Reduction of Soluble and Insoluble Iron Forms by Membrane Fractions of *Shewanella oneidensis* Grown under Aerobic and Anaerobic Conditions

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The effect of iron substrates and growth conditions on in vitro dissipatory iron reduction by membrane fractions of *Shewanella oneidensis* MR-1 was characterized. Membrane fractions were separated by sucrose density gradients from cultures grown with O₂, fumarate, and aqueous ferric citrate as the terminal electron acceptor. Marker enzyme assays and two-dimensional gel electrophoresis demonstrated the high degree of separation between the outer and cytosolic membrane. Protein expression pattern was similar between chelated iron- and fumarate-grown cultures, but dissimilar for oxygen-grown cultures. Formate-dependent ferric reductase activity was assayed with citrate-Fe³⁺, ferrozine-Fe³⁺, and insoluble goethite as electron acceptors. No activity was detected in aerobic cultures. For fumarate and chelated iron-grown cells, the specific activity for the reduction of soluble iron was highest in the cytosolic membrane. The reduction of ferrozine-Fe³⁺ was greater than the reduction of citrate-Fe³⁺. With goethite, the specific activity was highest in the total membrane fraction (containing both cytosolic and outer membrane), indicating participation of the outer membrane components in electron flow. Heme protein content and specific activity for iron reduction was highest with chelated iron-grown cultures with no heme proteins in aerobically grown membrane fractions. Western blots showed that CymA, a heme protein involved in iron reduction, expression was also higher in iron-grown cultures compared to fumarate- or aerobic-grown cultures. To study these processes, it is important to use cultures grown with chelated Fe³⁺ as the electron acceptor and to assay ferric reductase activity using goethite as the substrate.

In the absence of molecular oxygen, bacteria can use a variety of terminal electron acceptors for respiration. Many bacterial species have been identified that utilize insoluble metal oxides for respiration. Two such microorganisms that have received much attention are *Shewanella oneidensis* and *Geobacter sulfurreducens* (27). The genomes of both microbes have been sequenced. *S. oneidensis* MR-1, the focus of the present study, is highly versatile in its use of terminal electron acceptors (70). Acceptors include oxygen, fumarate, nitrate, nitrite, trimethylene N-oxide, dimethyl sulfoxide, sulfite, thiosulfate, and elemental sulfur, as well as solid mineral oxides including hydrous ferric oxide, goethite, hematite, and manganese oxide (32, 41, 70) and Fe(III), Mn(IV,III), Cr(VI), and U(VI) (4, 12, 26, 35, 41, 71).

The ability of *Shewanella* to utilize iron oxide as the terminal electron acceptor, a process referred to as dissipatory iron reduction (DIR), has been extensively studied. Due to the ease of genetic manipulation of *Shewanella*, the genes involved in DIR have been identified. These genes encode cytosolic membrane (CM), periplasmic, and outer membrane (OM) proteins, as expected for the inferred path of direct electron transfer from the cytoplasm to an insoluble extracellular substrate (7, 52, 59). Biochemical studies on DIR by *Shewanella* are complicated by the large number of proteins involved (58), the difficulty in separating the CM from the OM (36, 37), the variety of media used for the growth of *Shewanella* (7, 11, 34–36, 41, 43, 54), and the different methods used for monitoring ferric reductase activity (11, 36). Myers and Myers (36) separated the CM and the OM from *S. oneidensis* MR-1 cultures grown anaerobically with fumarate as the terminal electron acceptor. To assay for iron reduction, these workers used ferrozine-chelated ferric citrate (ferrozine-Fe³⁺) and formate or NADH as the reductant. Although the formate dehydrogenase (FDH) and NADH oxidase are CM localized, these researchers found their associated ferric reductase activity in the OM fraction. Dobbin et al. (11) studied the total membrane (TM) fraction from *Shewanella putrefaciens* grown aerobiocly. In contrast to the work of Myers and Myers, who found no ferric reductase activity in aerobically grown cells, these workers were able to demonstrate the reduction of chelated-Fe³⁺ aqueous species with formate as the electron donor. Finally, Beliaev et al. (7) used fumarate as the terminal electron acceptor to grow mutants of *S. oneidensis* MR-1. These authors, too, studied DIR by using the TM fraction, formate as the electron donor, and ferrozine-Fe³⁺ as the electron acceptor. In summary, although all of these researchers investigated the in vitro reduction of Fe³⁺, none grew cultures with Fe³⁺ as the terminal electron acceptor for protein isolation.

A number of different electron donors and acceptors have been used to grow cultures when investigating ferric reduction. Electron acceptors include ferric citrate (20, 35), fumarate (33, 36, 40), and even molecular oxygen (4, 11, 16, 24–26, 53–55, 68,
69, 72). Electron donors (and the carbon source) used for growth include tryptic soy broth, lactate (as the donor and carbon source), and H₂ (with malate as the carbon source) (10, 17, 25, 69). Although the effect of growth conditions has not been thoroughly examined, studies have indicated medium-dependent differences in protein expression (9, 15, 43).

In the present study, we demonstrate the importance of growing cultures with Fe³⁺ in an investigation of the role of CM and OM proteins in DIR. Growth conditions control the expression of OM and CM proteins. We first establish the degree of separation of CM and OM proteins by two-dimensional (2-D) gels and by marker enzyme assays. Ferric reduction assays were then performed on the membrane fractions using three different forms of Fe³⁺: aqueous citrate-Fe³⁺, aqueous ferrozine-Fe³⁺, and insoluble goethite (57). Importantly, our results also show that when goethite is used as the electron acceptor, kinetic properties are observed to be distinct from those observed for soluble forms of iron substrates. Thus, experiments utilizing an insoluble substrate must be completed to understand DIR in the absence of chelating ligands.

MATERIALS AND METHODS

Organism and growth conditions. Cultures of S. oneidensis MR-1 (ATCC 700550) were grown in defined medium as per Myers and Nealson (41) with the following modifications: 30 mM tri-lactate, 4 mM sodium phosphate, and 10 mM HEPES (pH 7.4) with 50 mM ferri citrate or 25 mM fumarate in 4-liter flasks under N₂ at 30°C. The medium used for aerobic and anaerobic cultures was identical except that oxygen, fumarate, or ferric citrate were substituted as the terminal electron acceptors as noted. Aerobic cultures were shaken (1 liter in 2.8-liter Bellco flasks) at 250 rpm at 30°C. Anaerobic cultures were stationary. Inocula for aerobic and anaerobic cultures were grown in Luria broth (shaken overnight at 24°C) and then centrifuged at 10,000 × g for 10 min at 4°C. The pellet was washed with 0.7% NaCl in 10 mM HEPES (pH 7.4) and then suspended in one-tenth the volume of buffer. Cultures were inoculated at 10⁶ cells/ml. Aerobic cultures were harvested when the absorbance at 600 nm was 0.6. Anaerobic iron-grown cultures were harvested at mid-log phase when the aqueous Fe²⁺ concentration reached 35 mM as measured with ferrozine (65). Fumarate cultures were harvested at mid-log phase when the absorbance at 600 nm was 0.15.

Membrane isolation and characterization. TM, CM, and OM fractions were isolated by using the EDTA-Brij-lysozyme method and sucrose density gradients (38) with the following modifications: cells were harvested (10,000 × g for 10 min at 4°C) and were suspended at 1 g of wet cells per 24 ml of 25% sucrose in Tris-Cl (pH 8.0) by using a glass homogenizer. Anaerobic cultures were homogenized with gentle shaking for 15 min, each of the following was added: (i) a one-tenth volume of lysosome (0.04 mg/ml [final]), (ii) a one-tenth volume of EDTA (5 mM final), (iii) 0.3% (wt/vol) Brij 58 (final) from a 5% stock, and (iv) 12 mM MgCl₂ (final) from a 1 M stock followed by the addition of a few crystals of DNP₆. Separation of the membrane fractions was performed on a sucrose density gradient from 30 to 55% (wt/wt). These modifications yielded improved separation of the CM and OM fractions and resulted in little or no intermediate fraction. The CM and OM fractions were visible as red bands in the sucrose gradient, and these corresponded to the predicted densities in the sucrose fractions. After centrifugation, the OM and CM pellets were stored in 20% glycerol in 10 mM HEPES (pH 7.5) at −80°C. Membrane fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (22). SDS-PAGE (12%) gels were visualized by Coomassie blue R250 or heme staining (31).

Enzyme assays. Iron oxide reduction assays with formate as the electron donor were performed according to the method of Ruebush et al. (57) in an anaerobic chamber under a N₂ atmosphere. Iron oxide reactions were conducted at room temperature and contained 0.1 mg of membrane protein and 4.5 mg of goethite/ml in 100 mM HEPES (pH 7.0) and were initiated by the addition of 10 mM sodium formate. At specified times, 100-μl aliquots of the membrane-membrane reaction mixtures were mixed with 33 μl of 2 N HCl (12, 28). The addition of HCl arrested the enzymatic reaction, stabilized the aqueous Fe²⁺, and solubilized the Fe³⁺ formed during DIR adsorbed to goethite (26). After acidification, samples were centrifuged to separate the mineral phase from the liquid (16,000 × g relative centrifugal force, 1 min). A 50-μl aliquot of the supernatant was then added to 950 μl of ferrozine (1 g/liter) in 100 mM HEPES (pH 7.0). The absorbance was measured as an ε₅₆₂₅ nm of 27.9 mM⁻¹ cm⁻¹ (65). To determine whether our assay measured total Fe³⁺ released by reduction (i.e., aqueous plus adsorbed Fe³⁺), samples from the enzymatic reaction were assayed immediately after HCl addition, while another set was incubated in the HCl for 22 h (a comparable length of time used to extract adsorbed Fe³⁺ with HCl after reduction). The Fe²⁺ content was identical irrespective of the length of the HCl extraction period.

The reduction of ferrozine-Fe³⁺ was measured by continuously monitoring the increase in absorbance at 562 nm according to the method of Myers and Myers (39). Reaction mixtures contained 1.2 mM citrate-Fe³⁺, 2 mM ferrozine, and 100 mM HEPES (pH 7.0) at room temperature, and the reactions were initiated by the addition of 10 mM sodium formate. Reaction mixtures for the reduction of citrate-Fe³⁺ (without ferrozine present in the reaction) contained 5 mM citrate-Fe³⁺ and 0.1 mg of membrane protein/ml in 100 mM HEPES (pH 7.0) at room temperature, and reactions were initiated by the addition of 10 mM sodium formate. Aliquots of 100 μl were removed at 5-min intervals. These samples were mixed with 33 μl of 2 N HCl. After acidification, 50 μl was added to 950 μl of ferrozine (1 g/liter) in 100 mM HEPES (pH 7.0). The absorbance was measured at 562 nm.

FDH activity was measured by the reduction of benzyl viologen by formate under N₂ (1). Reaction mixtures contained 10 mM sodium formate, 0.005 mg of TM/ml, 0.3 mM benzyl viologen, and 100 mM β-mercaptoethanol in 50 mM Tris-Cl (pH 8.0) at room temperature. Reactions were monitored at 555 nm (ε = 12 mM⁻¹ cm⁻¹). The activity is expressed as moles of formate oxidized rather than benzyl viologen reduced (1). The succinate dehydrogenase (SDH) activity was measured by the determining the reduction of dichloro-indolphenol (DCIP) at 600 nm (ε = 13 mM⁻¹ cm⁻¹) (2) at room temperature. The NADH oxidase activity was measured by monitoring the decrease in absorbance at 340 nm associated with aerobic oxidation of NADH (49) at room temperature (ε = 6.22 mM⁻¹ cm⁻¹).

2-D gel electrophoresis and protein identification. Membrane proteins were solubilized in 7 M urea, 2 M thiourea, 2% CHAPS [3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate], 1% Triton X-100, 18 mM dithiothreitol, and 280 mM β-mercaptoethanol. Isoelectric focusing (IEF; 0.1 mg of protein per gel strip) was performed with a LBK Multiphore II system and run for 30,000 V · h. A second-dimension SDS–10% PAGE was used to resolve the IEF-separated proteins. Coomassie blue R250-stained gels were analyzed with PDQuest software from Bio-Rad.

Purification of proteins. OM protein OmcA was purified according to the method of Myers and Myers (40) from S. oneidensis MR-1. CymA, MtrB, and OmcB were heterologously expressed in BL21(DE3)/pET9a and BL21(DE3)/pET15b. FDH activity was measured with the use of the formaconitase assay and both 1- and 2-D electrophoretic techniques. In the following discussion of the degree of separation of membranes, we also emphasize observed differences in membranes harvested from aerobic and anaerobic cultures.

Marker enzyme assays. The TM was isolated from triplicate cultures grown with O₂, fumarate, or chelated iron as the terminal electron acceptor. Enzyme activities of the TM fractions were analyzed prior to separation by sucrose density gradient ultracentrifugation, as were the resultant CM and OM
from the separation (Table 1 and Fig. 1). The specific activities of CM-associated marker enzymes such as NADH oxidase should increase in the CM relative to the TM. In the iron-grown cultures, the specific activity of NADH oxidase in the CM was more than four times greater than that measured in the TM (Fig. 1A and Table 1) and eight times greater than in the OM. Similar results were obtained with fumarate-grown cultures, where the CM/OM ratio of the specific activity was 9.5 (Fig. 1B and Table 1). For aerobically grown cultures, this ratio (CM/OM) was 11 (Table 1).

SDH activity, another marker enzyme for the CM, was also measured (Table 1). The SDH activity was too low in the anaerobic cultures for accurate assessment. In aerobic cultures, the ratio of SDH specific activity of CM to OM was 8.

These results document a high degree of separation of the CM and OM in our preparations.

The yield of the intermediate membrane (IM) fraction, observed by Myers and Myers (38), decreased as our preparations increased in degree of separation between the OM and CM. The IM fraction, which resembled mostly the OM but also contained CM components (38), could be visualized on the sucrose density gradient slightly above the OM band. Upon modifying the separation procedure (see Materials and Methods), which increased the CM and OM separation, most if not all of our preparation did not contain an IM fraction.

**SDS-PAGE of membrane proteins.** The TM, OM, and CM from oxygen-grown, chelated iron-grown, and fumarate-grown cultures were subjected to SDS-PAGE and visualized by Coomassie blue (Fig. 2A) and heme staining (Fig. 2B). Both the Coomassie blue and the heme staining revealed the high degree of separation of the CM and OM, as well as the effect of the growth medium on protein expression. The Coomassie blue visualization indicated that the proteins expressed in fumarate- and chelated iron-grown cultures were very similar. In contrast, proteins expressed in aerobic cultures shared very few expressed proteins (Fig. 2A). This difference was even more apparent with the heme-stained gel (Fig. 2B), where no heme bands were detected in the aerobically grown cultures. In contrast, heme proteins of 85, 75, and 35 kDa were visualized in the OM fraction of anaerobically grown cultures. The very low levels of these heme proteins detected in the CM fraction are consistent with a high degree of separation between the CM and OM fractions. A 20-kDa heme protein, with a molecular mass similar to that of CymA (34), was visualized in the CM fraction of anaerobically grown cultures. In summary, the pattern of membrane protein expression appeared similar between iron- and fumarate-grown cultures, but the amount of membrane-associated heme proteins was lower in the latter.

**TABLE 1. Marker enzyme assays of CM and OM fractions**

<table>
<thead>
<tr>
<th>Electron acceptor and membrane fraction</th>
<th>Activity (µmol/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Fe³⁺ citrate</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>0.012</td>
</tr>
<tr>
<td>CM</td>
<td>ND</td>
</tr>
<tr>
<td>OM</td>
<td>ND</td>
</tr>
<tr>
<td>Fumarate</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>0.009</td>
</tr>
<tr>
<td>CM</td>
<td>ND</td>
</tr>
<tr>
<td>OM</td>
<td>ND</td>
</tr>
<tr>
<td>Oxygen</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>0.014</td>
</tr>
<tr>
<td>CM</td>
<td>0.083</td>
</tr>
<tr>
<td>OM</td>
<td>0.009</td>
</tr>
</tbody>
</table>

a The terminal electron acceptor used for culture growth is indicated.
b Mean, mean value of assays from three replicate cultures; ND, no activity was detected.

**FIG. 1.** Bar graph plot of ferric reductase, NADH oxidase, and FDH of the TM, CM, and OM (data from Tables 1 and 4). NADH oxidase, FDH, and ferric reductase activity (µmol of Fe³⁺/min/mg of protein) was measured by using TM, CM, and OM isolated from iron-grown (A) or fumarate-grown (B) cultures. The FDH activities for both panels have been divided by 10 in order to plot them on the same scale (e.g., the actual values from the FDHs of panel A are 3.3, 6.6, and 0.69 µmol/min/mg of protein for TM, CM, and OM, respectively). The ferric reductase activity was measured with three different iron substrates: ferrozine-Fe³⁺ (FZ-Fe³⁺), ferric citrate (C-Fe³⁺), and goethite. The results show the means and standard deviations from three independent culture preparations. Note the differences in scale between panels A and B.
Detection of proteins implicated in DIR in the TM. To affirm that the heme proteins detected shown in Fig. 2B, are involved in DIR, we performed Western blot analysis. Antibodies specific for the proteins that have been identified to function in DIR were used. OM proteins OmcA, OmcB, and MtrB, CM protein CymA, and periplasmic protein MtrA were detected in the TM fraction (Fig. 3). This indicates that the TM fraction contains electron carriers that span the CM to the OM and includes components of the periplasmic fraction.

2-D gel electrophoresis. 2-D gel electrophoresis also shows that the CM and OM fractions were well separated, in addition to showing differences due to growth conditions (Fig. 4). Proteins were identified by peptide mass fingerprinting using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Table 2). From the 2-D gel of the CM from iron-grown cultures, well-characterized CM proteins ATPase subunits α and β, quinone-reactive Ni/Fe hydrogenase subunit, and nitrate-inducible formate dehydrogenase subunit H were identified. The well-characterized OM proteins MtrB (6), TolC (62), and porin were identified by MALDI-TOF. Interestingly, the OM heme proteins OmcA and OmcB were not identified in the 2-D gels. However, their presence in the OM was confirmed by the Western blots (Fig. 3). Their detection in 2-D gels may be problematic since other proteomic studies using 2-D gel electrophoresis also failed to identify these proteins (8, 14, 66). As a final method to determine the degree of separation between the CM and OM, protein spots from the 2-D gels were scanned, and the intensity was measured. This was done with triplicate gels each from a different preparation of the OM and CM (Table 3 and Fig. 5). The values listed in Table 3 are the average spot densities of the proteins of interest normalized by the total density of the gel. In agreement with the marker enzyme measurements showing a high degree of separation between the OM and CM, the ratio (OM/CM) of spot densities for selected OM proteins was always five or greater. As a trend, more OM proteins were found in the CM than CM proteins found in the OM fraction.

Iron substrates and partitioning of reductase activity in fumarate-grown cells. After we established the degree of separation of the CM and OM fractions, we examined how the ferric reductase activity of the TM partitioned into the CM and OM from fumarate-grown cultures. The ferric reductase activity was measured with the different iron forms (ferrozine-chelated Fe$^{3+}$/H$_{10}$$^{11}$001, citrate-chelated Fe$^{3+}$/H$_{11001}$, and insoluble goethite). With ferrozine-Fe$^{3+}$, the ferric reductase specific activity of the CM from fumarate-grown cultures was four times greater than the specific activity of the TM (Fig. 1 and Table 4). No activity could be detected in the OM fractions. With citrate-Fe$^{3+}$, the ferric reductase specific activity of the CM was again greater than that of the TM (Fig. 1 and Table 4). Again, little if any activity was observed in the OM.

With goethite as the iron substrate, a different pattern of activity partitioning was observed (Fig. 1 and Table 4). The specific activity of the CM was now lower than the activity of the TM. Again, little or no activity was observed in isolated OM.

Effect of growth conditions on ferric reductase activity. The experiments described above with fumarate-grown cultures were also performed with chelated iron- and aerobically grown cultures. With membrane fractions from iron-grown cultures, the iron-reductase activity was much higher (compare Fig. 1A versus B, where the scales of the y axes differ; see also Table 4).
With ferrozine-Fe$^{3+}$ as the substrate, the specific activity of the TM from iron-grown cultures was more than seven times higher than that from fumarate-grown cultures. Again, the CM exhibited the highest specific activity with little or no activity in the OM.

Similar trends were observed when citrate-Fe$^{3+}$/H11001 was used in the assay. As with the TM from fumarate-grown cultures, we observed slightly lower TM activity with the citrate-Fe$^{3+}$ as the substrate compared to ferrozine-Fe$^{3+}$ and goethite. Of significance and similar to that observed with the fumarate-grown cultures, the highest specific activity using goethite as the electron acceptor was in the TM and not the CM fraction.

No formate-dependent ferric reductase activity was observed in TM, OM, or CM from aerobic cultures using ferrozine-Fe$^{3+}$, citrate-Fe$^{3+}$, or goethite as substrates in any of the membrane fractions.

**Kinetic analysis of CM and OM iron reduction.** The results summarized in Fig. 1 suggest that components of both the CM and the OM (the TM) catalyze the maximal rate of goethite reduction. The activities of these two membrane fractions were further examined by using ferric citrate and goethite as substrates (Fig. 6). For ferric citrate reduction, the kinetic values obtained with the CM yielded a higher $V_{\text{max}}$ and $K_m$ than the TM (Fig. 6A). With the TM, $K_m$ and $V_{\text{max}}$ values of 0.17 mM...
and 0.94 μmol Fe(II)/min/mg of TM protein, respectively, were calculated (Fig. 6A). With the CM, $K_m$ and $V_{max}$ values of 0.50 mM and 3.34 μmol Fe(II)/min/mg of CM protein, respectively, were calculated (Fig. 6A). For goethite, the TM fraction yielded a $K_m$ of 53.6 mM and a $V_{max}$ of 0.19 μmol Fe(II)/min/mg of TM protein (Fig. 6B). For CM-catalyzed reduction of goethite, a much higher $K_m$ value of 238 mM was determined and also a slightly higher $V_{max}$ value of 0.31 μmol of Fe(II)/min/mg of CM protein (Fig. 6B).

Because the specific activity of FDH was also determined in the TM and the CM fraction, the rate of ferric citrate and ferric chelates, it would not be surprising to find that many if not all of the multiheme proteins of OM and CM isolated from iron-grown cultures. No expression of CymA was seen in TM fractions prepared from aerobically grown cells (Fig. 7).

**DISCUSSION**

**Physiologically relevant iron reduction.** Studies over the past 15 years have identified and localized proteins involved in DIR by *Shewanella*: OM proteins MtrB (6), OmcA (40), and OmcB (7); periplasmic proteins MtrA (7) and CctA (15); and CM protein CymA (34). OmcA, OmcB, and MtrA are decaheme proteins, whereas CymA and CctA are tetraheme proteins. Only a limited number of studies have attempted to determine the path of electron transfer to the terminal ferric reductase of DIR (3, 7, 11, 36). Central to identifying the terminal ferric reductase is the ability to distinguish between the physiologically relevant ferric reductase activity of the OM compared to iron reductase activity that maybe associated with the CM for other purposes. This is important since many oxidoreductases whose physiological function is not to reduce iron have been shown to possess fortuitous iron reduction activity (19, 67). Due to the relative ease which cytochromes reduce ferric chelates, it would not be surprising to find that many if not all of the multiheme proteins of DIR have ferric reductase activity. The use of a mineral oxide for the ferric substrate may help to distinguish between the relevant and fortuitous activities.

Until our most recent study with goethite (57), all in vitro studies of *Shewanella* components have used chelated forms of Fe(II). Although easier to work with experimentally, chelated forms of iron, due to variability in size, charge, and structure, may also yield artifacts for in vitro studies. Their smaller sizes...
relative to mineral oxides may allow them to gain access to enzyme active sites that may not be accessible to insoluble mineral oxides. Activity due to soluble ferric chelates may be exacerbated by in vitro separation of cellular components that expose enzymes that may never contact extracellular iron forms in vivo. For example, CM-associated ferric reductase activity cannot be attributed to the physiologically relevant terminal reductase on the OM surface that makes contact with mineral oxides since the OM is a barrier between the goethite and any CM enzymes in vivo. The ability to ascertain the difference between the multiple ferric reductase activities exhibited in an in vitro system would prove useful. As our results show in the next section, the use of goethite as the ferric substrate minimizes the contribution of chelated ferric reductase activity observed in vitro.

**Role of CM-localized FDH in iron reduction.** Scott and Nealson (61) discovered that formate is a central intermediate in the anaerobic metabolism of pyruvate by *Shewanella*. Formate is oxidized by CM-localized FDH, an entry point for electrons of respiration. In the present study, marker enzyme assays and 2-D gels show that our CM and OM preparations are highly separated. Similar to the findings of Myers and Myers (36), the FDH activity copurified with NADH oxidase, a CM-associated marker enzyme (1, 18, 21, 64). Thus, our OM preparations contain little FDH activity (Table 1) and, accordingly, the OM contains little if any formate-dependent ferric reductase activity.

However, our results on in vitro formate-dependent iron reductase activity is in contrast to those of Myers and Myers (36). Whereas we could detect ferric reductase activity in membrane fractions containing CM proteins (TM and CM), Myers and Myers (36) found that the formate-dependent reduction of

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**TABLE 4. Ferric reductase activities for membrane fractions isolated from cultures grown with different terminal electron acceptors**

<table>
<thead>
<tr>
<th>Electron acceptor and membrane fraction</th>
<th>Ferrozine-Fe$_{3+}$</th>
<th>Fe$_{3+}$ citrate</th>
<th>Goethite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (μmol/min/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>σ</td>
<td>Mean</td>
</tr>
<tr>
<td>Fe$_{3+}$ citrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>0.26</td>
<td>0.062</td>
<td>0.22</td>
</tr>
<tr>
<td>CM</td>
<td>0.60</td>
<td>0.165</td>
<td>0.57</td>
</tr>
<tr>
<td>OM</td>
<td>0.01</td>
<td>0.003</td>
<td>0.02</td>
</tr>
<tr>
<td>Fumarate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>0.04</td>
<td>0.001</td>
<td>0.09</td>
</tr>
<tr>
<td>CM</td>
<td>0.16</td>
<td>0.013</td>
<td>0.23</td>
</tr>
<tr>
<td>OM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Oxygen</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CM</td>
<td>ND</td>
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</tr>
<tr>
<td>OM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* The terminal electron acceptor used for culture growth is indicated.  
*b* The reaction mixture contained ferrozine, allowing continued monitoring of Fe$_{3+}$ formation.  
*c* The Fe$_{2+}$ concentration was measured after acidification by using ferrozine.  
*d* Mean, mean value of assays from three replicate cultures.  
*e* ND, no activity detected.
ferrozine-Fe$^{3+}$/H$_{11001}$ partitioned evenly between the CM and OM. Because the OM contained much more protein than the CM, these authors argued that the OM contained most of the total ferrozine-Fe$^{3+}$/H$_{11001}$ reductase activity (36). Thus, for formate to support reduction of ferrozine-Fe$^{3+}$/H$_{11001}$ in the OM, their OM fraction must have contained contaminating FDH-containing CM. This is not surprising since we have found that total separation of the CM and OM is difficult if not impossible by sucrose density gradient ultracentrifugation. Differences between that study and ours is presumed to reflect the higher degree of separation in our OM and CM fractions.

Reduction of soluble Fe$^{3+}$. Our previous study (57) showed that CM-localized FDH alone cannot reduce soluble or insoluble iron. Thus, other electron carriers are involved in chelated Fe$^{3+}$ reduction. The increase in ferric reductase specific activity of the OM over that of the TM (Fig. 1) for chelated Fe$^{3+}$ in the OM, their OM fraction must have contained contaminating FDH-containing CM. This is not surprising since we have found that total separation of the CM and OM is difficult if not impossible by sucrose density gradient ultracentrifugation. Differences between that study and ours is presumed to reflect the higher degree of separation in our OM and CM fractions.

In support of a mechanism involving soluble iron forms, researchers have noted organically solubilized iron in solutions of cultures of *Shewanella* and *Geothrix* spp. (46, 47). Other researchers have argued that the soluble factor is an electron shuttling compound and not a chelator (48). The results recently by Lies et al. (23) suggest the presence of at least two distinct mechanisms for the reduction of solid phase versus organically chelated Fe$^{3+}$. Consistent with this mechanism, our results show that solubilized iron can be reduced by oxidoreductases in the CM or periplasmic space.
Conclusions. Our results show for the first time the requirement of both the CM and the OM for maximal rates of reduction of a mineral oxide. These results also demonstrate using highly purified OM and CM that no formate-dependent ferric reductase activity is localized predominantly in the OM as reported previously (36). Our results clearly show that the CM-associated ferric reductase activity is highest with soluble forms of iron, which will only be physiologically relevant when ferric chelates are available. Finally, our results underscore the importance of the iron substrate for assays and medium used for growth for studying DIR. We demonstrate that the mechanism of goethite reduction is different from soluble iron reduction. Seemingly conflicting observations in the literature may therefore be the result of differences in growth conditions or ferric substrate.

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