been directly tested by incorporating electron-shuttling compounds or Fe(III) chelators as positive controls.

This potential shortcoming in previous studies with semipermeable membranes is significant in view of several recent studies that have suggested that direct contact between pure cultures of Fe(III) reducers and Fe(III) oxides is not necessary for Fe(III) oxide reduction. Selection for cells of Shewanella alga that did not attach to Fe(III) oxide did not reduce the rate of Fe(III) reduction, suggesting that attachment was not essential for Fe(III) reduction (4). Another study suggested that Geobacter sulfurreducens could reduce Fe(III) oxide by releasing an extracellular cytochrome that could act as an electron shuttle, thus eliminating the need for contact with the Fe(III) oxide (21). Therefore, the validity of the semipermeable-membrane approach was investigated further.

Potential for electron shuttling through semipermeable materials. Unless otherwise specified, G. metallireducens was grown in freshwater medium with acetate (20 mM) as the electron donor and poorly crystalline Fe(III) oxide (100 mmol/liter) as the electron acceptor under an atmosphere of N2-CO2 (80:20) (14). In order to evaluate the potential for electron shuttling through dialysis tubing, the Fe(III) oxide was entrapped in Spectra/Per dialysis tubing (molecular weight cutoff of 300,000; 18-cm length by 1-cm diameter), the tubing was rinsed and added to anaerobic sterile medium, and the culture vessels were aseptically flushed with N2-CO2 for 20 min for 3 consecutive days to ensure that all of the oxygen was removed. This dialysis tubing was chosen because it had the largest nominal pore sizes that were readily available and preliminary studies with tubing with smaller pore sizes gave comparable results.

When a 5% inoculum of G. metallireducens that had been grown on acetate-Fe(III) oxide medium was added to medium in which the Fe(III) oxide was free, G. metallireducens grew in and reduced the Fe(III), as evidenced by an accumulation of HCl-extractable Fe(II) over time (Fig. 1), as previously reported (14). In contrast, when the poorly crystalline Fe(III) oxide was retained within the dialysis membrane, little Fe(II) was detected outside the membrane. This was true even immediately after the membranes were ruptured with a needle to release the iron within (Fig. 1). However, once the Fe(III) was released, it was readily reduced over time (Fig. 1). This demonstrated that the lack of Fe(III) reduction in the presence of the membrane was not due to a toxic effect of the membrane on the organism.

The addition of anthraquinone-2,6-disulfonate (AODS; 50 μM) to the medium stimulated the reduction of the free Fe(III) oxide by G. metallireducens (Fig. 1), as expected from previous studies which have demonstrated that AODS serves as an electron shuttle between G. metallireducens and Fe(III) oxide (12, 13). The unexpected result was that the addition of AODS did not result in significant reduction of the Fe(III) oxide within the membrane, as evidenced by the fact that Fe(II) did not accumulate over time until after the membrane had been ruptured (Fig. 2). The membrane was ruptured to release the Fe(III), the Fe(III) was readily reduced. The finding that...
AQDS did not promote the reduction of Fe(III) in the dialysis membrane in this manner indicates that separation of Fe(III) oxide and Fe(III)-reducing microorganisms with a semipermeable membrane may not be an appropriate method for evaluating whether Fe(III)-reducing microorganisms release electron-shuttling compounds in order to reduce Fe(III).

In order to evaluate other potential separation mechanisms to test for the release of electron-shuttling compounds, poorly crystalline Fe(III) oxide was incorporated into microporous alginate beads with a nominal molecular mass cutoff of 12 kDa (5). The beads (diameter, 5 mm) were prepared as previously described (2, 5): except that Fe(III) oxide (500 mmol/liter) was added prior to the polymerization step. The amount of Fe(III) exposed on the surface of the beads was calculated to be 10% of the total Fe(III) added, based on the sizes of the beads and pores and the structure, shape, and size of the cross-linked alginate polymer. Beads were added to acetate-freshwater medium in order to provide Fe(III) at 50 mmol/liter. The production of Fe(II) was determined with ferrozine (14) after the beads had been extracted for 12 h in 0.5 N HCl.

G. metallireducens could only reduce less than 5 mmol of the added Fe(III) per liter (Fig. 2), which corresponds to the amount of the Fe(III) oxide that is calculated to be exposed on the surface of the beads. However, when 50 μM AQDS was included in the medium, nearly all of the Fe(III) in the beads was reduced. These results suggest that G. metallireducens reduced the AQDS to AHQDS, which then diffused into the bead, reducing the Fe(III) and regenerating AQDS. Each AQDS molecule must have been reduced multiple times, as the amount of Fe(III) reduced was ca. 500-fold higher than the electron-accepting capacity of the added AQDS.

These results indicate that incorporation of Fe(III) into microporous beads is a suitable strategy to test for the release of low-molecular-weight electron-shuttling compounds. The finding that G. metallireducens did not reduce the Fe(III) oxide within beads in the absence of AQDS suggests that G. metallireducens does not release low-molecular-weight electron-shuttling compounds in order to promote Fe(III) oxide reduction.

Alternative test for electron-shuttling compounds. The microporous beads would not be suitable for testing for the presence of high-molecular-weight electron-shuttling compounds and might be inappropriate for some compounds that might bind or otherwise react with the beads in unknown ways. As an alternative test for electron-shuttling compounds, the ability of culture filtrate of G. metallireducens to stimulate reduction of Fe(III) oxide by a washed cell suspension of G. metallireducens was evaluated. G. metallireducens was grown on poorly crystalline Fe(III) oxide (100 mmol/liter) and acetate (20 mM) in freshwater medium (14). Cells were removed via filtration (0.2 μm). This filtrate was oxidized by stirring for 1 h, diluted in various proportions with freshwater medium, and made anaerobic. Acetate (10 mM), Fe(III) oxide (10 mmol/liter), and a washed cell suspension of G. metallireducens were added. Over time, subsamples were taken anaerobically for analysis of HCl-
 extractable Fe(II). Filtrates of the culture did not stimulate the reduction of Fe(III) over the rate that was observed in fresh medium (Fig. 3). This contrasted with a significant stimulation of Fe(III) oxide reduction when only 10 μM AQDS was added. These results further suggest that *G. metallireducens* does not release an extracellular electron-shuttling compound in order to reduce Fe(III) oxides, in accordance with other recent studies (10).

**Evaluation of the presence of Fe(III)-chelating compounds.** Previous studies have demonstrated that the addition of 4 mM nitrilotriacetic acid (NTA) solubilizes Fe(III) from Fe(III) oxide and that this stimulates Fe(III) reduction by *G. metallireducens* (17, 18). However, the addition of NTA did not promote the reduction of Fe(III) within the semipermeable membrane (Fig. 1). As in the other studies, the Fe(III) was reduced once it was released from within the membrane.

In order to determine if incorporation of Fe(III) oxide into microporous beads would be a suitable strategy to test for the presence of Fe(III)-chelating compounds, NTA (4 mM) was added to cultures in which Fe(III) oxide was incorporated into the microporous beads described above. However, in contrast to the results with AQDS, the addition of NTA did not significantly stimulate Fe(III) reduction (K. Nevin, unpublished data). Studies in which the Fe(III)-containing beads were incubated in medium with NTA in the absence of cells demonstrated that the chelator only solubilized Fe(III) from the beads at a rate of approximately 10 μM/day. This rate of Fe(III) solubilization was apparently too slow to sustain the growth and activity of *G. metallireducens*. Thus, incorporation of Fe(III) oxide into microporous beads does not appear to be a suitable strategy for testing for the release of chelating agents by Fe(III)-reducing microorganisms.

As an alternative way to evaluate the possibility that *G. metallireducens* might solubilize Fe(III) from Fe(III) oxides prior to Fe(III) reduction, soluble Fe(III) in cultures of *G. metallireducens* was measured with ion chromatography in a anaerobic chamber. All supplies were placed in an anaerobic chamber for 24 h before use. The eluent was bubbled with nitrogen for 1 h and vacuum degassed for 20 min before use in analysis. Culture filtrates (0.2-μm-diameter pore size) were injected into a Dionex DX-500 Ion chromatograph, and Fe(II) and Fe(III) were separated on a Dionex IonPac CSSA column (7).

The standard culture medium for the growth of *G. metallireducens* (14) has an NTA-containing trace metal mixture that adds 59 μM NTA to the medium. Uninoculated medium that contained the trace metal mixture contained 1 to 3 μM soluble Fe(III), whereas no soluble Fe(III) was detected in medium to which the trace metal mixture had not been added. In order to avoid the apparent solubilization of Fe(III) by the NTA in the trace metal mixture, *G. metallireducens* was grown in a medium in which the NTA-containing trace metal mixture was replaced with a trace metal mixture with NTA omitted. The growth of *G. metallireducens* was not adversely affected by the lack of NTA in the trace metal mixture. Attempts to grow *S. alga* strain BrY (3) or *Geothrix fermentens* (6) in NTA-free medium with Fe(III) oxide as the electron acceptor were unsuccessful, although both grew in NTA-free medium with fumarate as the electron acceptor. This suggests that, unlike *G. metallireducens*, these two organisms required the presence of NTA, possibly to solubilize Fe(III), to grow on Fe(III) oxide.

During the growth of *G. metallireducens* in NTA-free medium, the accumulation of HCl-extractable total Fe(II) over time (Fig. 4A) was accompanied by an increase in soluble Fe(II) (Fig. 4B) for the first 3 days. After 3 days, soluble Fe(II) decreased even though the total Fe(II) increased over this period. The decline in soluble Fe(II) is attributed to increased formation of Fe(II) minerals such as siderite and magnetite, as previously reported (14, 16). The increase in dissolved Fe(II) was accompanied by an apparent increase in soluble Fe(III), which then decreased in parallel with the decrease in soluble

![Diagram](http://aem.asm.org/Downloaded_from)
Fe(II) (Fig. 4C). However, when the measurements of soluble Fe(II) and Fe(III) from this and similar experiments were plotted together, it was found that the measurements of soluble Fe(III) were highly correlated ($r^2 = 0.94$) with the measurements of soluble Fe(II) (Fig. 4D). When Fe(II) was added to uninoculated culture medium, there was also an increase in the measured dissolved Fe(III) that followed a similar pattern. These results suggest that the measurements of dissolved Fe(III) in the cultures are an artifact of the analysis. If Fe(III) was being solubilized as the result of $G.\ metalireducens$ releasing an Fe(III) chelator, it would not be expected that there should be such a consistent covariance of dissolved Fe(II) and Fe(III) concentrations. Furthermore, this would not explain the measurement of dissolved Fe(III) when Fe(II) was added to uninoculated medium. It may be that Fe(II) can form some type of defined, soluble complex with Fe(III), but we are unaware of any such soluble Fe(II)-Fe(III) complexes. A more likely explanation is that, despite the care taken to maintain anaerobic conditions and the fact that there was no evidence for on-column oxidation of Fe(II) standards, there was a small consistent oxidation of Fe(II) during the analysis or a less than perfect separation of Fe(II) and Fe(III) such that a very small fraction of the Fe(II) gave a secondary peak with a retention time similar to that of Fe(III). In any event, the strong covariance of measured dissolved Fe(III) with Fe(II) indicates that it is unlikely that Fe(III) is actually solubilized as the result of the growth and activity of $G.\ metalireducens$.

Implications for mechanisms for Fe(III) reduction. The results suggest that $G.\ metalireducens$ does not reduce poorly crystalline Fe(III) oxide by producing soluble reductants or electron-shuttling compounds or by solubilizing Fe(III) prior to reduction. This is the same conclusion that was previously reached on the basis of studies with semipermeable membranes. However, as shown here, such studies with semipermeable membranes are not suitable for testing for the presence of electron-shuttling compounds or Fe(III) chelators because $G.\ metalireducens$ did not reduce Fe(III) within the semipermeable membrane, even when an electron shuttle or chelator was added to the cultures.

The results do suggest that incorporation of Fe(III) oxide into alginate beads provides a suitable test for evaluating the release of electron-shuttling compounds. One limitation of this approach is that the nominal molecular mass cutoff of the beads (12 kDa) would restrict the movement of large electron-shuttling compounds. However, if there are Fe(III)-reducing microorganisms that do release electron-shuttling compounds, it seems probable that the electron-shuttling compounds would not be large. The larger the molecule, the greater the energy cost for synthesis. Thus, it would be expected that the most competitive Fe(III)-reducing microorganisms would make electron-shuttling compounds of low molecular weight, if they produced any shuttles at all.

The lack of evidence for the release of electron-shuttling or Fe(III)-chelating compounds suggests that $G.\ metalireducens$ directly transfers electrons to the surface of insoluble Fe(III) oxides. Both $G.\ metalireducens$ (9) and $G.\ sulfurreducens$ (8, 19) have membrane-bound Fe(III) reductase activities, and a cytochrome in the outer membrane of $G.\ sulfurreducens$ has recently been implicated in electron transfer to insoluble Fe(III) oxides (J. R. Lloyd and D. R. Lovley, unpublished data). The results presented here suggest that further studies on the mechanisms for electron transfer from the membrane-bound Fe(III) reductase components to the Fe(III) oxide surface are warranted.

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REFERENCES