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 dissimilatory 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, trimethylamine 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, 42, 71).

The ability of *Shewanella* to utilize iron oxide as the terminal electron acceptor, a process referred to as dissimilatory 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 aerobically. 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,

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69, 72). Electron donors (and the carbon source) used for growth include tryptic soy broth, lactate (as the donor and carbon source), and H_2 (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 Fe^{3+} 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 reductase assays were then performed on the membrane fractions using three different forms of Fe^{3+} : aqueous citrate- Fe^{3+} , aqueous ferrozine- Fe^{3+} , 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 D,L-lactate, 4 mM sodium phosphate, and 10 mM HEPES (pH 7.4) with 50 mM ferric citrate or 25 mM fumarate in 4-liter flasks under N_2 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 Belloco 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 21°C) and then centrifuged at $10,000 \times g$ 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 original volume of buffer. Cultures were inoculated at 10^6 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 Fe^{2+} 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 ($10,000 \times g$ 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. At room temperature with gentle shaking for 15 min, each of the following was added: (i) a one-tenth volume of lysozyme (0.64 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 $MgCl_2$ (final) from a 1 M stock followed by the addition of a few crystals of DNase I. 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 intermediary 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 $-80^\circ C$. 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 N_2 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- μ l aliquots of the mineral-membrane reaction mixtures were mixed with 33 μ l of 2 N HCl (12, 28). The addition of HCl arrested the enzymatic reaction, stabilized the aqueous Fe^{2+} , and solubilized the Fe^{2+} formed during DIR adsorbed to goethite (26). After acidification, samples were centrifuged to separate the mineral phase from the liquid

($16,000 \times g$ relative centrifugal force, 1 min). A 50- μ l aliquot of the supernatant was then added to 950 μ l of ferrozine (1 g/liter) in 100 mM HEPES (pH 7.0). The absorbance was measured as an $\epsilon_{562\text{ nm}}$ of $27.9\text{ mM}^{-1}\text{ cm}^{-1}$ (65). To determine whether our assay measured total Fe^{2+} released by reduction (i.e., aqueous plus adsorbed Fe^{2+}), 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 Fe^{2+} with HCl after reduction). The Fe^{2+} content was identical irrespective of the length of the HCl extraction period.

The reduction of ferrozine- Fe^{3+} was measured by continuously monitoring the increase in absorbance at 562 nm according to the method of Myers and Myers (36). Reaction mixtures contained 1.2 mM citrate- Fe^{3+} , 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- Fe^{3+} (without ferrozine present in the reaction) contained 5 mM citrate- Fe^{3+} 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 μ l were removed at 5-min intervals. These samples were mixed with 33 μ l of 2 N HCl. After acidification, 50 μ l was added to 950 μ l 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 N_2 (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 ($\epsilon = 12\text{ mM}^{-1}\text{ cm}^{-1}$). 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 ($\epsilon = 13\text{ mM}^{-1}\text{ cm}^{-1}$) (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 ($\epsilon = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$).

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 LKB Multiphore II system and run for 30,000 V $\cdot$ 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)/pLysS using 100 μ g of ampicillin and 34 μ g of chloramphenicol/ml as selection markers. MtrA was cloned into the pET21a expression vector and heterologously expressed in BL21(DE3)/pEC86 (5). MtrA was expressed and isolated according to the method of Pitts et al. (51), followed by His tag purification.

Western blot analysis. Membrane fractions from *S. oneidensis* MR-1 were resolved by SDS-PAGE under denaturing conditions (22). Western blots were prepared with antibodies heterologously expressed in New Zealand White rabbits by using a 77-day protocol (Covance, Inc.). The cross-reactivity of antibody preparations was eliminated by affinity purification (30).

RESULTS

To determine the role of OM and CM proteins in DIR, we first determined the degree of separation between the CM and the OM. Conventional sucrose density ultracentrifugation was used, and the degree of separation of the membrane fractions was determined by marker enzyme assays 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 O_2 , 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

TABLE 1. Marker enzyme assays of CM and OM fractions

Electron acceptor and membrane fraction ^a	Activity ($\mu\text{mol}/\text{min}/\text{mg}$ of protein) ^b					
	Succinate dehydrogenase		NADH oxidase		Formate dehydrogenase	
	Mean	σ	Mean	σ	Mean	σ
Fe ³⁺ citrate						
TM	0.012	0.0008	0.055	0.003	3.28	0.38
CM	ND		0.255	0.028	6.50	0.19
OM	ND		0.031	0.001	0.69	0.12
Fumarate						
TM	0.009	0.001	0.034	0.005	0.407	0.010
CM	ND		0.153	0.001	2.068	0.051
OM	ND		0.016	0.002	0.251	0.038
Oxygen						
TM	0.014	0.007	0.060	0.011	0.207	0.048
CM	0.083	0.009	0.254	0.059	0.445	0.130
OM	0.009	0.002	0.023	0.003	0.029	0.006

^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.

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.

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 $\text{Fe}^{2+}/\text{min}/\text{mg}$ 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 $\mu\text{mol}/\text{min}/\text{mg}$ of protein for TM, CM, and OM, respectively). The ferric reductase activity was measured with three different iron substrates: ferrozine- Fe^{3+} (FZ- Fe^{3+}), ferric citrate (C- Fe^{3+}), 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.

FIG. 2. SDS-PAGE profiles of membrane protein fractions from cultures grown with Fe^{3+} (Fe), fumarate (Fu), or oxygen (O_2) as the terminal electron acceptor. (Gel A) TM, OM, and CM proteins were subjected to SDS-PAGE, and the gel was stained with Coomassie blue R250. (Gel B) A gel loaded with the same samples as in gel A was stained for heme proteins with benzidine HCl. Each lane contained 20 μg of protein on a 12% acrylamide gel.

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 detec-

tion 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+} , citrate-chelated Fe^{3+} , 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).

FIG. 3. Western blot analyses of TM proteins with antibodies raised to OmcA (A), OmcB (B), MtrB (C), MtrA (D), and CymA (E). Cultures were grown with ferric citrate as the terminal electron acceptor. Panels A to D show gels loaded with 4 μg of TM protein, whereas panel E shows a gel containing 8 μg of TM protein.

FIG. 4. 2-D gel images of OM and CM protein fractions from cultures grown on different terminal electron acceptors. The gels shown are representative of three independent culture preparations from either oxygen-, iron-, or fumarate-grown cultures. (A) Oxygen-grown OM; (B) oxygen-grown CM; (C) Fe^{3+} citrate-grown OM; (D) Fe^{3+} -grown CM; (E) fumarate-grown OM; (F) fumarate-grown CM. The protein load for each gel was 0.1 mg.

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+} 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_{max} and K_m than the TM (Fig. 6A). With the TM, K_m and V_{max} values of 0.17 mM

TABLE 2. Proteins identified from 2-D gels of the OM and CM fractions from cultures grown on iron and fumarate as terminal electron acceptors

Gel of membrane fraction	Annotation	Mol wt ^a	pI ^b	Gene coverage ^c	TIGR locus ^d
Fe OM	MtrB	77,678	4.39	9 (5)	so1776
	TonB-dependent receptor	91,959	4.17	9 (6)	so2427
	Outer membrane porin, putative	39,898	4.47	33 (12)	so3896
	TolC	47,678	5.19	16 (6)	so3904
	Conserved hypothetical	24,958	4.58	3 (16)	so1824
	TonB-dependent receptor	95,762	4.31	9 (6)	so2907
	TonB-dependent receptor	97,761	4.25	14 (8)	so2469
	TonB-dependent receptor	71,558	4.53	26 (7)	so0815
	Bacterial surface antigen	92,518	4.67	22 (8)	so1637
	Outer membrane protein	74,498	4.60	7 (3)	so1428
Fumarate OM	TonB-dependent receptor	90,371	4.59	18 (8)	so1482
	OmpA family protein	40,182	4.54	21 (5)	so3545
	Heme transport	76,371	4.54	21 (10)	so3669
	Ferric alcaligin	77,997	4.51	15 (6)	so3033
	siderophore receptor				
	Peptidase M13 family	76,199	5.30	18 (7)	so0429
	Long-chain fatty acid transport protein	47,064	4.33	14 (3)	so3099
Fe CM	Formate acetyltransferase	84,861	5.58	9 (5)	so2912
	ATPase F1, β -subunit	49,774	4.62	24 (6)	so4747
	Quinone-reactive Ni/Fe hydrogenase, large subunit	62,346	6.17	14 (6)	so2098
	ATPase F1, α -subunit	55,228	5.12	33 (14)	so4749
	E1 pyruvate dehydrogenase	99,781	5.29	10 (8)	so0424

^a That is, the predicted molecular weight from the gene sequence.^b pI, the predicted isoelectric point from the gene sequence.^c Percentage of gene covered by peptides (number of peptides detected for the match).^d That is, the gene locus as given by The Institute for Genomic Research (www.tigr.org).

and 0.94 $\mu\text{mol Fe}^{2+}/\text{min/mg}$ of TM protein, respectively, were calculated (Fig. 6A). With the CM, K_m and $V_{\max}$ values of 0.50 mM and 3.34 $\mu\text{mol Fe}^{2+}/\text{min/mg}$ 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 $\mu\text{mol Fe}^{2+}/\text{min/mg}$ 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 $\mu\text{mol Fe}^{2+}/\text{min/mg}$ 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 goethite reduction, rather than being expressed per mg of protein, can also be expressed per amount of FDH activity. Here, the FDH activity is given as units (micromoles of product formed per minute) (Fig. 6C and D). For both goethite and ferric citrate, the activity was higher with TM than CM when calculated as per unit of FDH activity. The increase in TM over CM is much greater with goethite than with ferric citrate.

Expression analysis of heme protein CymA. The lower ferric reductase activity of fumarate-grown cultures is correlated with lower expression levels for heme proteins (Fig. 2). Western blot techniques were used to determine whether these heme proteins are the same as those involved in DIR. A candidate protein for the 20-kDa heme protein detected in the CM of anaerobically grown cells is CymA, which has been shown to be involved in anaerobic respiration (34, 45, 59, 60). With anti-

TABLE 3. Intensity of selected protein spots from 2-D gels of the OM and CM isolated from iron-grown cultures

Annotation	Mean spot density $\pm$ SD ^a		OM/CM ratio
	OM	CM	
OM proteins			
MtrB	24.6 $\pm$ 6.9	2.9 $\pm$ 1.7	8.3
TonB-dependent receptor	52.1 $\pm$ 17.8	3.3 $\pm$ 2.5	15.7
Outer membrane porin, putative	69.9 $\pm$ 9.5	11.9 $\pm$ 7.0	5.9
TolC	7.7 $\pm$ 1.5	1.1 $\pm$ 1.0	7.0
Long-chain fatty acid transport protein	7.1 $\pm$ 0.4	0.9 $\pm$ 0.9	7.9
CM proteins			
ATPase F1, α -subunit	3.7 $\pm$ 2.9	16.2 $\pm$ 10.3	0.2
ATPase F1, β -subunit	3.0 $\pm$ 2.0	11.4 $\pm$ 5.5	0.3
Formate dehydrogenase FdnH	4.5 $\pm$ 5.1	18.8 $\pm$ 9.3	0.2
Formate acyltransferase	ND	9.7 $\pm$ 5.7	<0.01
Quinone-reactive Ni/Fe hydrogenase	1.7 $\pm$ 1.6	7.5 $\pm$ 4.1	0.2

^a Spot density numbers are the average of the intensities from three gels from three separate culture preparations. ND, not determined.

bodies developed to heterologously express CymA, the expression of the total membrane preparation was investigated by Western blotting (Fig. 7). Consistent with the results obtained with heme staining (Fig. 2), greater expression of CymA was detected in iron-grown than in fumarate-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^{3+} . 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

FIG. 5. 2-D gel images of OM (A) and CM (B) protein fractions from iron-grown cultures. The numbers indicate proteins listed in Table 3. The protein load for each gel was 0.1 mg. (C) 3-D representation of the OM and CM merged to emphasize the unique proteins in each fraction represented by the red (OM) and green (CM) peaks.

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

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

Electron acceptor and membrane fraction ^a	Activity ($\mu\text{mol}/\text{min}/\text{mg}$)					
	Ferrozine- Fe^{3+} ^b		Fe^{3+} citrate ^c		Goethite	
	Mean ^d	σ	Mean	σ	Mean	σ
Fe^{3+} citrate						
TM	0.26	0.062	0.22	0.015	0.24	0.010
CM	0.60	0.165	0.57	0.017	0.16	0.001
OM	0.01	0.003	0.02	0.001	0.03	0.011
Fumarate						
TM	0.04	0.001	0.09	0.006	0.02	0.002
CM	0.16	0.013	0.23	0.027	0.01	0.001
OM	ND ^e		0.01	0.003	ND	
Oxygen						
TM	ND		ND		ND	
CM	ND		ND		ND	
OM	ND		ND		ND	

^a The terminal electron acceptor used for culture growth is indicated.

^b The reaction mixture contained ferrozine, allowing continued monitoring of Fe^{2+} 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.

FIG. 6. Effect of ferric citrate and goethite concentration on ferric reductase activity using TM (●) or CM (○). Panels A and B are plotted as ferric reductase activity for ferric citrate and goethite, respectively. Rather than the activity being expressed per milligram of protein, panels C and D express the activity per unit of FDH activity (in the TM or CM fractions). Each reaction contained 0.1 mg of protein/ml and 10 mM formate in 100 mM HEPES (pH 7.5) with concentrations of ferric citrate or goethite as indicated.

ferrozine-Fe³⁺ 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-Fe³⁺ reductase activity (36). Thus, for formate to support reduction of ferrozine-Fe³⁺ 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 Fe³⁺. Our previous study (57) showed that CM-localized FDH alone cannot reduce soluble or insoluble iron. Thus, other electron carriers are involved in chelated Fe³⁺ reduction. The increase in ferric reductase specific activity of the CM over that of the TM (Fig. 1) for chelated Fe³⁺ reduction indicates that removal of OM proteins (via sucrose density gradient ultracentrifugation) from the CM has minimal impact on soluble iron reduction. Thus, only the components of the CM are required for chelated Fe³⁺ reduction. Aside from soluble Fe³⁺, a number of other soluble electron carriers may also be reduced by the CM in the periplasm: fumarate (50), U⁶⁺ (13, 71), and trimethylamine N-oxide (63).

FIG. 7. Western blot of CymA TM from cultures grown with oxygen (lane 1), Fe³⁺ (lane 2), and fumarate (lane 3) as the terminal electron acceptors. Each lane contained 10 μg of protein.

The ability of the CM to reduce chelated Fe³⁺ in vitro does not preclude the reduction of chelated Fe³⁺ by OM or periplasmic electron transfer proteins in vivo. Evidence for involvement of periplasmic and OM protein electron carriers in reduction of chelated Fe³⁺ is more evident when the iron reduction rates are expressed per unit of FDH activity rather than per milligram of protein. The TM fraction actually exhibits a higher ferric reductase activity per unit of FDH than the CM when aqueous citrate-Fe³⁺ is used as the substrate. This indicates that the electron carriers downstream from FDH localized in the CM, periplasm, and the OM are facilitating, but are not required for, chelated iron reduction. Nevertheless, CM-dependent reduction of soluble metal forms may be relevant to environmental systems if Fe³⁺ is found in soluble form in the environment and if such soluble Fe³⁺ in the environment is transported through the OM.

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 Fe³⁺. Consistent with this mechanism, our results show that solubilized iron can be reduced by oxidoreductases in the CM or periplasmic space.

In addition, Magnuson et al. (29) isolated a ferric reductase complex from *Geobacter sulfurreducens* grown with either fumarate or ferric citrate. The complex catalyzed NADH-dependent ferric reductase activity using ferrozine-Fe³⁺ (36). These workers showed that the activity is membrane associated and the total activity is highest in the OM, similar to results by

Myers and Myers (36) with *S. oneidensis*. The study, however, did not report on the degree of separation of the CM and OM fractions. Nevertheless, the specific activities suggest that the ferric reductase activity is CM associated. This again suggests that the reduction of chelated iron involves CM components.

Reduction of insoluble Fe³⁺. When goethite is the iron substrate, the rate of reduction, when calculated per milligram of protein, is highest with the TM fraction from either iron- or fumarate-grown cultures (Fig. 1 and 6). At low concentrations of goethite, the TM catalyzed a higher rate of iron reduction. Only at the highest concentration of goethite used (30 mg/ml) was the CM-catalyzed rate slightly higher than the TM-catalyzed rate. When the rate of reduction is calculated as per unit of FDH activity, the TM is now almost four times higher than the CM. This is in contrast with the nearly twofold increase observed with ferric citrate (Fig. 6C). These results are consistent with the OM and periplasmic components being involved in insoluble iron reduction since mineral iron would be located outside of the cell envelope.

In the TM fraction, components of the CM and OM are retained, as well as periplasmic proteins that form a strong association with membrane proteins. Evidence that the TM contains not only CM and OM proteins but also periplasmic proteins comes from the SDS-PAGE gels stained for heme proteins and Western blots (Fig. 2B and 3). The prominent bands at 85, 75, 35, and 20 kDa correspond to the heme proteins OmcA, OmcB, MtrA, and CymA, respectively (7, 34, 44). MtrA is proposed to be a periplasmic protein involved in the iron reduction pathway (7) and is present in the TM fraction (Fig. 3). Candidate periplasmic electron carriers include CctA and MtrA, whereas candidate OM electron carriers are heme proteins OmcA and OmcB that are exposed on the cell surface (33).

Others have proposed that OmcB and other OM proteins are necessary for electron transfer to insoluble Fe oxides (6, 23, 39), whereas electron transfer to chelated Fe³⁺ species is possible for proteins in the CM and periplasm, as was seen with MtrA when expressed in *Escherichia coli* (51). Our results are also consistent with the mechanism of multiple reductase activities in *Shewanella* spp. (23).

Aerobic versus anaerobic growth. Many investigators have used aerobically grown cultures to study DIR in *Shewanella* (10, 11, 16, 17, 24–26, 53–56, 68, 69, 72). Our results clearly show that proteins expressed in oxygen-grown cultures differ most markedly from the protein profiles of anaerobic cultures. Major heme proteins involved in DIR are absent in aerobically grown cultures. Furthermore, no iron reductase activity was detected with membrane fractions from aerobically grown cultures.

Fumarate-grown versus Fe-grown cultures. Fumarate has frequently been used as the terminal electron acceptor in the growth medium for DIR studies. Whereas we and others have shown that the protein expression patterns of the CM and OM are similar, we observed major differences in the level of heme protein expression. In correlation with the lowered heme protein content of the TM from fumarate-grown cells, the ferric reductase activity was also lower in these TM fractions. Regardless of which iron substrate was used in the assay, the reduction activity was always higher with iron-grown cultures than with fumarate-grown cultures.

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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