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KINETIC AND BIOCHEMICAL ANALYSIS OF ELECTRON TRANSFER IN
DISSIMILATORY METAL REDUCTION BY SHEWANELLA ONEIDENSIS MR-1

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by

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ABSTRACT

Biochemical studies were used to further elucidate the pathway of electron transfer across the outer membrane (OM) of *Shewanella oneidensis* MR-1 and scaling kinetics were conducted to determine the dependence of two OM proteins, OmcA and MtrC, in dissimilatory metal reduction (DMR).

An outer membrane (OM) protein complex MtrC/A/B was purified from anaerobically grown *Shewanella oneidensis* MR-1. Analytical ultracentrifugation was used to characterize the complex and its molecular mass was determined to be approximately 198 kDa, which is consistent with a 1:1:1 stoichiometry of the individual subunits. This protein complex reduced both soluble and insoluble iron and manganese forms, with rates of reduction correlating to the metal species; i.e. soluble iron was reduced fastest while the most crystalline iron mineral goethite was reduced slowest. This trend is dependent upon the mineral reactivity and not enzyme specificity and therefore these proteins merely act as grounding wires to transfer electrons out of the cell.

Rates of iron reduction were determined at three scales: transient state with purified enzymes, steady state with total membrane (TM) fractions, and steady state with whole cells (WC). Transient state soluble iron reduction kinetic analysis was performed using stop flow techniques to determine molecular rate constants. The reactivity of soluble iron forms for both OmcA and MtrC follows the decrease in the association constant for ligand-metal complexation: EDTA-Fe$^{3+}$, NTA-Fe$^{3+}$, and Citrate-Fe$^{3+}$. Western blot analysis was used to determine the molar amounts of OmcA and MtrC in TM and WC samples in order to convert specific activity (moles Fe$^{2+}$ formed/min/mg protein) to a velocity (s$^{-1}$ : moles Fe$^{2+}$ formed/sec/ moles OmcA).

Comparison of rates between transient state and steady state revealed that OmcA and MtrC are kinetically competent to account for whole cell catalysis of soluble iron. When rates of reduction
of solid Fe-oxides (goethite) were compared at each kinetic scale, transient-state rate constants were 100 to 1000 times slower than steady-state rate constants. When flavins were included in the reaction mechanism, kinetic competence was exhibited. Thus, electron shuttles, such as flavins, are necessary to account for catalysis of goethite in whole cells.

As observed for iron oxides, OmcA and MtrC were not kinetically competent to account for physiological manganese oxide reduction via direct contact because the relevant rate constants were 3 orders of magnitude too slow. In the presence of flavins, the reaction rates were greatly increased and able to account for the reduction of insoluble manganese oxides in vivo. The extent of flavin stimulation was dependent upon the mineral reactivity, with regards to mineral composition (Fe versus Mn) and mineral structure.
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ABBREVIATIONS

AFM (Atomic Force Microscopy)
AQDS (Anthraquinone-2,6-disulfonate)
BET (Brunauer-Emmett-Teller)
CM (Cytoplasmic membrane)
DMR (Dissimilatory metal reduction)
ET (Electron transfer)
MK (Menaquinone)
Mtr (Metal reduction)
OM (Outer membrane)
Omc (Outer membrane cytochrome)
PCR (Polymerase chain reaction)
SDS-PAGE (Sodium dodecylsulfate-polyacrylamide gel electrophoresis)
TEA (Terminal electron acceptor)
TM (Total membrane)
WC (Whole cells)
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Chapter 1

DISSIMILATORY METAL REDUCTION

BIOGEOCHEMICAL CYCLING OF IRON AND MANGANESE

Abiotic mineral dissolution

Mineral dissolution is a complex and important process controlling the fate and transport of metals in the environment. In the simplest terms, mineral dissolution involves dissolving a solid species into a solvent to yield a solution. As this occurs, the mineral crystal structure breaks down as ions or molecules enter the solution. This process is controlled by a number of variables, which can be abiotic or biotic in nature. Rates of abiotic mineral dissolution are controlled by the rate of transport of reactants or products from the interface, the rate of back-reactions at the interface, or by reaction at the mineral interface (Brantley et al., 2006).

Reactions controlled at the mineral surface can be influenced by a variety of factors. For example, the rate-limiting step for surface reactions (interface control) is generally the detachment of the dissolving species at the interface. This detachment is thought to be determined by the number of sites (e.g. surface area) and the reactivity of these sites (Roden, 2003). The surface of a mineral can be quite heterogeneous in reactivity and contain a variety of energetically distinct sites. For example, reactivity of surface sites on minerals have been described as perfect surface, step and kink sites (Bandstra and Brantley, 2008). The reactivities of such sites are affected by the binding of organic and inorganic ligands to the surface (ligand-promoted dissolution) and pH (proton-promoted dissolution). Rates of ligand-promoted dissolution are dependent upon the chemical affinity and surface complexation of the ligand (Kraemer and
Hering, 1997). Ligands that affect metal oxide dissolution can be classified as either non-reducing or reducing (Brantley et al., 2006).

**Cycling of metals in the environment**

Mineral transformations caused by dissolution and precipitation reactions are responsible for the redox cycling of metals in the environment. These processes indirectly influence the (bio)geochemical cycles of many other elements, including the carbon cycle (Banfield, 1997). Likewise, organic carbon input is tightly coupled to metal reduction (Brannon et al., 1984; Hunt, 1983; Hunt and Kelly, 1988). This relationship has been examined for iron and manganese in the Amazon Basin and Panama Basin (Aller, 1990; Aller et al., 1991). For example, according to these workers, of the carbon oxidized in the entire Amazon Basin, iron and manganese accounted for 80% of the terminal electron acceptor, while in the Panama Basin, manganese was responsible for roughly all of the oxidation. This is not surprising since iron is the fourth most abundant element on Earth. Fe is essential for numerous cellular processes and prokaryotes require micromolar concentrations for biosynthesis (Frausto da Silva and Williams, 2001). Although less abundant, manganese is one of the strongest naturally occurring oxidants and is thus highly reactive. Complex interplay between cellular needs and environmental availability make cycling of metals, such as iron and manganese, an extremely interdependent and important process (Nealson et al., 2002) (Fig. 1-1).

**Ferric iron reduction**

At circumneutral pH in organic-free solutions, ferric iron is insoluble. As a consequence, in soils and sediments, Fe(III) is generally present as either organic complexes in solution or as the insoluble iron oxides. Reduction of iron oxides occurs in the sub-oxic zone in sediments. Under anoxic conditions, ferrous iron is relatively stable, and due to increased solubility of
ferrous oxide phases, more mobile than Fe(III). However, in the presence of molecular oxygen, Fe(II) can be oxidized to Fe(III) to form insoluble oxides (e.g. iron hydroxide, hematite or goethite). Iron is then transported back into the anoxic zone through sedimentation in what has been called “a gravity driven redox cycle” (Nealson et al., 2002). This redox cycling is directly linked to availability of oxygen (oxidant) and organic carbon (reductant). Furthermore, the high surface reactivity and surface-area-to-volume ratios of iron oxides promote adsorption of heavy metals and oxyanions (phosphate) (Stumm and Sulzberger, 1992). Redox transformations of iron can be abiotic (chemical) or biotic (Figure 1-2). Thus microbial influence on redox reactions involving iron controls heavy metal and trace element cycling in soils and aquatic sediments.

**Manganese reduction**

Manganese is the 10\textsuperscript{th} most abundant element in the Earth’s crust and of the heavy metals is the second most prevalent behind iron (Post, 1999). Manganese is ubiquitous in soils and sediments as mineral oxides. Manganese oxide minerals have high adsorption capacities far out of proportion to their concentrations and large surface areas, which contribute to their chemical reactivity. Due to its abundance and reactivity, Mn is important in control of heavy metal distribution between sedimentary and aqueous systems (Post, 1999). In sedimentary environments, the nonenzymatic reduction of Mn(IV) can occur via a number of metabolites including Fe(II), nitrite, hydrogen peroxide, and sulfide (Burdige and Nealson, 1986; Dollhopf et al., 2000; Lovley, 1991; Myers and Nealson, 1988).

**Microbial Fe and Mn reduction**

Micro-organisms often accelerate the rate of mineral transformations. Abiotic mineral dissolution rates are greatly enhanced due to the enzyme-catalyzed reactions inherent in microbial metabolism, especially at circumneutral pH, making microbial-mediated transformations much
faster than their chemical reaction counterparts (Kappler and Straub, 2005). For example, biological reduction of goethite at pH 7 is 100 times faster than ligand-promoted dissolution (Brantley et al., 2006). Therefore environmentally-relevant reduction rates are not controlled by chemical species alone but involve enzymes and other complex biological factors. Understanding the process of metal reduction and the inherent rates that control this process requires knowledge of the influence of microbial metabolisms as well as the underlying chemical mechanism occurring at or near the mineral surface.

**ANAEROBIC RESPIRATION USING ALTERNATIVE TERMINAL ELECTRON ACCEPTORS**

In the absence of oxygen, facultative and obligate anaerobes have the ability to obtain energy for growth and maintenance through respiration of alternative terminal electron acceptors (TEAs). Micro-organisms utilize a variety of TEAs (Figure 1-3), which include metals such as iron and manganese (Lovley and Phillips, 1988; Myers and Nealson, 1988). The use of metals in respiratory metabolism is defined as dissimilatory metal reduction (DMR). In dissimilatory reduction, electrons from carbon oxidation are transferred to various electron acceptors that are not produced by the cell. To do this, bacteria utilize electron carriers such as cytochromes, quinones, iron-sulfur proteins, flavoproteins and other electron transport proteins.

**Alternative electron acceptors**

In natural environments such as aquatic sediments, a hierarchy of electron acceptors exists based on the potential energy of the TEA. By sensing their surroundings, micro-organisms preferentially utilize TEAs whose reduction potential and abundance allow the highest energy yield. In other words, the largest energy difference between redox couples of electron donor and
acceptor will be most favorable and yield the greatest net energy gain for the cell. Oxygen is generally the most energetically favorable TEA, providing the highest energetic yield for a respiratory substrate \( E'_0 = +815 \text{ mV} \) (Madigan, 2000). Therefore, microorganisms capable of growing in the presence of oxygen -- aerobes and facultative anaerobes-- reduce oxygen. Once oxygen is depleted, alternative electron acceptors such as Fe(III), Mn(IV), nitrate, and fumarate are utilized. Moving deeper into sedimentary environments, into the anoxic zone, the distribution of major terminal electron-accepting processes from top to bottom define a “redox ladder”: nitrate, Mn(IV), Fe(III), fumarate, sulfate, and finally methanogenesis (Lovley, 1991). The implications of nitrate and fumarate respiration are discussed briefly below and the impacts of iron and manganese reduction will be examined in detail in subsequent sections.

Nitrate as a TEA in microbial respiration is reduced to nitrite, nitric oxide, nitrous oxide, and finally dinitrogen (Madigan, 2000). This process is known as denitrification and is a main branch of the global nitrogen cycle sustained by prokaryotes (Zumft, 1997). The enzymes that catalyze these reactions are central to the biogeochemical nitrogen cycle and structural, biochemical, and electrochemical studies of this system have yielded insight into the mechanisms of bacterial electron and proton transfer (Richardson and Watmough, 1999).

Fumarate is a metabolic intermediate of the citric acid cycle, which is formed by the oxidation of succinate (Campbell, 2009). Fumarate also serves as a terminal electron acceptor in anaerobic respiration, with an end product of succinate or propionate. Since fumarate can be formed from carbohydrates and proteins, fumarate reduction is the most widespread type of anaerobic respiration (Kroger et al., 1992). Microorganisms can also oxidize H\(_2\) (\textit{E. coli}) or formate (\textit{Wolinella succinogens}), the energetics of which is the same (Thauer et al., 1977).
**Electron carriers**

Electron transfer is dependent upon electron carriers that function to accept and donate electrons while conserving energy for synthesis of ATP (Madigan, 2000). These electron carriers include flavoproteins, cytochromes, and iron-sulfur proteins. Flavoproteins contain a flavin prosthetic group, either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), capable of transferring two electrons to an electron acceptor. One flavoprotein, fumarate reductase, contains a covalently-bound FAD cofactor involved in reduction of fumarate to succinate (Iverson et al., 1999). Cytochromes are proteins with heme prosthetic groups involved in single electron transfer reactions. The heme group consists of an iron atom coordinated within a tetrapyrrole (porphyrin ring) and based on the type of attachment to the protein backbone, is designated cytochrome $a$, cytochrome $b$ or cytochrome $c$ (Madigan, 2000). Iron-sulfur proteins, such as ferredoxin, contain clusters of iron and sulfur, the most common of which are Fe$_2$S$_2$ and Fe$_3$S$_4$. 

Various metals including reduced Fe and Mn and oxidized U and Cr adsorb to mineral surfaces. Reduction of these minerals through microbial processes liberates bound metals, which in turn can be reduced by dissimilatory metal reducing bacteria (DMRB). (Adapted from Nealson et al., 2002).

Figure 1-1: Metal oxide reduction/oxidation and the link to carbon and heavy metal cycling.
Figure 1-2: Chemical and microbial iron transformations. Both chemical and biological processes affect iron cycling yet at neutral pH, the highest rates of iron reduction are obtained through microbial-mediated electron transfer (Adapted from Kappler and Straub, 2005).
Figure 1-3: Summary of the most common types of anaerobic respiration. Standard redox potentials of various terminal electron acceptors are placed in context of most energetically favorable or electropositive (bottom) to least energetically favorable or electronegative (top) (Adapted from Madigan et al., 2000).
DISSIMILATORY METAL REDUCING MICROORGANISMS

The dissimilatory reduction of ferric iron by bacteria was first demonstrated in *Pseudomonas* isolated from marshy soil (Balashova and Zavarzin, 1979). Cultures were able to reduce ferric hydroxide and ferrihydrite with molecular hydrogen as an electron donor. The linkage of metal reduction to metabolism and growth was examined further with an isolate, designated GS-15, from freshwater sediments from the Potomac River, MD by Lovley and Phillips (1988) and with a Mn-reducing bacterium *Alteromonas putrefaciens* MR-1, isolated from anaerobic sediments of Oneida Lake, NY by Myers and Nealson (1988). Phylogenetically, a broad range of micro-organisms are capable of dissimilatory metal reduction (DMR) (Lonergan et al., 1996). However, much of what is known about this process has been through studies with *Geobacter* and *Shewanella* species, considered the model organisms for this process. While both *Shewanella* and *Geobacter* have the ability to reduce soluble and insoluble substrates, the mechanisms for reduction of solid-oxide minerals is quite different between these two species.

**Shewanella species**

*Shewanella*, a γ-proteobacteria, was first isolated from dairy spoilage and fish and has been found in marine, freshwater and terrestrial environments (Black Sea, Oneida Lake), and uranium-contaminated aquifers (New Mexico) (Shewan et al., 1960; Venkateswaran et al., 1999; Wildung et al., 2000). As a facultative anaerobe found at the oxic/anoxic interface, *Shewanella*, in addition to respiring oxygen, is also able to reduce a wide range of alternative TEAs including fumarate, nitrite, nitrate, dimethylsulfoxide, trimethylamine N-oxide, thiosulfate, sulfite, elemental sulfur; soluble and insoluble metals Fe(III) and Mn(IV); and toxic metals U(VI), Cr(VI), and Tc(VII) (Marshall et al., 2006; Payne and DiChristina, 2006; Wildung et al., 2000). This respiratory plasticity and the ability to culture *Shewanella* under a variety of growth conditions...
conditions, combined with a sequenced genome, makes this bacterium ideal for studying dissimilatory iron reduction (DIR).

While the proton motive force-generating electron transport for energy conservation occurs at the cytoplasmic membrane (CM) in Gram-negative bacteria, early biochemical studies of *Shewanella* revealed the majority of cytochromes (80% of total membrane bound) were localized to the OM in anaerobically-grown cells (Myers and Myers, 1992). A number of S. *oneidensis* mutant strains lacking specific c-type cytochromes show greatly diminished activity for various TEAs. For instance, *S. putrefaciens* strains lacking OM hemeproteins MtrA (metal reduction), and MtrC or OM non-heme MtrB, or a combination of the three, were markedly deficient in Fe(III) reduction (Beliaev et al., 2001). Likewise, mutants lacking MtrB were unable to properly localize OmcA (outer membrane cytochrome) and MtrC, suggesting MtrB is required for proper incorporation of these proteins in the OM (Myers and Myers, 2002). Analysis of the *Shewanella* genome identified 39 c-type cytochromes with 14 containing four or more heme-binding sites (Heidelberg et al., 2002). Thus biochemical and genomic data corroborate the importance of heme proteins in electron transport for metal reduction and as potential metal reductases in extracellular electron transfer (ET). These hemeproteins along with other proteins involved in ET will be discussed in greater detail in subsequent sections.

**Geobacter species**

*Geobacteraceae* are a family of Fe(III)-respiring δ-proteobacteria. Recent interest in *Geobacter* is due to its potential use in bioremediation, and electricity generation (Lovley et al., 2004; Ren et al., 2008). *Geobacter metallireducens*, isolated as strain GS-15, was one of the first microorganisms found to couple complete oxidation of organic compounds to metal reduction (Gorby and Lovley, 1991; Lovley et al., 1993). Another species, *G. sulfurreducens*, has served as the model organism for extracellular ET studies. It was the first genome from the
*Geobacteraceae* to be sequenced. Surprisingly, this genome encodes for 103 putative c-type cytochromes (Caccavo et al., 1994; Coppi et al., 2001; Methe et al., 2003). Recently, the genome of *G. metallireducens* has been sequenced and shows differential gene expression compared to *G. sulfurreducens*, suggesting dramatic differences in metabolism and physiology of this bacterium compared to other *Geobacteraceae* (Aklujkar, 2009).

As c-type cytochromes have been implicated in extracellular electron transfer (e.g. MacA, OmcB, OmcE, OmcS and OmcT), genetic and biochemical analysis has involved targeted knockouts of the genes encoding these proteins (Butler et al., 2004; Kim et al., 2005; Leang et al., 2003; Lloyd et al., 2003; Mehta et al., 2005). For example, MacA is a 36-kDa diheme c-type cytochrome localized to the periplasm of *G. sulfurreducens* and proposed to be an intermediate in ET to Fe(III). A *macA* mutant was generated by single-step gene replacement and exhibited greatly reduced growth rates with Fe(III) citrate as the TEA when compared to wild-type (Butler et al., 2004).

While cytochromes represent the major ET components of DMR, other non-heme proteins in *Geobacter* are required. One defining factor for DMR in *Geobacter* is the requirement of direct contact with insoluble substrates (Nevin and Lovley, 2000). Reduction can occur without prolonged attachment but growth on oxide surfaces requires biofilm formation and elicits maximal current production on electrodes (Reguera et al., 2007). Biofilm development is mediated by formation of pili structures which enhance cell-surface and cell-cell interactions. Of the known pili genes in *G. sulfurreducens*, *pilA* and *pilT* have been implicated in pili production and Fe(III) and Mn(IV) oxide reduction (Reguera et al., 2005; 2007). Recent work has focused on growth on electrode surfaces for biotechnological applications. Through mini*Himar* transposon mutagenesis, proteins important for biofilm formation and respiration other than cytochromes (e.g. transporters, two-component signaling proteins) have been identified (Rollefson, 2009). Furthermore, engineered strains of *G. sulfurreducens* show increased rates of
respiration for more effective bioremediation strategies (Izallalen et al., 2008). Thus future work with Geobacteraceae will undoubtedly lead to developments in biotechnology and bioremediation.

MECHANISMS OF ELECTRON TRANSFER TO FE AND MN OXIDES

Three potential mechanisms for reduction of extracellular solid-phase electron acceptors have been proposed: reduction through direct contact between OM cytochromes and mineral surface (Lower et al., 2007; Lower et al., 2001; Xiong et al., 2006), reduction at a distance through cyclic reduction/oxidation of electron shuttles (Marsili et al., 2008; Ross, 2009; von Canstein et al., 2008), and reduction at a distance through ligand-promoted solubilization of metal oxides with metal chelators (Taillefert et al., 2007) (Figure 1-4). To distinguish the latter two mechanisms, metal chelators act at the mineral surface, solubilizing metals at the mineral surface, which can then be reduced at the cell surface, while electron shuttles are first reduced by the cell and then proceed to “shuttle” electrons between the bacterial cell and mineral surface.

Direct contact

The requirement for direct contact between bacterial cells and the mineral surface in mineral reduction was first examined by Munch and Ottow (Munch and Ottow, 1983) with B. polymyxa and C. butyricum. This concept was further examined by Arnold and coworkers (Arnold et al., 1986) using cell suspensions of Pseudomonas sp. 200. Separation of the cells from goethite using a dialysis membrane (MWCO of 12-14 kDa) yielded no goethite reduction, suggesting the requirement for direct cell/mineral contact (Arnold et al., 1986). Early studies with Shewanella oneidensis MR-1 supported a mechanism of physical contact for growth on insoluble MnO₂ (Myers and Nealson, 1988).
In vitro, albeit indirect, experimental evidence for this mechanism in *Shewanella* as the route of electron transfer first came from Atomic Force Microscopy (AFM) studies by Lower et al. (Lower et al., 2001). In contrast to aerobically grown cells, these workers were able to show preferential binding of goethite to *Shewanella* whole cells that were anaerobically grown. They argued on the basis of AFM force curves that a 150 kilodalton protein was involved as a “putative reductase” critical for such binding. Likely candidate proteins for this “putative reductase” in *S. oneidensis* include OM multi-heme cytochromes OmcA and MtrC, which are localized to the outer membrane and extracellularly exposed (Myers and Myers, 2004). Thus, these two proteins are capable of interacting directly with extracellular substrates, such as solid-oxide minerals.

More recently, Lower et al. (Lower et al., 2007) again used AFM, to study the specific interaction of OmcA and MtrC with hematite. They showed on the basis of unique force signatures that the OmcA-hematite interaction is twice the strength of the MtrC-hematite interaction and they proposed that these specific binding interactions allowed for direct electron transfer to the iron oxide TEA.

The direct contact mechanism was also supported by a study by Xiong et al. (Xiong et al., 2006) using dynamic light scattering and fluorescence correlation spectroscopy (FCS) to measure the binding affinity between OmcA and hematite. Briefly, purified OmcA was labeled with the chromophore Alexa-488 and intensity fluctuations were measured using FCS to determine the internal dynamics of OmcA upon association with hematite. Low-amplitude bursts from fluorescent intensity traces were characteristic for labeled OmcA alone but upon addition of hematite, a dramatic increase in brightness of these bursts was observed; indicative of OmcA binding to hematite.

Recent work by Ruebush et al. (Ruebush et al., 2006) using an *in vitro* model system of TM fractions from *Shewanella oneidensis* MR-1 was also used as an argument for the direct contact mechanism. They proposed that the most likely mechanism for insoluble iron reduction
by *S. oneidensis* in their *in vitro* experiments was through direct contact since they assumed that small molecules had been removed from the TM sample after centrifugation and dialysis. Ruebush et al. therefore argued that reduction of insoluble metal oxides by *S. oneidensis* was possible through direct contact as well as through the electron shuttle and metal ligand mechanisms.

One specific mechanism that has been proposed for direct contact in *Shewanella* is the nanowire mechanism proposed by Gorby et al. (Gorby et al., 2006). Microbial nanowires or monolateral pili are produced in other organisms such as *Geobacter* sp. and aid in establishing contact with Fe(III) oxides (Reguera et al., 2005). It has been hypothesized that outer membrane cytochromes, due to their localization (OmcA and MtrC in the case of *S. oneidensis*) are the conductive species of these nanowires, yet their role in these appendages is not fully understood. MR-1 double mutants (ΔOmcA/MtrC) with in-frame deletions of both *omcA* and *mtrC* reduced a third of the total ferrihydrite after 24 h compared to WT and had about 5 times less electrochemical activity (Gorby et al., 2006). While experimental evidence supports a link between OmcA and MtrC and nanowires, conclusive evidence for the conductive nature of nanowires has not been demonstrated. Furthermore, it is unknown whether or not OmcA and MtrC localize to the nanowires and are responsible for electron transfer. It is also unknown whether these structures are produced under anaerobic conditions, since nanowire production in *S. oneidensis* only occurred under electron acceptor limiting conditions (O₂ < 2% of air saturation) (Gorby et al., 2006).

**Electron shuttles**

Another possible mechanism of electron transfer to extracellular solid-phase substrates involves the use of electron shuttles to mediate ET at a distance. Electron shuttles can be exogenous, e.g. humic acids, plant exudates, and antibiotics or endogenous, such as melanins and
flavins (Lovley et al., 2000; Lovley and Woodward, 1996; Marsili et al., 2008; Turick et al., 2003; Turick et al., 2008; Turick et al., 2002; von Canstein et al., 2008). The possible involvement of secreted (endogenous) electron shuttles in reduction of poorly soluble minerals was first proposed by Newman and Kolter (Newman and Kolter, 2000). Experimental evidence, although indirect, for participation of electron shuttles in DIR by dissimilatory Fe(III)-reducing microorganisms was further examined by the work of Nevin and Lovley (Nevin and Lovley, 2002). They were able to show reduction of iron oxide trapped in porous alginate beads and they concluded from this experiment that *G. fermentans* produced an electron shuttle with characteristics of a water-soluble quinone. Thus, in the absence of direct contact with the solid-phase Fe oxides, a shuttling mechanism was employed by this bacterium. In a similar experiment, Lies et al. (Lies et al., 2005) demonstrated reduction of iron (hydr)oxides precipitated in nanoporous glass beads by *S. oneidensis* MR-1. CymA and MK were required for direct and indirect mineral reduction, while MtrB and MtrC were not completely necessary for reduction at a distance. One interpretation of this finding involves a freely-diffusible electron shuttle capable of accepting electrons from CM localized electron carriers. Nonetheless, as in *Geothrix*, *S. oneidensis* utilizes an electron shuttle mechanism in the absence of direct contact. Recently the identity of an endogenous electron shuttle has been discovered in experiments with *S. oneidensis* (Marsili et al., 2008; von Canstein et al., 2008) and the implications of these findings are presented in subsequent sections on flavins.

**Chelators**

DMR studies have demonstrated enhanced Fe(III) reduction in subsurface sediments upon addition of synthetic chelators, yet direct experimental evidence for microbially synthesized chelators is limited (Arnold et al., 1986; Dichristina, 1992; Dollhopf et al., 2000; Lovley and Woodward, 1996). Nonetheless, using voltammetric techniques, soluble organic-Fe(III)
complexes have been detected in anaerobic cultures of *S. putrefaciens* strain 200 incubated with iron oxides in the absence of exogenous chelating compounds (Taillefert et al., 2007). Soluble organic-Fe(III) was detected before Fe(II) formation: this observation is consistent with ligand-promoted dissolution of Fe(III) prior to reduction (Taillefert et al., 2007). The previous studies by Nevin and Lovley (Nevin and Lovley, 2002) that supported iron oxide dissolution at a distance (trapped in porous alginate beads) by *Geothrix fermentans* were unable to distinguish between the mechanisms of electron shuttling and Fe(III) chelation. Furthermore, while flavins serve as electron shuttling agents in *S. oneidensis*, their capacity for metal chelation is also evident (Marsili et al., 2008). Therefore, it is likely electron shuttles may have the ability to chelate Fe(III), and act as endogenous metal chelators.

Siderophores, another type of endogenous iron-binding agent, are small chelating compounds produced by various bacteria and fungi in response to iron limitation and have a high affinity for Fe(III) (Madigan, 2000). Three major groups of siderophores exist and include hydroxamates, catecholates, and carboxylates. While *S. putrefaciens* produces a hydroxamate type siderophore, putrebactin, during aerobic growth, the impact of siderophores in dissimilatory metal reduction may be negligible (Gram, 1994; Ledyard and Butler, 1997).
Figure 1-4: Model of electron transfer mechanisms employed to reduce insoluble mineral oxides.

(1) Direct contact of the microbe to the mineral surface and subsequent electron transfer through OM cytochromes or electrically conductive pili. Strong attachment may not be required for catalysis but the microbe surface interacts directly with the mineral. (2) Reduction of the mineral surface using secreted small redox-active molecules (electron shuttles). The secreted flavins reduce iron at the mineral surface, become oxidized, and return to the cell for another round of reduction. (3) Metal chelates secreted by the cell form a complex with Fe(III). The chelated iron is soluble and therefore can be reduced at the mineral surface or transported through the OM and reduced by the microbe in the periplasm.
PATHWAY OF ELECTRON TRANSFER TO EXTRACELLULAR SUBSTRATES

In Gram-negative bacteria, the proton motive force-generating electron transport for energy conservation occurs at the cytoplasmic membrane (CM) (Scott and Nealson, 1994). A number of these microorganisms have the ability to link anaerobic growth to the respiration of Fe(III) and Mn(IV) despite the fact that at circumneutral pH, iron and manganese are insoluble and thus cannot freely diffuse into the bacterial cell. Therefore electrons generated from dissimilative metabolism must traverse the CM, periplasm, and OM. In light of such limitations, specific electron transfer mechanisms have been developed for respiration of extracellular terminal electron acceptors.

While most soluble electron acceptors are reduced in the periplasm by soluble or CM bound reductases, reduction of extracellular substrates requires multiple ET proteins able to transfer electrons considerable distances (Figure 1-5). One pathway has been extensively studied in Shewanella for the dissimilatory reduction of iron using a variety of genetic (e.g. knock-out studies) and biochemical techniques (e.g. protein localization). The majority of components involved have been identified (Table 1-1) and will be discussed below.

Electron transfer at the cytoplasmic membrane

Electron transfer at the CM involves the linkage of primary dehydrogenases (e.g. NADH dehydrogenase, formate dehydrogenase or hydrogenase), responsible for oxidation of carbon sources, to intermediate electron transfer proteins through a lipid soluble quinone pool (Richardson and Sawers, 2002). This electron transport chain creates a proton-electrochemical gradient to generate ATP from carbon oxidation (Scott and Nealson, 1994).
Figure 1-5: Anaerobic respiration. Schematic of electron transfer to various soluble and insoluble TEAs. Soluble substrates diffuse into the periplasm and are reduced by periplasmic electron carriers. Insoluble substrates that cannot diffuse into the cell are reduced by extracellularly exposed electron carriers. Adapted from Richardson et al., 2008.
Table 1-1: Proteins involved in dissimilatory metal reduction.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass</th>
<th>Theoretical pI value</th>
<th>Cofactors</th>
<th>Localization</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CymA</td>
<td>21</td>
<td>8.7</td>
<td>4 hemes</td>
<td>CM</td>
<td>ET to various reductases</td>
<td>Myers and Myers, 2000; Schwalb et al., 2003</td>
</tr>
<tr>
<td>CctA (Stc)</td>
<td>13</td>
<td>4.6</td>
<td>4 hemes</td>
<td>P</td>
<td>Implicated in Fe reduction</td>
<td>Gordon et al., 2000</td>
</tr>
<tr>
<td>MtrA</td>
<td>32</td>
<td>7.8</td>
<td>10 hemes</td>
<td>P/OM</td>
<td>Implicated in Fe reduction</td>
<td>Beliaev et al., 2001; Pitts et al., 2003</td>
</tr>
<tr>
<td>MtrB</td>
<td>76</td>
<td>4.4</td>
<td>n.d.</td>
<td>OM</td>
<td>Localization of OmcA and MtrC</td>
<td>Myers and Myers, 2002</td>
</tr>
<tr>
<td>MtrC/OmcB</td>
<td>75</td>
<td>5.5</td>
<td>10 hemes</td>
<td>OM</td>
<td>Potential terminal Fe reductase</td>
<td>Beliaev et al., 2001; Myers and Myers, 2003b</td>
</tr>
<tr>
<td>OmcA</td>
<td>83</td>
<td>6.2</td>
<td>10 hemes</td>
<td>OM</td>
<td>Potential terminal Fe reductase</td>
<td>Myers and Myers, 2001; 2003b</td>
</tr>
<tr>
<td>GspD</td>
<td>76.7</td>
<td>5.24</td>
<td>--</td>
<td>--</td>
<td>T2SS</td>
<td>Heidelberg et al., 2002</td>
</tr>
<tr>
<td>GspE</td>
<td>57.5</td>
<td>5.13</td>
<td>n.d.</td>
<td>CM</td>
<td>T2SS</td>
<td>Heidelberg et al., 2002</td>
</tr>
<tr>
<td>GspG</td>
<td>16</td>
<td>4.57</td>
<td>--</td>
<td>--</td>
<td>T2SS</td>
<td>Heidelberg et al., 2002</td>
</tr>
<tr>
<td>TolC</td>
<td>47</td>
<td>5.21</td>
<td>n.d.</td>
<td>OM</td>
<td>Efflux protein</td>
<td>Shyu et al., 2002</td>
</tr>
</tbody>
</table>

Abbreviations: cytoplasmic membrane (CM), outer membrane (OM), periplasm (P), not identified (n.d.)
Menaquinone

Menaquinone (MK) is a membrane-associated electron carrier. It is the substrate for formate dehydrogenase and also for CymA, acting as an electron shuttle for the two proteins. It is a required component for respiratory metabolism on both soluble and insoluble Fe(III). *S. oneidensis* mutants lacking MK and methylmenaquinone were unable to reduce nitrate, Fe(III), or fumarate and were severely but not completely deficient in Mn(IV) reduction (Myers and Myers, 1993). Likewise, mutants generated from Tn5 mutagenesis containing insertions in genes involved in menaquinone biosynthesis, *menD* and *menB*, revealed deficiencies in trimethylamine N-oxide (TMAO), dimethyl sulfoxide (DMSO) and Fe(III) reduction (Saffarini et al., 2002). Addition of menaquinone (vitamin K₂) to these mutants restored Fe(III) reductase activity. The secretion of a menaquinone-like compound produced by wild-type *Shewanella* restores MK-negative mutants (and corresponding membrane fractions) and this compound is thought to act as an electron shuttle for metal oxide reduction at a distance (Myers and Myers, 2004; Newman and Kolter, 2000).

CymA

CymA is a 21-kDa CM-localized tetraheme *c*-type cytochrome in *Shewanella*. The CymA protein was found in the cytoplasmic membrane and soluble fraction of *S. oneidensis* (Myers and Myers, 1997). Deletion of the gene encoding this protein severely affects reduction of a variety of substrates including nitrate, nitrate, DMSO, fumarate, and Fe(III)/Mn(IV) oxides (Myers and Myers, 1997; Myers and Myers, 2000). The *cymA* knockout strain, MR1-CYMA, was unable to grow using fumarate at a TEA and lost the ability to reduce Fe(III), Mn(IV) and nitrate (Myers and Myers, 2000). Redox properties were first determined in a homolog of CymA from *S. frigidimarina* NCIMB400 and experimental evidence suggests the presence of four low-
spin c-hemes, each with bis-His axial ligation, and midpoint potentials of +10, -108, -136, and -229 mV (Field et al., 2000). Redox titrations of a truncated form of CymA in *Shewanella oneidensis* MR-1 yielded midpoint potentials of -175 and -261 mV for two pairs of hemes (Schwalb et al., 2002).

CymA has been identified as a member of the NapC/NirT protein family, and therefore is postulated to be a quinol oxidase. As such, it is a potential electron transfer intermediate between the quinone/quinol pool and various periplasmic ET proteins. This activity was substantiated by Field et al. (Field et al., 2000) in experiments using a homolog of CymA from *S. frigidimarina* NCIMB400. These workers showed that CymA can be reduced by either duroquinol or menaquinol. Furthermore, kinetic data supports facile electron transfer between CymA and the soluble fumarate reductase (Fcc3) in MR-1. The fumarate-dependent oxidation of reduced CymA was observed under steady-state conditions and the rate constant was determined to be $1.9 \times 10^7$ M$^{-1}$s$^{-1}$ (Schwalb et al., 2002). Recently, expression of CymA in *E. coli* using a plasmid containing the *cymA* gene from *S. oneidensis* MR-1, imparted soluble ferric iron reduction capabilities in this organism that were necessary and sufficient for growth (Gescher et al., 2008).

Based upon its location and requirement for *in vivo* reduction of various substrates, CymA has been postulated to be the terminal CM electron carrier and a branch point for ET at the CM (Gescher et al., 2008; Richardson, 2008).

**Electron transfer across the periplasm**

In reduction of substrates that diffuse into the periplasm, reducing equivalents generated at the CM are directed to intermediate electron carriers (e.g. NrfAB for nitrite reduction) and subsequently passed to a variety of terminal electron acceptors such as trimethylamine N-oxide (TorCA), fumarate (fcc$_3$), tetraethionate (Ttr), nitrite (NrfAB), and nitrate (NapAB) that are reduced in the periplasmic space (Figure 1-5). The reductases for these molecules are localized to
the periplasmic space and due to their proximity to the CM, the reduction of soluble periplasm-localized TEAs can be directly linked to the proton motive force (PMF) generated across the cytoplasmic membrane (Richardson, 2008). For insoluble TEAs, electrons must be transferred through the periplasm by intermediate electron transfer proteins. The major periplasm-localized heme protein implicated in iron reduction in *Shewanella* is CctA (Gordon et al., 2000; Tsapin et al., 1996; Tsapin et al., 2001).

**CctA**

CctA (also named STC) is a small soluble tetraheme cytochrome involved in electron transfer to iron. CctA from *S. putrefaciens* was first purified and characterized using a variety of biochemical and electrochemical techniques (Tsapin et al., 1996; Tsapin et al., 2001). It has also been found to be induced by anaerobiosis. Electrospray mass spectrometry and two-dimensional gel electrophoresis was used to determine the molecular mass and pI of CctA, 12.2 kDa and 5.8, respectively (Tsapin et al., 1996). Electrochemical characterization of the heme groups revealed a peak potential position of -233 mV *versus* the standard hydrogen electrode (SHE). Not only do the heme groups have bis-histidine coordination, but the reduced hemes are oxidized by ferric citrate (Tsapin et al., 1996). Comparison of $^1$H NMR spectra of STC to a cytochrome *c*$_3$ from *D. vulgaris* revealed all four hemes are paramagnetic and low spin (Tsapin et al., 2001).

A homolog of this protein in *S. frigidimarina* NCIMB400 was also characterized. This cytochrome *c*$_3$ is a tetraheme c type cytochrome localized to the periplasmic space and shows sequence homology to STC from *S. oneidensis* MR-1 Gordon et al. (Gordon et al., 2000). Including heme groups, its molecular mass is approximately 11.8 kDa. The heme groups have a redox potential range from -240 mV to -58 mV at pH 7. Insertion of a spectinomycin/streptomycin resistance cassette into the *cctA* gene encoding this protein, creating
a knockout mutant, caused a severe impairment of ferric citrate reduction (Gordon et al., 2000), thus implicating it as an integral component in the iron respiration pathway.

A high resolution crystal structure of STC (CctA) from *S. oneidensis* MR-1 was obtained for an oxidized orthorhombic crystal (0.97 Å). The structure revealed a semilinear heme motif involved in promiscuous electron harvesting which differs from the classical $c_3$ heme core pattern. With multiple redox centers located at the surface of the protein, the chances of productive collisions increases and therefore may represent a new structural heme motif that provides a more efficient strategy for electron transfer to heterogeneous surfaces such as solid-phase minerals (Leys et al., 2002) and should be examined for a more physiologically-relevant terminal reductase, such as OmcA or MtrC. CctA could be the ET component responsible for passing electrons across the periplasmic space, from the CM to the OM, and thus further studies are needed to determine this function.

**Electron transfer across the outer membrane**

In order to reduce insoluble TEAs, electrons must traverse the OM and reach the extracellular milieu. The majority of the cytochrome proteins in *S. oneidensis* MR-1 are localized to the OM during anaerobic growth (Myers and Myers, 1992) and through genetic knock-out studies, three major OM proteins have been determined to be required for maximal rates of iron reduction, MtrA, MtrB and MtrC. Sequence analysis of *mtrC*, *mtrA*, and *mtrB* revealed they are part of an operon, are cotranscribed in the order of *mtrC, mtrA, and mtrB*, and that they are induced under anaerobic conditions (Beliaev et al., 2001). Therefore MtrC, MtrB, and MtrA may represent electron carriers for ET across the OM.
MtrA

MtrA is a 32 kDa periplasmic decaheme cytochrome containing 10 putative heme-binding sites -- all 10 have bis-histidine axial ligation (Beliaev and Saffarini, 1998; Pitts et al., 2003). Electron paramagnetic resonance (EPR) spectroscopy was used to determine the coordination of the heme groups and EPR potentiometric redox titrations showed the hemes reduce over a potential range of -100 mV to -400 mV (Beliaev and Saffarini, 1998; Pitts et al., 2003). Recently the reduction potential window was studied using protein film voltammetry (PFV) and the midpoint potential was determined to be -100 mV (Firer-Sherwood et al., 2008).

MtrA was first identified through isolation of mutants deficient in Fe(III) and Mn(IV) reduction (Beliaev and Saffarini, 1998). Mutants were generated using Tn5 mutagenesis and the observed phenotype was determined to be the result of an insertional inactivation in the mtrB gene. Examination of DNA upstream of mtrB led to the discovery of mtrA and the amino acid sequence was found to have a putative signal sequence indicative of secreted proteins (Beliaev and Saffarini, 1998). To determine the importance of MtrA in metal reduction, mutants with a disruption of the mtrA gene were constructed using a suicide vector (pVIK165). Loss of MtrA resulted in a lack of metal reduction activity in whole cells but not in membrane fractions, suggesting MtrA is not necessary for activity of the terminal reductase nor is it involved in the final reduction step (Beliaev et al., 2001). Another interpretation would be exposure of various CM and periplasmic-localized electron carriers contributes to the overall reduction and therefore can compensate for the loss of MtrA.

MtrB

MtrB is localized at the OM, partially exposed on the cell surface and required for proper incorporation of OmcA and MtrC into the OM (Myers and Myers, 2002; Myers and Myers, 2003). Based upon its primary amino acid sequence, MtrB is proposed to be an integral OM beta-barrel
protein (Hartshorne et al., submitted). The role of MtrB in Fe(III) and Mn(IV) reduction was first examined using transposon mutagenesis to create a Tn5 inactivation of mtrB. Compared to wild-type MR-1, mutant strain SR-21 (∆MtrB) lost the ability to reduce Fe(II) and Mn(IV) but not soluble TEAs nitrate, nitrite, fumarate, DMSO, TMAO, thiosulfate or sulfite (Beliaev and Saffarini, 1998). MtrB is also required for the reduction of humic acids (Shyu et al., 2002). Using transposon mutagenesis, Shyu and coworkers (Shyu et al., 2002) made a mutant, AQ-38, that was unable to reduce anthraquinone-2,6-disulfonate (AQDS). The mutant contained an insertion in the mtrB gene. While MtrB contains one CXXC motif, which resembles a heme-binding motif (CXXCH), no data exists to support the use of this motif in binding heme for electron transfer. Therefore, while MtrB may not be directly involved in electron transfer through heme cofactors, it is important for the reduction of insoluble extracellular substrates and thus a major component of the ET pathway.

MtrC

MtrC (previously OmcB) is a 75-kDa extracellularly exposed OM lipoprotein (Beliaev et al., 2001; Myers and Myers, 2002; Myers and Myers, 2003; Myers and Myers, 2001). It contains 10 low-spin c-type magnetically isolated and interacting hemes that titrate over a broad potential range (+100 to -500 mV versus SHE) (Hartshorne et al., 2007).

MtrC was first discovered to be an integral component in Fe and Mn reduction through genetic studies by Myers and Myers (Myers and Myers, 2001) and Beliaev and co-workers (Beliaev et al., 2001). Myers and Myers (Myers and Myers, 2001) created an omcB mutant that caused a 75% decrease in MnO2 reduction but was not hindered in its ability to reduce ferric citrate and FeOOH. Although initial conclusions suggested MtrC is not involved in Fe(III) reduction, the mutant was markedly deficient in other OM cytochromes and total cytochrome content was <15% compared to WT, thus complicating the interpretation to include the
possibility of the disruption of the cytochrome c-maturation genes or other factors integral to expression of c-type cytochromes under these conditions. Furthermore, membrane fractions of an *mtrC* mutant showed decreased iron reduction (Beliaev et al., 2001).

MtrC has the ability to directly bind to hematite surfaces; the strength of binding was interrogated using atomic force microscopy (AFM) (Lower et al., 2007). Unique force curves with sawtooth features were generated between hematite and MtrC compared to featureless force curves for control experiments with an uncoated gold substrate and hematite-functionalized tip or an MtrC-functionalized gold substrate with a bare AFM tip. These results were interpreted as formation of a stable bond between MtrC and the hematite surface. This technique was recently employed to determine the surface distribution of MtrC on live *S. oneidensis* MR-1 cells using antibody-functionalized AFM tips (Lower et al., 2009). Using an antibody recognition force microscopy (Ig-RFM) technique, MtrC was found uniformly distributed across the cell surface, associated with the extracellular polymeric substance (EPS) with Fe(III) as the TEA. Due to its localization and association with the OM, MtrC is considered capable of aiding in transfer of electrons to extracellular substrates. MtrC has also been hypothesized to be integral to the production of putative nanowires, and these observations may lend further support to a role in extracellular electron transfer under specific growth conditions (Gorby et al., 2006). The obligate use of said nanowires will be discussed further in Chapter 3.

Not only does MtrC reduce various iron and manganese species, but it has been shown to reduce aqueous U(VI), forming UO₂ nanoparticles as a product (Marshall et al., 2006). This was demonstrated with *in vitro* reduction studies and *in vivo* work with an *mtrC* mutant. Furthermore, using high-resolution immune-electron microscopy, MtrC was found to associate with extracellular UO₂ particles (Marshall et al., 2006).
OmcA

The 83 kDa OM-associated lipoprotein, OmcA, is expressed under anaerobic conditions when iron(III) or fumarate are present as the TEA, with specific content of aerobically grown cells being 14% of that of fumarate-grown cells (Myers and Myers, 2004; Myers and Myers, 1997; Myers and Myers, 2003). The *omcA* gene sequence encodes for a protein of 734 amino acids (78.6 kDa) and contains a leader sequence, a lipoprotein consensus sequence and 10 putative heme binding sites (Myers and Myers, 1998). Through use of magneto-optical spectroscopy, OmcA was found to contain 10 low-spin bis-His ligated c-hemes and a midpoint potential between –234 and –324 mV (Field et al., 2000). Mutants lacking the *omcA* transcript retained the ability to grow on the soluble TEAs trimethylamine N-oxide (TMAO), nitrate, fumarate, thiosulfate, dimethyl sulfoxide, and tetrathionate. Furthermore, reduction of nitrate, nitrite, ferric citrate, FeOOH, and anthraquinone-2,6-disulfonic acid (AQDS) was not hindered but reduction of MnO₂ was 45% slower than wildtype (Myers and Myers, 2001).

Localization of OmcA is affected by the absence of MtrC and MtrB. In *mtrB* mutants, OmcA was predominantly found localized to the CM and soluble fraction (Myers and Myers, 2002; Myers and Myers, 2002). Not only are MtrC and MtrB important for OmcA localization, but the type II secretion system has also been implicated and will be discussed in detail in subsequent sections. Furthermore, using antibody-functionalized AFM tips, Lower and coworkers (Lower et al., 2009) showed OmcA localization at the cell/mineral interface in association with extracellular polymeric substance (EPS).

Due to its localization, it is hypothesized that OmcA can directly bind and therefore transfer electrons to insoluble substrates. To test this hypothesis, atomic force microscopy (AFM) was used to determine specific bonds between OmcA and hematite (Lower et al., 2007; Xiong et al., 2006). AFM force spectra for approach and retraction of recombinant OmcA with the hematite surface displayed sawtooth force signatures indicative of formation of a specific
bond (Lower et al., 2007). The specifics of this interaction were further probed using phage-display technology and a hematite-binding consensus sequence was identified in OmcA (Lower et al., 2008). This sequence, Ser/Thr-Pro-Ser/Thr, was found adjacent to the terminal heme-binding domain in the published sequence data for OmcA.

Soluble iron reductase activity of OmcA has been examined in vivo by Borloo et al. (Borloo et al., 2007) and with purified protein by Wang et al. (Wang et al., 2008). Rate constants for OmcA with various soluble iron species were determined in both studies. Transient-state rates with purified OmcA were $4 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ for Fe-citrate, $2 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ for Fe-NTA, and $6.2 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ for Fe-EDTA (Wang et al., 2008). Rates from in vivo studies of OmcA-dependent Fe-NTA reduction were $1.17 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ (Borloo et al., 2007). OmcA also plays a role in U(VI) reduction (Marshall et al., 2006). Marshall and coworkers (2006) showed that while OmcA does not directly reduce uranyl citrate, an omcA deletion mutant exhibited decreased rates of U(IV) reduction.

Electron transfer at the micro-organism/mineral interface

Once localized to the OM, electrons must transport across the divide between the micro-organism and mineral surface. The heterogeneity of both surfaces complicates the study of extracellular electron transfer: the determination of the true pathway is difficult. Two putative terminal reductases, OmcA and MtrC, are localized to the OM, partially exposed on the outer surface of the cell, and have the ability to interact with extracellular substrates (Myers and Myers, 2003). One important aspect of this process involves the proper localization of proteins to the outer leaflet of the OM; the type II secretion system (T2SS) has been implicated (Shi et al., 2008). Furthermore, secreted endogenous electron shuttles (e.g. flavins; (Marsili et al., 2008; von Canstein et al., 2008)) may also aid in mineral reduction. The elucidation of the intricacies
responsible for such processes have led to a better understanding of the potential strategies for extracellular respiration and will be discussed in subsequent sections.

**Type II secretion system (T2SS)**

The type II secretion pathway (or main terminal branch of general secretory pathway) is responsible for translocation of proteins to the OM in gram-negative bacteria (Cianciotto, 2005). Proper localization of proteins to the OM is required for reduction of extracellular TEAs and therefore a prerequisite for insoluble mineral reduction. The role of two T2SS proteins, GspD and GspG, has been examined with respect to proper localization of two OM cytochromes from *S. oneidensis*, OmcA and MtrC (Shi et al., 2008). Mutants with deletions in *gspD* (ΔGspD) or *gspG* (ΔGspG) exhibited a loss of pink color in the culture supernatant compared to WT. This lack of color was attributed to the loss of OmcA and MtrC and confirmed by Western blot. Furthermore, OmcA and MtrC in these mutants had a lack of accessibility to Proteinase K treatment, further supporting their absence in the OM (Shi et al., 2008). In another study, an *E. coli* strain expressing OmcA from *Shewanella* acquired soluble and insoluble iron reduction capabilities (Donald et al., 2008). UV-visible spectroscopy was used to monitor heme absorbance changes of whole cells, and a *gspD* mutant expressing OmcA exhibited no heme oxidation upon addition of iron oxide, indicative of a loss of surface exposure of OmcA (Donald et al., 2008). Thus GspD and GspG are required for the proper localization of OM proteins OmcA and MtrC.

**Flavins**

The ability of flavins to enhance iron reduction was first examined by Myers and Myers (Myers and Myers, 1993). These workers showed that addition of flavin mononucleotide (FMN) increased ferric reductase activity in purified membrane fractions from *S. oneidensis* MR-1. The involvement of flavins as electron shuttles would explain reports that *Shewanella* secretes a small
quinone-like compound involved in electron transfer (Newman and Kolter, 2000) and can utilize sterically sequestered iron located in alginate beads (Nevin and Lovley, 2002). In a similar experiment, Lies et al. (Lies et al., 2005) demonstrated reduction of iron (hydr)oxides precipitated in nanoporous glass beads by *S. oneidensis* MR-1. These studies demonstrated the phenomenology of electron transfer without direct contact for *S. oneidensis*, but more substantive evidence was not provided until very recently when two groups independently discovered that flavins were responsible for this phenomenon (Marsili et al., 2008; von Canstein et al., 2008). In *Shewanella* biofilms, increased current production was attributed to the presence of riboflavin. In addition, for planktonic cells, an addition of riboflavin, FMN or FAD increased iron reduction rates with goethite but not with ferric citrate (Marsili et al., 2008; von Canstein et al., 2008). Therefore flavins are inferred to play a major role in extracellular electron transfer at the microbe/mineral interface in this organism.

**SUMMARY**

Much work has been done on identifying the proteins required for dissimilatory metal reduction. Since iron and manganese are predominantly found as insoluble minerals at circumneutral pH in the environment, bacteria have evolved a mechanism to link reduction of extracellular metals to energy conservation for growth. The mechanisms of such reactions have been examined using genetic knockout studies, *in vitro* iron reduction systems, *in vitro* AFM studies and various other biochemical and kinetic techniques. It is proposed that extracellular solid-phase TEAs can be reduced by various mechanisms including direct contact, electron shuttles, or iron chelators. While *Geobacter* requires direct contact for growth on solid-oxide minerals, *Shewanella* can utilize both direct and indirect contact. The purpose of this thesis is to
further characterize the pathway of electron transfer from the CM, across the periplasmic space, through the OM and finally to the TEA using biochemical and kinetic techniques.

This dissertation addresses the following questions:

1. What protein(s) are involved in transferring electrons from the periplasm, through the OM, and finally to an insoluble substrate?

2. Is it possible to identify, purify and kinetically characterize the protein(s) involved?

3. What proteins are responsible for the iron reductase activity observed in anaerobically grown cells?

4. Can we infer a mechanism by kinetically examining purified proteins?

5. Does the mechanism of manganese reduction differ from iron reduction?

Chapter 2 of this thesis addresses the question of electron transfer through the OM. Multiple OM reductases have been examined but no mechanism has been determined. In this chapter, I purified an OM protein complex MtrC/A/B, and determined its molecular mass, stoichiometry, and metal reductase capacity. This work, in concert with native page and protein cross-linking studies performed by Dr. Shane Ruebush (Ruebush, 2006) has shed light on the possible pathway of electron transfer through the OM in *S. oneidensis* MR-1. The combination of work from Chapter 4 in Ruebush’s Ph.D thesis (Ruebush, 2006) with the work described here was published in Applied and Environmental Microbiology (Ross et al., 2007). I performed AUC experiments at East Anglia University under the supervision of Dr. David Richardson and all other experiments under the supervision of Professors Ming Tien and Susan Brantley.

Chapter 3 examined the kinetics of electron transfer to various Fe(III) species. This work reveals the power of the kinetic scaling approach in elucidating the three possible mechanisms for electron transfer (metal chelation, electron shuttle, direct contact). The work has eliminated direct contact as a plausible mechanism of electron transfer from OmcA or MtrC to iron oxides in vivo and fully supports the involvement of electron shuttles in DIR. Flavins are particularly implicated. I performed kinetic studies at three different scales: a) transient state analysis of purified enzymes and steady state analysis of purified b) TM and c) WC. I performed all of the experiments in Chapter 3 and this work was described in a paper accepted for publication in Applied and Environmental Microbiology.


In Chapter 4 I investigated the role of OM cytochromes OmcA and MtrC in electron transfer to manganese oxides. Scaling studies, performed at three kinetic scales, revealed the importance of flavins in manganese oxide reduction. The extent of stimulation by flavin addition in mineral reduction studies with whole cells was inversely related to mineral reactivity; the less reactive mineral bixbyite was accelerated to greater extent by added flavin as compared to the more reactive mineral birnessite. Overall, flavins were necessary for physiologically-relevant
manganese oxide reduction, supporting the electron shuttle hypothesis, and the impact of these small molecules was shown to be dependent upon mineral structure and reactivity.
Chapter 2

CHARACTERIZATION OF PROTEIN-PROTEIN INTERACTIONS INVOLVED IN IRON REDUCTION BY SHewanella Oneidensis MR-1

INTRODUCTION

Environmental bacteria utilize a wide range of terminal electron acceptors (TEAs) during respiratory metabolism, generally depleting the environment in electron acceptors in a sequential pattern or "redox ladder" starting with acceptors at higher potential followed by those at lower potential (Madigan, 2000). For example, facultative anaerobes utilize O\textsubscript{2} when present before nitrate, Mn, and Fe species as TEAs. This redox ladder has been attributed to the competition among microorganisms and the energetic benefit of utilizing TEAs yielding higher $\Delta G$ per mole. Under anaerobic conditions, microbes can utilize over 20 elements as TEAs (Madigan, 2000). Of these, six elemental systems are known to be respired in solid form external to the outer membrane or external to the peptidoglycan layer for gram-positive bacteria: S, As, Se, U, Fe, and Mn (Fredrickson et al., 1998; Herbel et al., 2003; Lovley and Phillips, 1988; Moser and Nealson, 1996; Myers and Nealson, 1988; Newman et al., 1997).

Reduction of soluble or insoluble metals as TEAs is referred to as respiratory or dissimilatory metal reduction (DMR) (for a review, see (Nealson et al., 2002)). Our understanding of DMR is largely derived from studies of the Shewanella genus (Lovley, 2002). The Shewanella genus is comprised of gram-negative facultative anaerobes able to utilize a large number of aqueous organic and inorganic metal complexes as well as solid oxides as TEAs. The latter include hydrous ferric oxide, goethite, hematite, and manganese oxides (Myers and Nealson,
1988; Venkateswaran et al., 1998), Fe(III), Mn(IV,III), Cr(VI), and U(VI) (Arnold et al., 1986; Fredrickson et al., 1998; Liu et al., 2001; Myers and Myers, 1994; Myers and Nealson, 1988; Wade and Dichristina, 2000).

The respirome of *Shewanella oneidensis* is rich in respiratory electron transport proteins, including 42 putative e-type cytochromes. Previous work has identified some of the potential electron transport proteins involved in DMR (Beliaev and Saffarini, 1998; Beliaev et al., 2001; Gordon et al., 2000; Myers and Myers, 2001). However, these genetic studies yield little biochemical information on the sequence of electron transfer from the cytoplasmic membrane (CM), through the periplasm, and across the outer membrane (OM) to the extracellular mineral oxide. At the CM, one source of electrons in lactate-grown cells is formate (Scott and Nealson, 1994) where oxidation of formate by formate dehydrogenase (FDH) reduces menaquinone to menaquinol. Menaquinol is proposed to reduce CM-localized tetraheme protein CymA. CymA is believed to be the terminal CM electron carrier. Electron transport through the CM results in proton translocation at the level of menaquinone reduction by the proton motive FDH. Reduced CymA passes its electrons to unidentified carriers in the periplasm (e.g., possibly CctA [(Beliaev et al., 2001)]). Electron carriers located in the OM include decaheme proteins OmcA and MtrC. Both of these proteins have been shown to be extracellularly exposed (Myers and Myers, 2003). These extracellular heme proteins function to pass the electrons to the external environment (Xiong et al., 2006). MtrB is also essential to DMR and localized in the OM but has no known cofactors (Beliaev and Saffarini, 1998; Myers and Myers, 2002; Shyu et al., 2002).

In the work described here, we use protein purification, analytical ultracentrifugation, and formaldehyde cross-linking to determine protein-protein interactions of the electron transfer to TEAs operating in *S. oneidensis*. For the first time, a complex composed of MtrC/MtrA/MtrB is demonstrated to be involved in OM electron transfer. Based upon its location, this complex acts
only as a grounding wire and cannot act as part of the proton motive energy-conserving system of the cell which is confined to the inner membrane.

MATERIALS AND METHODS

Organism and growth conditions

*S. oneidensis* MR-1 (ATCC 700550) was grown by the method of Myers and Nealson (Myers and Nealson, 1988) with the following modifications: 30 mM DL-lactate, 4 mM sodium phosphate, and 10 mM HEPES, pH 7.4, with 50 mM ferric citrate in 4-liter flasks under N₂ at 30°C. Inoculum was prepared by growth of *S. oneidensis* in Luria-Bertani (LB) broth (shaken overnight at 21°C) and then centrifuged at 10,000 x 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 1/10 original volume of buffer. Cultures were inoculated with 1 x 10⁶ cells/ml. Anaerobic iron-grown cultures were as previously described (Ruebush et al., 2006), using lactate as the carbon source and ferric citrate as the TEA. Cultures were harvested at mid-log phase when the aqueous Fe²⁺ concentration reached 35 mM as measured with ferrozine (Stookey, 1970).

Metal oxides

Goethite was synthesized by the method of Schwertmann and Cornell (Schwertmann, 1991). Birnessite was synthesized by the method of Lopano et al. (2007). The six-line ferrihydrite was synthesized by the method of Guyodo et al. (2006). The specific surface area was estimated by the method of Brunauer-Emmett-Teller (BET) (Brunauer, 1987) for six-line ferrihydrite (174 m²/g) and goethite (30 m²/g).
Membrane isolation and characterization

The total membrane (TM), CM, and OM fractions were isolated as previously described (Ruebush et al., 2006) using a modified version of Myers and Myers (1992). These modifications resulted in improved separation, yielding CM and OM with no intermediary fraction. After centrifugation, the OM and CM pellets were stored in 20% glycerol in 10 mM HEPES, pH 7.5, at −80°C. Separation of CM and OM was assessed by NADH oxidase activity assay (41). Protein concentration was measured by the method of Lowry et al. (1951).

The periplasm was isolated using osmotic shock of *S. oneidensis* cultures (16). Cells were suspended in 50 mM Tris-Cl, pH 8.0, 250 mM sucrose, 2.5 mM EDTA for 5 min at room temperature. Cell suspensions were centrifuged for 10 min at 14,000 rpm at 4°C. The pellet was suspended in ice-cold 5 mM MgSO₄ and kept on ice with occasional swirling. The soluble periplasmic fraction was obtained from the supernatant after centrifugation for 10 min at 14,000 rpm. The spheroplast pellet was suspended in 50 mM Tris-Cl, pH 8.0.

Heterologous expression

Antibodies were raised to *S. oneidensis* proteins by using proteins expressed in *Escherichia coli* as antigens. Genes encoding electron transport proteins were obtained by PCR. Oligonucleotide primers were synthesized (Integrated DNA Technologies) for PCR amplification of *mtrC*, *mtrB*, *mtrA*, *cymA*, and *cctA* (Table 1). Chromosomal DNA was isolated from *S. oneidensis* MR-1 using DNA Easy (QIAGEN). The *Taq* polymerase-amplified PCR product was subcloned into pGEM-T Easy vector, digested with appropriate restriction endonucleases, and then ligated into pET15b, pET21a, or pET25b (Novagen).

*E. coli* BL21(DE3)/pLysS (Stratagene) cells were used for the expression of MtrC, MtrB, and CctA. MtrA and CctA was expressed in *E. coli* BL21(DE3)/pEC86 which contained the *ccmABCDEFGH* genes (cytochrome *c* maturation) (Arslan et al., 1998).
Cells containing the mtrC-pET21a construct were grown in Luria broth containing 100 μg ampicillin and 34 μg chloramphenicol to an absorbance at 600 nm of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was then added. Cells were harvested by centrifugation after incubation for 3 h at 37°C. MtrC was expressed as inclusion bodies and purified (54).

Cells containing the mtrA- and cctA-pET21a construct were heterologously expressed and purified by the method of Pitts et al. (Pitts et al., 2003) with modifications. CctA was purified as described above for MtrA but without detergent. After the polymyxin B sulfate treatment, the cell pellet was homogenized in 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 2% laurylsarcosine. MtrA was most abundant in the detergent-extracted fraction and affinity purified using a Ni-Sepharose column with buffers containing 2% laurylsarcosine.

Cultures containing mtrB-pET25b were grown in LB medium at 37°C to an optical density at 600 nm of 0.6 at 200 rpm. Cultures were then induced to express MtrB by adding 1 mM IPTG and incubating for 4 h at 37°C. Cultures were collected by centrifugation, suspended in 20 mM Tris-Cl, pH 8.0, plus 100 mM NaCl, and frozen. MtrB was expressed as inclusion bodies and purified (54).

*E. coli* BL21(DE3)/pLysS cells containing the cymA-pET15b construct were grown in LB broth containing 50 μg ampicillin and 34 μg chloramphenicol. At an absorbance at 600 nm of 0.6, IPTG (1 mM) was added, and then the cells were incubated for 3 h at 37°C. Cells were collected by centrifugation and then washed with binding buffer (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, and 10 mM imidazole) two times. The pellet was suspended and sonicated three times for 1 min each time. Inclusion bodies were collected by centrifugation at 14,000 x g for 45 min. The pellet was suspended in binding buffer plus 6 M urea and 2% Trition X-100 at room temperature for 30 min. The protein solution was loaded onto a Ni-Sepharose column equilibrated with binding buffer plus 6 M urea. Four column volumes of binding buffer
containing 6 M urea were passed through the column. The column was then washed with 3 volumes of wash buffer (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 500 mM imidazole, and 6 M urea. The eluted protein was concentrated and buffer exchanged into 10 mM HEPES, pH 7.5, using an Amicon ultrafiltration (10-kDa cutoff).

**Antibody production**

Polyclonal antibodies to OmcA, MtrC, MtrA, CymA, MtrB, and CctA were raised in New Zealand White rabbits (Covance Custom Immunology Services, Inc.). The immunoglobulin G fraction was further purified by affinity methods using the purified protein antigens on nitrocellulose membranes (Marchese-Ragona et al., 1988).

**MtrC, MtrA, and MtrB (MtrC/A/B) purification**

(i) **Anion-exchange chromatography**

TM fractions were isolated (Ruebush et al., 2006) and solubilized in 5% Triton X-100 in 50 mM HEPES, pH 7.5. The protein sample was loaded onto a Q-Sepharose (Amersham) column and eluted with a 0 to 0.5 M NaCl gradient (total, 150 ml). Heme proteins were eluted at 32% and 46% NaCl in the gradient.

(ii) **Gel permeation chromatography**

Fractions containing MtrC/A/B from the anion-exchange column were purified on a Sephacryl S-300 column (3-cm diameter x 1 m) at room temperature. The buffer was 50 mM HEPES, pH 7.5, containing 250 mM NaCl, and 0.9% N-octylglucoside. The void volume was determined using blue dextran.
### Table 2-1: Oligonucleotides used for PCR.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtrA1</td>
<td>CATATGAAGAACTGCTAAAAATGAAAAA</td>
</tr>
<tr>
<td></td>
<td>ACCTACTGCC</td>
</tr>
<tr>
<td>MtrA2</td>
<td>CTCGAGGCGCTGTAATAGCTTGCCAG</td>
</tr>
<tr>
<td>MtrCl</td>
<td>CATATGTTAACCGGCTGTGGTGGGAAG</td>
</tr>
<tr>
<td>MtrC2</td>
<td>CTCGAGTTACATTTTCACATTAGTGAT</td>
</tr>
<tr>
<td></td>
<td>CTGC</td>
</tr>
<tr>
<td>MtrB1</td>
<td>GAATTCATGAAATTTAAGCTC</td>
</tr>
<tr>
<td>MtrB2</td>
<td>CTCGAGGAGTTTGAACCTAGCTCA</td>
</tr>
<tr>
<td>CymA1</td>
<td>CATATGTTGTTGGCTATTTTGCAAC</td>
</tr>
<tr>
<td></td>
<td>TCAG</td>
</tr>
<tr>
<td>CymA2</td>
<td>CTCGAGTTATCTCTTTTGGATAGGGGTGAG</td>
</tr>
<tr>
<td>CctA1</td>
<td>CATATGGAAAGTCACCCCTGACACAG</td>
</tr>
<tr>
<td>CctA2</td>
<td>CTCGAGCTTCTCAGAACAGAGCG</td>
</tr>
</tbody>
</table>

a The underlined regions indicate the following restriction endonuclease sites engineered into the oligonucleotides: Ndel sites in set 1 and XhoI sites in set 2 (indicated by 1 and 2, respectively, at the end of oligonucleotide name).

b This sequence is for the restriction endonuclease EcoRI.
**Native gel electrophoresis**

Proteins from the TM and the anion-exchange chromatography-purified fractions containing MtrC, MtrA, and MtrB were separated by 5 to 10% gradient native PAGE containing 0.1% Triton X-100 using Tris-glycine buffer, pH 8.8. Gels were stained using Coomassie blue R and heme stain or transferred to nitrocellulose membranes for Western blot analysis.

**Sedimentation equilibrium analysis of MtrC/A/B**

Experiments were performed using a Beckman Optima XL-I analytical ultracentrifuge equipped with scanning absorbance optics and an An50Ti rotor. The sample density and the MtrC/A/B partial specific volume of 0.72 ml g\(^{-1}\) were estimated from the amino acid sequence using SEDNTERP software (Philo, 1997). MtrC/A/B samples were diluted to appropriate concentrations with 50 mM HEPES, 200 mM NaCl, 5% (wt/vol) Triton X-100, pH 7.6. A sample volume of 100 µl was loaded into charcoal-filled Epon double-sector cells fitted with quartz windows. Reference sectors were filled with 110 µl buffer. Sedimentation equilibrium experiments were performed at 20°C using speeds of 6,000, 8,000, and 10,000 rpm. Concentration profiles were measured at absorbance wavelengths of 530 nm (2.5 µM) and 430 nm (0.25 µM) for each MtrC/A/B sample. MtrC/A/B sample concentrations, prior to analysis, were estimated based on experimentally derived Soret band extinction coefficients for MtrA (1,320 mM\(^{-1}\) cm\(^{-1}\)) (Pitts et al., 2003) and MtrC (1,260 mM\(^{-1}\) cm\(^{-1}\)) (Hartshorne et al., 2007) and on the assumption of 1:1:1 MtrC:MtrA:MtrB stoichiometry (as suggested from SDS-PAGE analysis). Scans were recorded every 4 hours, and equilibrium was considered to have been reached when the absorbance values remained constant over a 4-h period. Once equilibrium was attained, five scans were recorded for each sample. The program ULTRASCAN 8.0 ([http://www.ultrascan.uthscsa.edu/](http://www.ultrascan.uthscsa.edu/)) was used to simultaneously fit the obtained sedimentation equilibrium profiles obtained at the three different speeds to a single non-interacting species. The
solution molecular mass of the MtrC/A/B complex (plus adsorbed detergent) was determined by fitting the absorbance data of the equilibration runs using the exponential equation:

$$C(r) = C(r_0) \cdot e^{\left[\frac{(1-\nu\rho)\omega^2}{2RT}M(r^2-r_0^2)\right]}$$  \hspace{1cm} (1)

where $C(r)$ is the concentration at radius $r$, $C(r_0)$ is the concentration at reference radius $r_0$, $M$ is the solution molecular mass, $R$ is the universal gas constant, $T$ is the temperature, $\omega$ is the angular velocity, $\rho$ is the density of the solution, and $\nu$ is the partial specific volume of the protein complex. Rearranging this equation gives:

$$\frac{d\ln(Cr)}{dr^2} = \frac{M(1-\nu\rho)\omega^2}{2RT}$$  \hspace{1cm} (2)

For visualizing the data graphically, measured absorbance can be substituted for concentration and plots of ln absorbance versus $(r^2 - r_0^2)/2$ for a single macromolecular species are expected to give a straight line, the slope of which is proportional to the solution molecular mass.

**Kinetics of MtrC/A/B oxidation with various Fe and Mn forms**

Samples of purified MtrC/A/B were chemically reduced with dithionite. Concentrated dithionite was added at small intervals until no further increase was observed at 550 nm. To this solution, ferric citrate, six-line ferrihydrite, goethite, and birnessite were added as described in the figure legends. Heme oxidation was monitored by UV/visible spectroscopy.
Formaldehyde cross-linking

*S. oneidensis* MR-1 cells were grown to mid-log phase in modified M1 medium (Ruebush et al., 2006) with 30 mM lactate and 50 mM ferric citrate as the electron donor and acceptor, respectively. The cross-linking was performed by the method of Higgs et al. (1998). Cells were centrifuged at 16,000 × g for 1 min, then washed with an equal volume of phosphate-buffered saline (PBS), pH 6.8, and suspended in phosphate-buffered saline to an optical density at 550 nm of 0.4. Cells were cross-linked for 0, 30, 60, and 120 min at room temperature with 1% formaldehyde. Cross-linked cells were centrifuged 16,000 × g for 1 min, and the pellet was suspended in 50 µl of 1× denatured gel sample buffer (Laemmli, 1970) for 5 min at 60°C. Control samples were boiled at 100°C for 10 min for 0- and 120-min samples. A total of 0.1 A_{550} unit was loaded per well (12.5 µl) on a 6 to 13% gradient SDS-PAGE. Proteins were then transferred to nitrocellulose membranes, and the blots were visualized with the Immobilon Western AP substrate (Millipore) according to the manufacturer's protocol. Molecular masses were determined using Benchmark protein standards (Invitrogen, Inc.). Prior to visualization with antibodies or by heme staining, each blot was first visualized for protein (including molecular mass standards) using Ponceau S. Heme proteins were detected by heme staining with West Pico chemiluminescent substrate (Pierce) by incubating the membrane for 5 min in a 1:1 ratio of the peroxide and chemiluminescence solutions. The membrane was then exposed to CL film (Pierce) for imaging.

**RESULTS**

**Demonstration of antibody specificity**

To monitor the interaction of selected proteins thought to be involved in DMR, we obtained antibodies to these proteins. Polyclonal antibodies were raised in rabbits against purified
OmcA, MtrC, MtrB, MtrA, CctA and CymA. The antibodies were affinity purified, and their specificity was examined with Western blots. Our previous work had shown the specificity of the antibodies to OmcA, MtrC, MtrB, MtrA, and CymA (Ruebush et al., 2006). In the present paper, we show the specificity of the antibody to CctA. Separate SDS-polyacrylamide gels were loaded with the same whole-cell lysate of *S. oneidensis* grown with ferric citrate as the TEA. The proteins were then transferred to a nitrocellulose membrane, and the blot was visualized with purified antibody to CctA (Fig. 2-1). Only a single cross-reacting band was detected, demonstrating the high degree of specificity of the antibody to CctA.

**Co-purification of MtrC, MtrA, and MtrB**

In purification of OM heme proteins, we found OmcA to be easily purified but MtrC was difficult to purify. Not only was MtrC unstable to purification but it was also difficult to purify away from MtrB under these conditions. Using antibodies to MtrC, mtrA and MtrB, we discovered that these three proteins copurify as a complex (Fig. 2-2). The TM fraction was detergent solubilized with 2% Triton X-100 and then subjected to anion exchange chromatography on Q-sepharose. Monitoring heme absorbance at 410 nm, the elution profile showed two major peaks (Fig. 2-2A). The fractions from these peaks were then subjected to SDS-PAGE, and the proteins were visualized either by heme staining or by Western blotting. The heme-stained gels showed that the first heme peak off the Q-Sepharose column had a molecular mass of 83 kDa, identical to that of OmcA (data not shown). The second heme peak from the Q-Sepharose column contained two heme bands on the SDS-polyacrylamide gel, and their molecular weights were identical to those of MtrC and MtrA (data not shown). SDS-PAGE and Western blot analysis showed that the first heme peak was OmcA (Fig. 2-2B). The second heme peak contained MtrC, MtrA, and MtrB (MtrC/A/B). Despite different pI values, these three proteins coeluted from the anion-exchange column. Little or no difference was observed in the
distribution of MtrC and MtrB in the fractions. Some MtrA eluted from the column slightly before MtrC and MtrB, suggesting its interaction with MtrB and MtrC to be slightly weaker and that it merely associates with the OM. Nevertheless, the majority of MtrA coeluted with these two proteins.

The fractions containing MtrC/A/B (fractions 42 to 54) were pooled, concentration by Amicon ultrafiltration (10-kDa cutoff), and then subjected to size exclusion chromatography on Sephacryl S-300 column using 0.9% N-octylglucoside as the detergent. A major heme peak eluted after the void volume (Fig. 2-3A) and was analyzed by SDS-PAGE (Fig. 2-3B). Coomassie blue staining showed two major proteins which corresponded to the molecular masses for MtrA and MtrC. These two major bands were shown to be heme proteins as demonstrated by heme staining (Fig. 2-3B, lane 2). Mass spectroscopy showed that the protein band just above MtrC is MtrB, and in fact, the two were not well resolved by SDS-PAGE. The presence of MtrC, MtrA, and MtrB in the major heme peak was also confirmed by Western blotting with the respective antibodies.
Figure 2-1: Western blot of whole-cell extracts from *S. oneidensis* MR-1 using affinity-purified antibodies to CctA. Whole cells were sonicated, and 8-μg samples of protein were loaded onto 12% SDS-polyacrylamide gels. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel.
Figure 2-2: (A) Q-Sepharose anion-exchange chromatography profile of TM fractions from *S. oneidensis* MR-1 monitored at heme-associated absorbance at 410 nm. (B) Western blots of protein fractions from Q-Sepharose column indicating the elution profiles of OmcA, MtrC, MtrB, and MtrA. Four replicate gels containing 12-μl amounts of protein from fractions 26 to 60 (even-numbered fractions correspond to those labeled on the elution profile) were run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Detection of antibodies used Immobilon Western AP substrate. Note that fraction 50 for the MtrC visualization is lower in intensity due to leakage of the well.
Figure 2-3: Gel permeation chromatography of the MtrC/A/B peak fractions and analysis by SDS-PAGE. Fractions from anion exchange chromatography of TM samples were pooled, concentrated, and applied to a gel filtration column. (A) Elution profile of MtrC/A/B from gel filtration (Sephacryl S-300 HR resin; Amersham Biosciences) as monitored from the heme absorbance at 409 nm. The arrow indicates the void volume (blue dextran). (B) 10% SDS-PAGE of MtrC/A/B fraction taken from the peak fraction after gel filtration and visualized with either silver staining (lane 1) or heme staining (lane 2). The predominant heme peaks correspond to MtrC (69 kDa) and MtrA (30 kDa). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel.
Native gel electrophoresis of the MtrC/A/B complex

After chromatography on Q-sepharose, the MtrC/A/B complex was subjected to native gel electrophoresis, which separates proteins based on both size and charge (Fig. 2-4A). Fractions 38 to 56 (shown in Fig. 2-3) were pooled, and 15-μl samples were loaded onto three adjacent lanes of the native gel. After electrophoresis, the gel was Western transferred to a nitrocellulose membrane. One of the lanes was visualized with anti-MtrC antibody, another with anti-MtrB antibody, and the third was visualized with anti-MtrA antibody. The nitrocellulose strips were then reassembled \textit{in silico} to reconstruct the original blot (Fig. 2-4A). MtrC in lane 1 in Fig. 2-4A was detected in three bands. As shown by the Western blots in adjacent lanes, two of the bands near the top of the gel share the same electrophoretic mobility as MtrB and MtrA. This is consistent with MtrC, MtrA, and MtrB forming a complex.

To demonstrate the specificity of the interaction between MtrC, -A, and –B on the native gel, we ran a similar gel with TM (7.5 μg protein). The TM, in addition to MtrC/A/B, contained OmcA. Probing adjacent lanes with anti-OmcA and anti-MtrC showed that these two OM proteins did not comigrate on the native gel.

Molecular mass determination of the MtrC/A/B complex

To determine the MtrC/A/B solution molecular mass in 5% (wt/vol) Triton X-100, analytical ultracentrifugation (sedimentation equilibrium) analysis was employed. Sedimentation equilibrium profiles of MtrC/A/B samples were collected at three rotor speeds (6,000, 8,000, and 10,000 rpm) for two MtrC/A/B concentrations (2.5 and 0.25 μM). At each MtrC/A/B concentration, the sedimentation equilibrium data collected at the three rotor speeds were simultaneously fitted to a single-species, non-interacting model and yielded molecular masses of 198 and 197 kDa for the 2.5 and 0.25 μM MtrC/A/B samples, respectively. A plot displaying
sedimentation behavior of the 2.5 µM MtrC/A/B sample at the three rotor speeds is displayed in Fig. 2-5A.

A plot of ln absorbance versus \((r^2 - r_0^2)/2\) (Fig. 2-5B) is expected to give a straight line, the slope of which is directly proportional to the solution molecular mass of the complex (plus adsorbed detergent) and is independent of concentration for a homogeneous single-component system (Horan et al., 1995). A straight line plot obtained for MtrC/A/B in 5% (wt/vol) Triton X-100 at 6,000 rpm is displayed (Fig. 2-5B) and demonstrates almost identical gradients at both MtrC/A/B concentrations. This analysis suggests an unchanging molecular mass over the 10-fold concentration range examined and is suggestive of a homogeneous, single-component sample.

**Electron transfer from reduced MtrC/A/B complex to various TEAs**

The reactivity of the MtrC/A/B putative complex was demonstrated by spectroscopic studies. MtrC/A/B was purified by anion-exchange chromatography and then gel filtration (Figs. 2-2 and 2-3). Incremental amounts of dithionite were added to MtrC/A/B until the complex was fully reduced as monitored by absorbance at 550 nm. This fully reduced MtrC/A/B was stable for over 40 min with no auto-oxidation in the glove box (Fig. 2-6). However, it was rapidly oxidized by aqueous ferric citrate and by the solid-phase six-line ferrihydrite, goethite, and birnessite (Fig. 2-6). While the ferric citrate resulted in oxidation of the complex in less than 15 s, complete oxidation of the complex was much slower with the solid phases: birnessite (4 min), six-line ferrihydrite (10 min), and goethite (35 min).
Figure 2-4: (A) Western blots of MtrC/A/B proteins separated by native PAGE. Fractions 38 to 56 shown in Fig. 2-3 containing the peak MtrC/A/B fractions were pooled, and 15-μl samples were applied to the gel. OM proteins MtrC, MtrB, and MtrA were probed with polyclonal antibodies. (B) TM proteins (15 μg per lane) separated using native PAGE and probed for OmcA and MtrC. Protein bands are indicated with arrows at the sides of the gels.
Figure 2-5: Analytical ultracentrifugation (sedimentation equilibrium) analysis of MtrC/A/B. A representative plot of absorbance profiles (measured at an absorbance value of 530 nm) of 2.5 μM MtrC/A/B following centrifugation runs at 6,000 (squares), 8,000 (triangles), and 10,000 (diamonds) (16 h at 20°C). The data from all three centrifugation speeds were simultaneously fitted to the equation for a single-species, non-interacting model (solid lines). Conditions of measurement were MtrC/A/B samples in 50 mM HEPES, 200 mM NaCl, 5% (wt/vol) Triton X-100, pH 7.6. Residuals between the experimental data and the fitted lines are shown in the graph at the top of the figure. (B) Representative visualization of sedimentation equilibrium profiles of two concentrations of MtrC/A/B, 2.5 μM (squares) and 0.25 μM (circles) measured at absorbance values of 530 nm and 440 nm, respectively, following centrifugation at 6,000 rpm for 16 h at 20°C. Plots of ln absorbance versus \((r^2 - r_r^2)/2\) of both MtrC/A/B samples display similar straight-line slopes, demonstrating a single-species, homogeneous system and a derived molecular mass that is independent of concentration in the range examined. Conditions of measurement were MtrC/A/B samples in 50 mM HEPES, 200 mM NaCl, 5% (wt/vol) Triton X-100, pH 7.6. Residuals between the experimental data and the fitted lines are shown in the graph at the top of the figure.
Figure 2-6: Reduction of various iron and manganese forms with chemically reduced MtrC/A/B. MtrC/A/B from the Sephacryl S-300 column was reduced by dithionite. To the reduced complex, we added no iron (control), ferric citrate (1 mM), goethite (1 mg/ml), ferrihydrite (2 mM), or birnessite (1 mg/ml). The no-iron addition was scanned at 0, 10, 20, 30, and 40 min, whereas the other samples were scanned at the time intervals shown in the figure. The arrow indicates direction of spectral change going from fully reduced to fully oxidized MtrC/A/B complex.
Localization of MtrA

Our work suggests that MtrA, while weakly associated with MtrB/C is associated with the OM through these protein-protein interactions. Beliaev et al. (5) have shown more of a periplasm localization. To investigate this possibility, TM, OM, CM, and the periplasm were characterized. The TM, OM, and CM fractions were isolated from ferric citrate-grown cultures of *S. oneidensis* MR-1. Marker enzyme assays were used to determine the degree of separation between the OM and CM. The ratio of NADH oxidase (marker enzyme for the CM) activities in the CM to OM was $0.38 \mu$mol/min/mg protein to $0.05 \mu$mol/min/mg protein. This ratio of 7.6 is comparable to previous results (8), showing that the membrane fractions were well separated. The TM, OM, and CM fractions were then subjected to SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Figure 2-7A shows that MtrA and MtrB were both found in the TM fraction and the OM fraction. In contrast, the CM protein, CymA, was found in the CM and TM fractions.

To isolate the periplasmic fraction, cells were osmotically shocked and then centrifuged. The supernatant contained the periplasmic fraction and the pellet contained the whole spheroplasts. Heme staining of Western blots from SDS-PAGE of the periplasmic fraction showed no heme bands with a molecular mass equal to that of MtrA (Fig. 2-7B). The periplasm did contain heme bands with molecular masses of 10 kDa and 60 kDa, which are consistent with previous results (59). In contrast, heme staining of lanes containing whole cells or spheroplasts (prior to osmotic shock) showed a 32-kDa heme band (Fig. 2-7B). Western blot analyses of the samples indicated that MtrA is found in whole cells and in spheroplasts. These results are consistent with MtrA being associated mainly with the OM through weak protein-protein interactions at the periplasmic face.
Formaldehyde cross-linking

Protein-protein interactions were further probed by formaldehyde cross-linking. Whole cells were applied to SDS-polyacrylamide gels and antibodies were used to identify the cross-linked proteins. The gel showed four prominent bands which correspond to the molecular masses of OmcA, MtrC, MtrB, MtrA and CymA (Table 2-2). Using antibodies specific for each of these proteins, identical bands were detected at the corresponding molecular masses (Figure 2-8). Addition of 1% formaldehyde to the whole cells resulted in formation of new heme bands of approximately 160, 151, 136, and 59 kDa (Fig. 2-8). The intensity of the new cross-linked bands increased with duration of formaldehyde treatment (Fig. 2-8). The rightmost lane of the gel shown in Figure 2-8 (and also Fig. 2-9) is the final time point boiled to demonstrate the reversibility of the formaldehyde cross-linking.

Antibodies were used to identify the cross-linked proteins. OmcA cross-reactive material was detected in bands at 83 kDa (non-cross-linked OmcA) and approximately 130 and 160 kDa (Fig. 2-9). Two other bands were detected at molecular masses higher than 220 kDa, beyond the resolution range of our gels. MtrC cross-reactive material was detected at the 75-kDa band (non-cross-linked) and at the 160-kDa band. This 160-kDa band is identical in size to the OmcA cross-reactive band and is thus consistent with a one-to-one complex of OmcA and MtrC (calculated molecular mass of 158 kDa).

MtrB cross-reactive material was detected at 76 kDa (native non-cross-linked), 151 and 136 kDa (Fig. 2-9). The cross-linking was extensive with MtrB. The monomer proteins disappeared during the time course of the experiment. Western blots using MtrA antibody show the same higher-molecular-mass complexes detected with anti-MtrB: approximately 136 and 151 kDa (Fig. 2-9). The 136-kDa band, although cross-reactive with both MtrA and MtrB, was larger than a one-to-one complex (which would be 108 kDa). Thus, proteins of approximately 28 kDa and 15 kDa appear to be found in the complex. These two bands (136 and 151 kDa) were also
detected in the heme-stained cross-link gel blot (Fig. 2-8). The monomeric form of MtrA was completely cross-linked during the time course of the experiment.

Cross-linking of CM and periplasmic proteins CymA and FDH was also examined. CymA cross-reactive material was detected at 19 (non-cross-linked CymA), 32, 54, 95, and 116 kDa (Fig. 2-9). With FDH, we used antibodies raised to the H-subunit of the respiratory formate dehydrogenase (FdnH). While we were able to detect cross-linking of the H-subunit with what would be the G- and I-subunits by molecular mass, no other cross-reactive bands could be detected (data not shown). Since these proteins are thought to exchange electrons via the menaquinone pool rather than direct protein-protein interactions, this result is expected and gives confidence in the specificity of the interactions detected by our cross-linking experiments.

To further examine electron transfer at the CM using formaldehyde cross-linking, the interaction of CymA and CctA was examined. Periplasm-localized CctA has a molecular mass of 12.1 kDa. If CctA is cross-linked to CymA, it would yield a complex with a molecular mass of approximately 30 kDa, which is close to the 32-kDa band observed using the CymA antibody (Fig. 2-9). Using a higher percentage SDS-polyacrylamide gel, we examined more closely the possible cross-linking between CctA and CymA. The results clearly show that although the molecular masses of the two cross-reactive bands are close, they are slightly different. The results with the CctA antibody also did not reveal interactions between CctA and MtrA (Fig. 2-10).
Figure 2-7: (A) Separation of proteins from *S. oneidensis* MR-1 cultures into the CM, OM, and TM fractions. MtrA is shown to fractionate with the OM as does MtrB. CymA associates with the cytosolic membrane. (B) Heme staining of *S. oneidensis* cell extracts (lane 1), supernatant of washed cells (lane 2), and periplasmic fraction (lane 3). (C) Western blot using anti-MtrA of the same fractions as in panel B. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel in panels B and C.
Table 2-2: Physical properties of selected proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (kDa)</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CctA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.4</td>
<td>4.6</td>
</tr>
<tr>
<td>CymA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21</td>
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</tr>
<tr>
<td>MtrA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32</td>
<td>7.8</td>
</tr>
<tr>
<td>MtrB</td>
<td>76</td>
<td>4.4</td>
</tr>
<tr>
<td>MtrC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75</td>
<td>5.5</td>
</tr>
<tr>
<td>OmcA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83</td>
<td>6.2</td>
</tr>
<tr>
<td>FdnH</td>
<td>33</td>
<td>5.2</td>
</tr>
</tbody>
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<sup>a</sup> Heme protein.
Figure 2-8: Heme staining of cells cross-linked by 1% formaldehyde and transferred to a nitrocellulose membrane. Lane 1, untreated cells that were boiled; lane 2, cells treated for 0 min; lane 3, cells treated for 30 min; lane 4, cells treated for 60 min; lane 5, cells treated for 120 min; lane 6, cells treated for 120 min and boiled. Each lane was loaded with 1 absorbance unit of cells to increase the concentration of low-abundance heme proteins. The arrows on the left indicate the bands that are observed, and the heme proteins OmcA, MtrC, MtrA, and CymA are identified. The new heme protein bands as a result of treatment with formaldehyde are indicated by arrows without labels. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the membrane.
Figure 2-9: In vivo cross-linking of cells grown with ferric citrate as the terminal electron acceptor probed for complexes containing OmcA (A), MtrC (B), MtrB (C), MtrA (D), and CymA (E). Lanes 1, untreated cells that were boiled; lanes 2 to 5, cells treated with 1% formaldehyde for 0 min (lanes 2), 30 min (lanes 3), 60 min (lanes 4), and 120 min (lanes 5); lanes 6, cells treated with 1% formaldehyde for 120 min and boiled. The positions of formaldehyde cross-linked complexes are indicated by arrows to the left of the gels, and the molecular mass markers are indicated to the right of the gels.
Figure 2-10: In vivo cross-linking of cells grown with ferric citrate as the terminal electron acceptor probed for complexes containing CctA and CymA. Lanes 1 and 7, untreated cell and boiled; lanes 2 and 8, cells treated with 1% formaldehyde for 0 min; lanes 3 and 9, cells treated with 1% formaldehyde for 30 min; lanes 4 and 10, cells treated with 1% formaldehyde for 60 min; lanes 5 and 11, cells treated with 1% formaldehyde for 120 min; lanes 6 and 12, cells treated with 1% formaldehyde for 120 min and boiled. The positions of formaldehyde cross-linked complexes are indicated by arrows at the sides of the gels, and the positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel.
DISCUSSION

In the present study, we have characterized protein-protein interactions of the OM, periplasm, and CM of *S. oneidensis* using protein purification and cross-linking methods. Our results show the following for the first time. (i) MtrC, MtrA, and MtrB exist as a complex in the OM. (ii) The purified MtrC/A/B complex has a molecular mass of 198 kDa, consistent with a 1:1:1 stoichiometry of the MtrC, MtrA, and MtrB subunits. (iii) MtrC/A/B has aqueous Fe and Fe and Mn solid-oxide reduction capabilities. (iv) MtrA is associated with the OM, presumably at the periplasmic face but is not freely diffusible in the periplasm. (v) MtrC weakly interacts with OmcA and does not form a complex that is stable during purification using the methodologies that yield a stable MtrC/A/B complex. While our negative results do not negate such interactions, we have not been able to demonstrate (i) interaction between CctA and CymA and (ii) interaction between CctA and MtrA. The implications of these findings are discussed below.

Electron transport through the CM

A model of the protein-protein interactions consistent with these new and previously published results is shown in Figure 2-11. Proton motive force-generating respiratory electron transfer in the CM is initiated by oxidation of periplasmic formate to CO₂ by FDH (Scott and Nealson, 1994). Reduced FDH then reduces menaquinone by two electrons to menaquinol (translocating 2 H⁺ to the periplasm), which in turn transfers its electrons to CymA (Richardson and Sawers, 2002). Our cross-linking experiments did not reveal any protein partners for FDH or CymA, which is consistent with these proteins interacting with the MQ/MQH₂ pool rather than directly with each other. CymA, though, plays a central role in respiration, since knockouts of CymA are defective in utilization of all TEAs except for trimethylamine N-oxide by *Shewanella* (Myers and Myers, 1997; Myers and Myers, 1993).
Figure 2-11: Model depicting the interactions of OM proteins as a result of cross-linking and native protein methods. Interactions of CymA and CctA are yet to be defined. Menaquinone is shown in the membrane and shown as “Q” in the figure. The interaction of MtrC/A/B as a complex has been demonstrated in this work. We and others (Shi et al., 2006) have demonstrated the interaction of OmcA and MtrC. MtrA faces the periplasm, while OmcA and MtrC are extracellularly exposed lipoproteins and may potentially contact mineral oxides. The reduction potentials of various proteins and electron acceptors are shown to the left. Reduction potentials were obtained from the following: formate (White, 2000), menaquinone (Patel and Willson, 1973), MtrA (Pitts et al., 2003), CctA (Gordon et al., 2000), EDTA-Fe³⁺ (Schwarzenbach and Heller, 1951), NTA-Fe³⁺, goethite and citrate-Fe³⁺ (Thamdrup et al., 2000), and pyrolusite (Stone and Ulrich, 1989). Decoupling of the electron transfer to insoluble TEAs from the proton pump at the CM implies that electron transfer to solid oxides does not provide energy to the cell but serves only to ground the cell by dumping electrons.
Electron transport through the periplasm

For soluble TEAs, electron transfer has been proposed to occur at the CM in the periplasm (Maier et al., 2004; Myers and Myers, 2000), which is consistent with our in vitro iron reduction studies (Ruebush et al., 2006). For solid-phase TEAs, electrons of CM-localized CymA must traverse the periplasm and through the OM. Electron transfer through the periplasm occurs either by continuous contact of proteins spanning the periplasm or by shuttling electron carriers in the periplasm. Two candidate proteins that can span the periplasm or can act as shuttles are MtrA and CctA. Our cross-linking experiments with the CymA antibody, while not excluding the possibility, were not able to show any interaction between these two proteins. In contrast, high-molecular-mass complexes are observed with the CymA antibody (34, 54, 95, and 116 kDa) that might be involved with electron shuttling in the periplasm; however, these complexes could not be identified.

The apparent lower affinity of MtrA for the MtrC/A/B complex could confer upon this protein the ability to act as a reversible periplasmic electron shuttle (i.e., MtrA reversibly associates/dissociates with MtrB/C depending on the redox state of the protein). However, if MtrA served as a shuttle between the OM and CM, one would predict major localization as a freely diffusible protein in the periplasm, and this was not observed. Our results therefore argue against, but do not definitively preclude, MtrA being a diffusible periplasmic electron shuttle. The periplasm has an estimated inner membrane-to-OM width of around 150Å. Recent structures of the 20-kDa pentaheme NrfB protein reveal an electron wire of around 40Å (Clarke et al., 2007). Thus, the 40-kDa decaheme MtrA could conceivably have a heme wire of some 80 Å allowing it to penetrate some distance from the OM into periplasm, but possibly insufficient to directly contact CymA at the inner membrane. Our observations are then at present best interpreted as revealing that MtrA, being part of the MtrC/A/B complex, serves as the protein that receives electrons at the OM.
Another possible periplasmic electron shuttle is CctA which is periplasm localized based on signal sequence predictions (Beliaev et al., 2001). Our cross-linking experiments were not able to identify protein-protein partners with CctA. Its molecular mass (10 kDa) is approximately consistent with the difference between the 151- and 136-kDa bands which are known to contain MtrA and MtrB. However, further characterization with a CctA antibody revealed only a 28-kDa cross-reactive band. In regard to its interaction with CM proteins, the 28-kDa protein also does not correspond with a complex which would be formed with CymA. Thus, our experiments were not able to identify the electron transfer chain through CctA.

Electron transport through the OM

In contrast to the periplasm, our results provide a well-defined model for the OM (Fig. 2-11). In terms of OM proteins, OmcA could be easily purified; in contrast, MtrC was not only difficult to purify, it was also unstable with decreasing yields during the purification process. This is consistent with an obligate association with other protein-forming complexes. Anion-exchange chromatography suggests that these other subunits are MtrA and MtrB. These proteins coeluted with MtrC despite pI values of 7.78 (MtrA), 4.42 (MtrB), and 5.48 (MtrC). With a pI of 6.23 for OmcA, the elution order from an anion-exchange column should be MtrA, OmcA, MtrC, and then MtrB (first to last). In contrast, OmcA consistently eluted first, before MtrA. Furthermore, despite a calculated pI difference of 1.06, MtrC and MtrB coeluted as one peak, consistent with a strong complex between the two proteins. Although some MtrA eluted before MtrB and MtrC, most eluted together with those proteins, consistent with a three-subunit OM complex.

The existence of MtrC/A/B as a complex is also consistent with native gel electrophoresis. Separating based on size and charge, MtrC, MtrA, and MtrB migrated as (at least) two common bands. The presence of additional bands indicates the presence of more than one form of the protein in addition to potential monomers. Finally, an MtrC/A/B complex is
consistent with the fact that these three proteins are expressed as part of an operon (Beliaev et al., 2001).

The observed molecular mass of the MtrC/A/B complex (197 to 198 kDa) is close to that predicted for a 1:1:1 MtrC:MtrA:MtrB stoichiometry based on primary sequence analysis and sedimentation equilibrium analysis of purified MtrC (~85 kDa) and MtrA (~40 kDa) (data not shown) determined in identical buffer/detergent conditions. MtrB has not yet been characterized by analytical ultracentrifugation, as pure protein is not available, but from primary sequence analysis, a molecular mass of 75 kDa is predicted. Thus, the total predicted mass of a 1:1:1 complex of MtrC/A/B is ~200 kDa, which is very close to the experimentally derived figure. When purified, this complex has the ability to reduce multiple metal forms, including aqueous and solid species (ferric citrate, six-line ferrihydrite, goethite, and birnessite). The reactivity of the metal forms vary where the amount of time needed to fully oxidize the MtrC/A/B complex span from <15 seconds for the aqueous species to up to 35 min for solid phases.

Formaldehyde protein cross-linking experiments indicated little, if any, cross-linking between MtrB and MtrC, though these two proteins where shown to exhibited the highest degree of association in chromatographic and electrophoretic separation. The lack of cross-linking between MtrB and MtrC does not negate interactions, since the cross-linking reaction is dependent upon basic side chain residues in close enough proximity for formaldehyde cross-linking to occur (Prossnitz et al., 1988). In contrast, although MtrA and MtrB were extensively cross-linked, MtrA exhibited a lower binding affinity, as it was partially separated from MtrB and MtrC. Even though the binding affinity of MtrA is lowest with the putative MtrC/A/B complex, it is the most efficiently cross-linked, which is consistent with MtrA and MtrB having reactive amines in close proximity.

Our results also show that MtrC/A/B interacts with OmcA through MtrC, in agreement with the findings of Shi et al. (2006). These workers reported the interaction between OmcA and
MtrC as a high-affinity complex characterized by a dissociation constant of less than 0.5 μM. While our results support an interaction between these two proteins, under our experimental conditions a less stable interaction was observed compared to that of MtrC with MtrA and with MtrB. For example, our results show that the interaction between OmcA and MtrC is disrupted by anion-exchange chromatography, whereas the MtrC/A/B interaction is not. Also, native gel electrophoresis suggests that the interaction between these two proteins is low in affinity and transient in nature. Thus, the stability of the interaction between OmcA and MtrC may be dependent on growth and/or experimental conditions, and this requires further study.

**Electron transfer to solid-phase oxides**

In the absence of soluble TEAs, electrons are transported across the periplasm to the OM, where their interactions with the TEAs most likely occur through OmcA and MtrC. Based on current knowledge OM-localized electrons cannot participate in proton motive force-generating electron transfer (Fig. 2-11). Thus, these OM electron carriers serve only to ground the cell by transferring electrons out. To act as effective grounding wires, the electron carriers across the OM would exhibit very low substrate specificity. Such may be the case, since *Shewanella* can apparently reduce both solid Mn and Fe oxides with the same suite of enzymes. Furthermore, global transcriptome analysis shows no significant differences in gene expression by *S. oneidensis* when grown on solid ferrihydrite, MnO₂, or colloidal Mn (Beliaev et al., 2005). In addition, no mutants that can use Mn solid oxide as a TEA but not Fe solid oxide have been isolated (T. J. DiChristina, personal communication). Efficient grounding for the cell may also occur if both of the OM heme proteins provide separate routes of electron transfer (Lies et al., 2005).

Importantly, if the reduction of insoluble TEAs at the OM is decoupled from proton pumping at the CM, then microbes cannot exploit the greater reduction potential of solid-phase TEAs for energy generation. Consistent with this, Kostka et al. (1995) found no difference in cell
yield (measured as carbon yield) for \textit{S. oneidensis} grown on soluble ferric citrate versus insoluble iron oxides when calculated per mole of electrons utilized. Furthermore, based upon graphed data reported by DiChristina et al. (2002), approximately identical yields (~4.1 \textit{versus} 4.9 \times 10^{13} cells/mol of electrons) were observed when \textit{S. oneidensis} was grown on ferric citrate versus Mn oxide, respectively.

Despite the observations of apparently equal growth yields for aqueous versus solid-phase TEAs in the laboratory, in nature, Mn oxides are generally reduced earlier (e.g., at shallower depths) than Fe oxides (Stumm, 1996). This has been explained by arguing that organisms that utilize TEAs with higher reduction potential outcompete organisms utilizing TEAs lower on the redox ladder due to higher growth yields. However, based upon Figure 2-11, utilization of Mn before Fe oxides must only reflect that solid-phase metal oxides with higher reduction potential are used up first simply due to the faster kinetics of reduction of these phases. To maximize cell yield, microorganisms can regulate the use of soluble TEAs through gene expression, but cell yield is not affected by gene regulation of the use of solid-phase TEAs. Transfer of electrons occurs faster into metal oxides with higher reduction potential due to kinetics (kinetic control) but not through gene-enabled substrate-specific binding between the OM proteins and the insoluble oxides (regulatory control).
Chapter 3

KINETIC CHARACTERIZATION OF OMCA AND MTRC, TERMINAL REDUCTASES INVOLVED IN RESPIRATORY ELECTRON TRANSFER FOR DISSIMILATORY IRON REDUCTION IN SHEWANELLA ONEIDENSIS MR-1

INTRODUCTION

Microbial processes play a central role in the cycling of organic and inorganic compounds on Earth. A select number of these compounds are assimilated by organisms and used as building material for the cell. In addition, a large number of organic and inorganic compounds are used as electron donors and acceptors for respiratory metabolism. Iron, one of the most abundant elements on the Earth’s crust, is both assimilated by life and utilized as an electron donor (Fe^{2+}) and acceptor (Fe^{3+}) in respiratory metabolism. Due to the importance of iron, microorganisms directly impact the fate and transport of iron, and these Fe impacts indirectly influence the (bio)geochemical cycles of many other elements, including the carbon cycle (Banfield, 1997). Furthermore, respiratory metabolism evolved in microorganisms prior to the emergence of oxygenic photosynthesis and was very versatile in using a large number of both organic and inorganic TEAs (Madigan, 2000). Therefore, the use of iron as a TEA predates the use of molecular oxygen among organisms on earth. When metals are used as the TEA, this process is referred to as dissimilatory metal reduction (DMR), or in the case of iron, dissimilatory iron reduction (DIR). Interest in microbial DMR has intensified upon discovery of the ability of DMR bacteria to catalyze reactions of environmentally-relevant contaminants, including radionuclides such as U(VI) (Anderson et al., 2003; Liu et al., 2002; Liu et al., 2006; Lovley et al., 1992; Marshall et al., 2006; Sani et al., 2004; Shelobolina et al., 2007).
DMR has been extensively studied in the facultative anaerobe *Shewanella oneidensis* MR-1 due to its respiratory versatility (for review, see-(Lovley, 1993; Lovley et al., 2004; Nealson et al., 2002; Shi et al., 2007)). This bacterium also has the ability to utilize soluble and insoluble forms of iron and manganese as TEAs (Fredrickson et al., 2000; Klonowska et al., 2005; Lovley and Phillips, 1988; Madigan, 2000; Moser et al., 1996; Myers and Nealson, 1988). Genetic and biochemical studies show that reduction of the soluble TEAs occurs at the cytoplasmic membrane (CM), the location of the proton motive force-generating electron transport. However, in utilization of insoluble oxides, CM-localized electrons need to traverse the periplasm and the outer membrane (OM) to the extracellular matrix. Three mechanisms of transfer of OM-localized electrons to metal oxides have been hypothesized: 1) electron transfer through direct contact to the metal oxide, 2) cyclic reduction/oxidation of electron shuttles and 3) solubilization of the metals with chelators followed by diffusion to and across the OM to the CM. For review of proposed mechanisms, see (Gralnick and Newman, 2007).

While there are very few reports of bacteria producing chelators for DIR (Taillefert et al., 2007), there are more data to support the other two mechanisms. For example, using atomic force microscopy (AFM), Lower et al. (2007) measured binding between both OM heme-proteins OmcA and MtrC with the iron oxide hematite and reported that OmcA shows a greater affinity toward hematite than does MtrC. These workers suggested that this tight binding is a pre-requisite for direct electron transfer. Xiong et al. (2006) also showed tight binding between OmcA and hematite by using dynamic light scattering and fluorescence correlation spectroscopy. Previous work from our laboratory (Ruebush et al., 2006a; 2006b) demonstrating the rates of metal oxide reduction by membrane fractions from *Shewanella* also supported a direct contact mechanism. Direct contact between cellular components and the iron oxides is also consistent with the nanowire mechanism proposed by Gorby et al. (2006).
In support of the electron shuttle hypothesis, two laboratories independently showed that *S. oneidensis* produces extracellular flavins, which may act as electron shuttling agents (Marsili et al., 2008; von Canstein et al., 2008). The involvement of flavins as an electron shuttle would explain previous reports that *Shewanella* secretes a small quinone-like compound involved in electron transfer (Newman and Kolter, 2000) and can utilize sterically sequestered iron located in alginate beads (Lies et al., 2005).

While the previous studies described above provide compelling evidence for their respective mechanisms, none of the mechanisms have been subjected to detailed kinetic analysis. Kinetic studies while not able to prove a mechanism, can be used to eliminate proposed mechanisms if rates of a reaction measured with purified enzymes, as determined *in vitro*, cannot account for the rates observed in the whole cells. We previously described the importance of kinetic studies and how to apply these kinetic studies to DIR (Brantley et al., 2006; Ruebush et al., 2006a; 2006b). Briefly, rate constants are determined with *in vitro* studies using purified enzymes. The kinetics of the enzyme of interest is then studied in the whole cell (or in subcellular fractions). The rate constants are then compared between these systems to determine whether the rate of the reactions catalyzed by the enzyme can account for the rates observed in the cell.

In our previous work, we performed kinetic studies on membrane fractions of *S. oneidensis* to develop an *in vitro* system for examination of solid-Fe-oxide reduction (Ruebush et al., 2006a; 2006b). In the present study, this kinetic characterization is extended to purified enzymes and with whole cells for the purpose of determining kinetic competence. While our major focus is on the reduction of insoluble iron forms, for comparative purposes and as a control to validate our studies with the insoluble oxides, our study also includes kinetic analysis of soluble chelated iron forms. We have focused our kinetic studies on two OM-localized hemeproteins, OmcA and MtrC. Previous genetic knock-out studies show the importance of these enzymes in metal oxide
reduction as potential terminal metal reductases (Beliaev et al., 2001; Myers and Myers, 2004; Myers and Myers, 2003). We describe both transient- and steady-state kinetic analyses. While both hemeproteins have been studied by stop flow (Shi et al., 2006; Wang et al., 2008) and by steady-state methods in whole cells (Borloo et al., 2007), no study has yet attempted to infer mechanisms from scale-up kinetic studies (where kinetic constants from transient-state are compared with those obtained from steady-state). Our studies are thus performed at three different kinetic scales: 1) transient-state iron reduction using purified OmcA and MtrC, 2) steady-state kinetics with purified total membrane (TM) fractions, and 3) steady-state whole cell kinetics.

MATERIALS AND METHODS

Materials

Xanthine oxidase from buttermilk, Triton X-100 SigmaUltra, ferric citrate, NTA (N,N-bis[carboxymethyl]glycine) and riboflavin were purchased from Sigma. CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) was purchased from Soltec Ventures, Inc. EDTA ((ethylenedinitrilo)tetraacetic acid disodium salt, dihydrate) was purchased from EMD Chemicals Inc.

Bacterial growth

Shewanella oneidensis MR-1 (ATCC 700550) was maintained on Luria-Bertani (LB) agar. Single colonies were used to inoculate 5 ml aerobic LB media and then allowed to grow at 30°C to an optical density of 0.6 at 600 nm. The culture was centrifuged at 10,000×g for 15 minutes and the pellet was washed with HEPES buffered saline (HBS, 0.7% NaCl in 10 mM HEPES, pH 7.4) and then re-suspended in one tenth the volume in the same solution. This cell
suspension (1 ml) was added to a 4.5 L of defined argon-purged M1 minimal medium as per Myers (Myers and Nealson, 1988) and harvested when the ferrous ion concentration reached 35 mM (Ruebush et al., 2006).

**Total membrane isolation**

The TM fraction was isolated according to Ruebush et al. (2006) modified from (Myers and Myers, 1992) using lysozyme, EDTA and 5% Brij.

**OmcA and MtrC purification**

TM (10 mg/mL) fractions were solubilized with 50 mM HEPES pH 7.5 and 5% Triton X-100 and subjected to anion exchange chromatography (Q-sepharose fast flow, Amersham) using a linear gradient of 0-100% B at 1 ml/min over 160 min (Buffer A= 50 mM HEPES pH 7.5, 5% Triton X-100, Buffer B = Buffer A + 0.5 M NaCl). The OmcA fraction, as determined by Western blot analysis was isolated and subjected to MonoQ chromatography. OmcA was loaded onto a MonoQ column (Amersham) and eluted with a linear gradient of 0-100% buffer B at 1 ml/min over 160 min (Buffer A= 50 mM HEPES pH 7.5, 0.2% Triton X-100, Buffer B = Buffer A + 0.5 M NaCl).

While the first heme peak (as detected by 409 nm absorbance) contained OmcA, the second heme peak that eluted from the Q-sepharose column contained the MtrC/A/B complex (Ross et al., 2007) as visualized by heme staining. To isolate MtrC from the purified complex, we first exchanged detergents by binding the complex to a Q-Sepharose column (Q-sepharose fast flow, Amersham) and then washed with three column volumes of buffer A (50 mM HEPES pH 7.5, 0.5% CHAPS). MtrC was eluted with a linear gradient of 0-100% buffer B at 1 ml/min over 160 min (Buffer B = Buffer A + 0.5 M NaCl).
Extinction coefficients for OmcA and MtrC were determined with the pyridine hemochromogen assay (Berry and Trumpower, 1987). The extinction coefficient for OmcA at 409 nm is equal to 1,670,000M⁻¹cm⁻¹ and for MtrC, ε₄₀⁹ = 1,440,000M⁻¹cm⁻¹.

For standards on Western blots, recombinant MtrC was purified as inclusion bodies as previously described by Ross et al. (2007). MtrC content in the purified inclusion bodies was determined by its UV absorbance associated with its aromatic amino acids. The extinction coefficient at 280 nm (ε₂₇₆ = 48,800 M⁻¹cm⁻¹) was determined as per Edelhoch (Edelhoch, 1967).

**Transient-state kinetics**

OmcA and MtrC were reduced enzymatically with xanthine oxidase (Cheng et al., 2007). Stock solutions of xanthine oxidase (2 units/mL), xanthine (0.5 mM) and benzyl viologen (1 mM) were purged with oxygen-free argon and transferred to a Coy anaerobic chamber (3% hydrogen, N₂ atmosphere). Reaction mixtures contained purified OmcA (or MtrC) (0.5 μM), xanthine (0.15 mM), benzyl viologen (1.5 μM), and xanthine oxidase (0.01 units/ml).

Reduction was monitored by UV/Vis spectroscopy and was complete after approximately 2 hours. Fully reduced protein was then loaded into an Applied Photosystems (Surrey, U.K.) SX.18MV stopped flow apparatus. Heme oxidation was monitored with a diode array detector and rates were obtained by monitoring the decrease in 550 nm absorbance associated with the ferroheme oxidation.

**Western blot quantification of MtrC and OmcA**

Purified MtrC or OmcA was used as a protein standard for SDS-PAGE/Western blots and ranged from 1.8 x 10⁻¹¹ to 1.8 x 10⁻¹² moles per lane. OmcA and MtrC were quantified in TM fractions (0.5 μg per lane) and whole cell (7.5 μl of cell suspension (2.25 x 10⁷ total cells)). After
separation of the proteins using SDS-PAGE the samples were transferred to a nitrocellulose membrane (Whatman, Protran BA 83) and protein bands visualized with alkaline-conjugated anti-rabbit IgG and AP substrate (Immobilon™ Western, Millipore). Blots were then scanned and analyzed using ImageJ64 public software from the NIH (http://rsbweb.nih.gov/ij/).

Iron reduction assays with whole cells and TM fractions

Reduction assays using TM fractions were performed as described previously (Ruebush et al., 2006a; 2006b). Reactions contained 0.1 mg/ml TM protein, 10 mM formate and Fe(III) forms as specified in the figure legends, in 100 mM HEPES pH 7.0. Reactions were initiated by addition of formate. At specified times, 100 μl aliquots were removed and quenched with 33 μl of 2 N HCl. Acidified samples (50 μl) were added to 950 μl of a ferrozine solution (1g/L ferrozine in 100 mM HEPES pH 7) and ferrous ion concentration was determined spectrophotometrically at 562 nm using an extinction coefficient of 27.9 mM⁻¹ cm⁻¹ (Stookey, 1970).

Whole cell experiments were identical to TM incubation except that whole cells replaced the TM fraction. Isolation of whole cells was performed anaerobically at 4°C. The cells were centrifuged at 10,000 × g for 15 minutes. The cells were then washed with M1 minimal medium and then suspended in 1/30 of the original volume in defined medium. From this stock, 100 μl (or 3 x 10⁸ cells) was added to the iron-reduction reaction mixtures.

In experiments in which spent medium was added (see below for spent media preparation), either 450 μl or 900 μl were added to the reaction mixture.

Flavin quantification

TM or whole cells were added to an equal volume of 5% (w/v) trichloracetic acid, mixed for 30 s, and placed on ice in the dark for 60 min. Samples were centrifuged at 10,000 × g for 12 minutes to pellet the precipitated protein, and the supernatant was analyzed using HPLC (Midttun
et al., 2005). Samples were separated by reversed-phase (HP 1090 Liquid chromatogram) using a Discovery C18 column (Supelco, 15 cm x 4.6 mm, 5 μm) with a 2 cm guard column (Pelliguard LC-18, Supelco). Flavin content was determined by monitoring its associated fluorescence (HP 1046A Programmable fluorescence detector) with an excitation wavelength of 440 nm and emission wavelength of 525 nm (Woodcock et al., 1982). A gradient of methanol versus 20 mM ammonium acetate, pH 5.4 was used to determine the presence of flavin (von Canstein et al., 2008). The following gradient timetable was used to separate samples: 0-6 min (5% methanol), 6-28 min (increase from 5% to 37% methanol), 28-33 min (increase from 37% to 99% methanol), 33-35 min (hold at 99% methanol), and 35-36 min (rapid decrease from 99% to 5 % methanol). Samples were analyzed for fluorescence at 440 nm using Chemstation (HP) and compared to a riboflavin standard (Sigma).

**Spent medium preparation**

After anaerobic cultures (described above) reached 35 mM Fe(II), and cells were removed, the spent ferric citrate medium was placed over a Chelex® 100 Resin column (Biorad, 100-200 mesh, sodium form) to remove any excess iron. The column was equilibrated with oxygen-free buffer (100 mM HEPES, pH 7.5) in the anaerobic chamber. Once equilibrated, spent medium was added to the column; collection started after two column volumes passed through the column.

**Miscellaneous methods**

Protein content was determined using the method of Lowry (Lowry et al., 1951) with bovine serum albumin (BSA) as a standard. Goethite was synthesized according to the method of Schwertmann and Cornell (Schwertmann, 1991) and the Brunauer-Emmett-Teller (BET) surface area was estimated to be 33.9 m²/g (Brunauer, 1987).
RESULTS

Purification of native OmcA and MtrC

For transient-state kinetic studies, OmcA and MtrC were purified from *S. oneidensis*. SDS-PAGE analysis showed that OmcA (Fig. 3-1) was purified to greater than 95% homogeneity as demonstrated by silver (Fig. 3-1A) or heme staining (Fig. 3-1B). MtrC is equally pure (Coomassie blue, Fig. 3-1C and heme, Fig. 3-1D).

Transient-state kinetic studies of soluble iron forms with OmcA and MtrC

Kinetic constants between soluble iron and OmcA or MtrC were measured with a stopped flow kinetic apparatus. Reaction rates were obtained by monitoring the absorbance change associated with oxidation of the ferroheme to the ferriheme either at 419 nm or 550 nm (Fig. 3-2). While both OmcA and MtrC contain 10 hemes/molecule, the data did not indicate significant differences in heme reactivity and thus the results were fit with a single exponential. Rate constants were obtained from plots of observed rates versus Fe concentration: the slopes of linear fit lines are equal to forward rate constants (k₁) and the y intercepts are equal to the reverse rates (k₋₁). The reaction scheme below depicts the interaction of reduced enzyme (ferroheme) with the oxidized iron chelate (encircled Fe³⁺).

\[
\text{Fe}^{3+} + \text{Ferroheme} \xrightleftharpoons[k_-]{k_1} \text{Fe}^{2+}\text{Ferriheme}
\]

Rate constants obtained for both OmcA and MtrC with the three ferric chelates, citrate, NTA and EDTA, (Fig. 3-2), decreased in the order: EDTA-Fe³⁺ > NTA-Fe³⁺ > citrate-Fe³⁺ (Table 3-1 and Table 3-2).
Quantification of OmcA and MtrC

We previously performed steady-state kinetic analysis of iron reduction by membrane fraction and expressed rates as mol Fe reduced per mg of protein per min (Ruebush et al., 2006). To obtain molecular rate constants from such studies (e.g. mol Fe reduced per mol enzyme per min), the molar concentration of enzyme must be determined. Western blots were used with OmcA and MtrC standards to quantify the concentration of these proteins in TM and whole cell preparations. The specificity of these antibodies has been previously demonstrated (Ross et al., 2007). A sample Western blot of MtrC is shown in Fig. 3-3. Quantification by such methods yielded a concentration in TM preparations of $2.2 \times 10^{-10} \pm 2.5 \times 10^{-12}$ moles OmcA and $1.9 \times 10^{-10} \pm 4.3 \times 10^{-12}$ moles MtrC per mg of TM protein. For whole cells, our Western blots yielded $7.9 \times 10^{-20} \pm 9.5 \times 10^{-21}$ moles of OmcA and $1.2 \times 10^{-19} \pm 4.5 \times 10^{-20}$ moles of MtrC per cell which is similar to what was determined by Borloo et al. (Borloo et al., 2007) ($4 \times 10^{-21}$ moles of OmcA per cell) based on heme content of OmcA and MtrC mutants.
Figure 3-1: SDS-PAGE of purified OmcA and MtrC. OmcA (Lanes 1 and 2) and MtrC (Lanes 3 and 4) were subjected to SDS-PAGE on 10% polyacrylamide and visualized using silver staining (lane 1), Coomassie blue (lane 3) or heme staining (lanes 2 and 4). Molecular mass markers and their mass (in kilodaltons) are to the left of the gel.
Figure 3-2: Reaction of ferroOmcA with ferric iron chelates. The reaction between reduced OmcA (0.35 μM) with 0.1 mM NTA-Fe$^{3+}$ was monitored by rapid scan every 20 ms in a stop flow (total of 10 scans). Arrows indicate direction of spectral change. Inset: Plot of rate ($k_{obs}$) versus various concentrations of EDTA-Fe$^{3+}$ (■), NTA-Fe$^{3+}$ (●), and citrate- Fe$^{3+}$ (▲).
Figure 3-3: Western blot analysis of MtrC standard, TM and whole cells (WC). MtrC content was visualized using affinity-purified polyclonal antibodies to MtrC. MtrC protein standards ranged from $1.8 \times 10^{11}$ to $1.8 \times 10^{12}$ moles, and were loaded onto a 10% SDS-PAGE gel along with TM fractions (0.5 μg per lane) and whole cell samples (7.5 μl of cell suspension ($2.25 \times 10^{7}$ total cells)).
**Kinetic analysis of iron reduction**

Iron reduction kinetic studies were performed with both soluble and insoluble forms of iron. The electron donor for in vitro TM incubations was formate. For in vivo whole cell incubations, the electron donor was the same as the carbon source used for growth, lactate. The reaction kinetics were fit to the Michaelis-Menten equation to determine $K_m$, $k_{cat}$ and $k_{cat}/K_m$ for EDTA-$\text{Fe}^{3+}$, NTA-$\text{Fe}^{3+}$ and citrate-$\text{Fe}^{3+}$ and either TM (Fig. 3-4A) or whole cells (Fig. 3-4B). $K_m$ is the half saturation constant, $k_{cat}$ is the maximal velocity ($V_{max}$) expressed as turnover number and $k_{cat}/K_m$ is the apparent second order rate constant, or catalytic efficiency of the enzyme which can be experimentally determined by the slope at low substrate concentration for a plot of rate versus substrate concentration. By quantifying the amount of OmcA and MtrC in these preparations, the velocities could be expressed as turnover number: moles of $\text{Fe}^{2+}$ produced per second per mole of either MtrC or OmcA (thus simplifying to units of s$^{-1}$). Fig. 3-4 shows data only for OmcA (the MtrC-normalized velocity plots are similar and therefore not shown). The second-order rate constants ($k_{cat}/K_m$) are listed in Table 3-2. For the TM fraction, $k_{cat}/K_m$ values obtained with the soluble iron form followed the same trend as that observed from stop-flow experiments: EDTA-$\text{Fe}^{3+}$$>$NTA-$\text{Fe}^{3+}$$>$citrate-$\text{Fe}^{3+}$. For whole cells, EDTA-$\text{Fe}^{3+}$ and NTA-$\text{Fe}^{3+}$ exhibited similar $k_{cat}/K_m$ values and were greater than citrate-$\text{Fe}^{3+}$.

Steady-state kinetic experiments were also performed with goethite and either TM or whole cells (Fig. 3-5). Quantification of OmcA and MtrC by Western blot allowed calculation of $k_{cat}/K_m$ expressed as per mole or per unit surface area of goethite. The $k_{cat}/K_m$ values, expressed per mole goethite (formula, $\text{FeOOH}$), are much lower than values calculated per mole Fe for the soluble Fe experiments (Table 3-1 and 3-2).
Figure 3-4: Steady-state kinetic analysis of soluble iron reduction. Rates of TM-mediated (A) or whole cell-mediated (B) iron reduction are expressed as moles iron reduced per mole of OmcA per second (simplifying to s⁻¹). TM fractions (0.1 mg/ml) or whole cell suspensions (see Materials and Methods) were added to 1 ml reaction mixtures containing buffer or defined media and the specified concentrations of chelated iron species. The reaction was initiated upon the addition of 100 μl of 100 mM formate or 100 μl of the cell suspension. At 0, 15 and 30 min, 100 μl aliquots were removed and analyzed for ferrous iron (nmol Fe²⁺ formed/min/mg TM). Panel A shows results from TM incubations and panel B from whole cell incubations with EDTA-Fe³⁺ (■), NTA-Fe³⁺ (▽), or citrate-Fe³⁺ (●). Note the difference in scales between A and B.
Figure 3-5: Steady-state kinetic analysis of iron oxide reduction. TM- (●) and whole cell-mediated (○) goethite reduction rates are expressed as moles Fe$^{2+}$ per mole of OmcA per second. TM fractions (0.1 mg/ml) or whole cell suspensions (100 μl) were added to 1 ml reaction samples containing buffer or defined media and various concentrations of goethite. The reaction was initiated upon the addition of 100 μl of the cell suspension and 100 μl aliquots were taken at 0, 15 and 30 min for Fe$^{2+}$ quantification.
Single-turnover experiments were also performed with goethite. Again, OmcA and MtrC were reduced with xanthine oxidase and the reactions with goethite were monitored by heme oxidation at 550 nm. These reactions are much slower than the reaction with the soluble iron forms yielding much slower second-order rate constants. Furthermore, of significance, the rate constants ($k_{cat}/K_m$) are up to 3 orders of magnitude lower than the rate constants obtained from steady-state kinetics with TM or whole cells (Table 1 and Table 2).

**Flavin content of ferric-citrate-grown cultures**

Because flavins (flavin adenine dinucleotide, flavin mononucleotide and riboflavin) have been proposed to act as the shuttling agent for iron oxide reduction (von Canstein et al., 2008), we investigated whether flavins were produced in ferric citrate-grown cultures. Total flavin content was measured by HPLC separation and its associated fluorescence (Table 3-3). The flavin concentration of the extracellular medium (spent culture supernatant with cells removed) was measured to be $0.30 \pm 0.02 \mu M$. When calculated per gm whole cell protein, our value of $0.38 \mu mol$ flavin/gm protein is within error of the value of $0.4 \mu mol/gm$ protein measured by von Canstein et al. (von Canstein et al., 2008) for fumarate-grown cultures of *Shewanella*. Because the flavin in the extracellular medium has been hypothesized to act as an electron shuttle, we also analyzed whether our TM fraction contained flavin: $0.12 \mu mol$ flavin was measured per gm protein in the TM fractions. This is perhaps not surprising due to the hydrophobic nature of flavins and the likelihood of membrane association. It follows that flavin was found in washed whole cells as well. The percentage that was extracellular could not be determined.
Table 3-1: Kinetic constants for MtrC with soluble and insoluble iron forms.*

<table>
<thead>
<tr>
<th></th>
<th>Purified Enzyme Experiments</th>
<th>TM Experiments</th>
<th>Whole cell Experiments</th>
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<tbody>
<tr>
<td>EDTA-Fe^{3+}</td>
<td>(9.2 \times 10^5 \pm 3.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1})</td>
<td>(1.9 \times 10^5 \pm 2.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1})</td>
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<td>NTA-Fe^{3+}</td>
<td>(2.4 \times 10^5 \pm 3.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1})</td>
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<td>(1.1 \times 10^4 \pm 1.7 \times 10^3 \text{ M}^{-1}\text{s}^{-1})</td>
</tr>
<tr>
<td>Citrate-Fe^{3+}</td>
<td>(3.9 \times 10^4 \pm 3.3 \times 10^3 \text{ M}^{-1}\text{s}^{-1})</td>
<td>(1.2 \times 10^3 \pm 3.0 \times 10^2 \text{ M}^{-1}\text{s}^{-1})</td>
<td>(6.7 \times 10^3 \pm 4.4 \times 10^2 \text{ M}^{-1}\text{s}^{-1})</td>
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<tr>
<td>Goethite</td>
<td>(0.0033 \pm 0.0003 \text{ (m}^2/\text{mL})\text{ s}^{-1})</td>
<td>(4.9 \pm 0.9 \text{ (m}^2/\text{mL})\text{ s}^{-1})</td>
<td>(0.45 \pm 0.06 \text{ (m}^2/\text{mL})\text{ s}^{-1})</td>
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<td>(0.0099 \pm 0.0007 \text{ M}^{-1}\text{s}^{-1})</td>
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<td>(1.37 \pm 0.17 \text{ M}^{-1}\text{s}^{-1})</td>
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*Trends for rates of reduction with ferric chelates correlate to the binding affinity of the ligand.
Table 3-2: Kinetic constants for OmcA with soluble and insoluble iron forms.*

<table>
<thead>
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<th>Purified Enzyme Experiments</th>
<th>TM Experiments</th>
<th>Whole cell Experiments</th>
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<tr>
<td>EDTA-Fe³⁺</td>
<td>$3.0 \times 10^5 \pm 7.4 \times 10^3 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$1.5 \times 10^4 \pm 2.1 \times 10^3 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$9.2 \times 10^3 \pm 1.1 \times 10^3 \text{M}^{-1}\text{s}^{-1}$</td>
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<tr>
<td>NTA-Fe³⁺</td>
<td>$1.7 \times 10^5 + 2.9 \times 10^4 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$1.4 \times 10^4 + 4.0 \times 10^3 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$1.1 \times 10^4 + 1.5 \times 10^3 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>Citrate-Fe³⁺</td>
<td>$3.7 \times 10^4 \pm 3.2 \times 10^4 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$1.2 \times 10^5 \pm 3.0 \times 10^4 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$7.0 \times 10^4 \pm 4.6 \times 10^4 \text{M}^{-1}\text{s}^{-1}$</td>
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<tr>
<td>Goethite</td>
<td>$0.0066 \pm 0.0003 \text{(m}^2/\text{mL})^{-1}\text{s}^{-1}$</td>
<td>$8.1 \pm 0.09 \text{(m}^2/\text{mL})^{-1}\text{s}^{-1}$</td>
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<td>$0.019 \pm 0.003 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$24.2 \pm 5.5 \text{M}^{-1}\text{s}^{-1}$</td>
<td>$1.15 \pm 0.2 \text{M}^{-1}\text{s}^{-1}$</td>
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*Trends for rates of reduction with ferric chelates correlate to the binding affinity of the ligand.
Table 3-3: Flavin content of various preparations.

<table>
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<th>Preparation</th>
<th>µmol flavin/g protein</th>
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</tr>
<tr>
<td>Total membrane</td>
<td>0.12 ± 0.018</td>
</tr>
<tr>
<td>Spent culture medium</td>
<td>5.8 ± 0.70</td>
</tr>
</tbody>
</table>
Flavin-dependent stimulation of goethite reduction

To assess whether the flavin-containing medium could stimulate iron reduction, we examined the effect of adding spent medium containing the low-molecular-weight redox-active fraction to our iron-reducing reaction mixtures. The spent medium was first passed through a Chelex® column to remove all of the soluble iron and added back to whole cell incubations. As shown in Table 3-4, addition of Chelex®-treated spent culture medium caused a 10-fold increase in the rate of goethite reduction. Similar stimulation was observed with the TM fraction (data not shown).

Reaction of riboflavin with OmcA and MtrC

The reaction of OmcA or MtrC with riboflavin was studied by stop flow. Similar to studies using soluble iron, the rate of decrease in 550 nm absorbance was used to monitor the oxidation of ferroheme to ferriheme. The second-order rate constants for OmcA and MtrC measured in the presence of riboflavin were $5.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $1.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ respectively.

We then determined the reactivity of flavins with goethite. Riboflavin was reduced by the xanthine oxidase system and its reaction with goethite was monitored by the increase in 450 nm absorbance associated with oxidized flavin. These single turnover experiments performed at different goethite concentrations yielded a second-order rate constant of $0.22 \text{ M}^{-1}\text{s}^{-1}$.

Simulation of transient- and steady-state rates with calculated rate constants

Our results suggest that the sluggish reactivity between OmcA and MtrC with goethite can be compensated by the introduction of flavin into the mechanism. To further assess the role of flavins in whole cell catalyzed DIR, we performed some additional experiments with flavin for the purpose of kinetic simulations. As described above, we monitored the oxidation of OmcA by
goethite, except this time, we added flavin at a physiologically-relevant concentration (Fig. 3-6). The added flavin increased the rate of OmcA oxidation. The data were then simulated using Kinsim and our experimentally-determined rate constants (Fig. 3-6). The simulation showed that our rate constants are able to fit the data at three different concentrations of goethite with satisfactory accuracy.

We then simulated steady-state results from whole cell experiments using a flavin-dependent mechanism. The simulation was performed using a range of flavin concentrations slightly higher than our measured value of 0.30 μM for bulk phase. We reasoned that the effective concentration of flavin in proximity of the cell would be higher than that measured in the bulk phase. As shown in Fig. 3-7, using our experimentally-determined rate constants for individual steps of electron transfer between OmcA and flavin and between flavin and goethite, a concentration of 1.35 μM flavin perfectly simulated the time course of iron reduction by whole cells.
Table 3-4: Stimulation of whole cell goethite reduction by spent culture medium.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Iron reduction rate mmol Fe$^{2+}$/min/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells with defined medium</td>
<td>0.033 ± 0.007</td>
</tr>
<tr>
<td>Whole cells with defined medium and spent medium</td>
<td>0.18 ± 0.016</td>
</tr>
<tr>
<td>Whole cells with spent medium</td>
<td>0.32 ± 0.058</td>
</tr>
</tbody>
</table>
Figure 3-6: Kinetic simulation of OmcA-mediated reduction of goethite in the presence of riboflavin. The reaction was monitored by oxidation of ferroOmcA at 550 nm. Data points are from experiments containing 0.47 μM ferroOmcA, 1 μM flavin and varying goethite concentrations: 1.9 mg (○), 3 mg (■), or 10 mg (▲). Lines represent simulated data for the reaction mechanism shown in Fig. 8A with the following rate constants: OmcA and flavin (5.9 x 10^5 M^-1 s^-1), flavin and goethite (0.22 M^-1 s^-1) and the following concentration of reactants: OmcA (0.476 μM), riboflavin (1 μM) and the three goethite concentrations indicated above.
Figure 3-7: Simulation of steady-state whole cell-mediated goethite reduction using experimentally-determined rate constants between OmcA and riboflavin (5.9 x 10^5 M⁻¹s⁻¹), and riboflavin and goethite (0.22 M⁻¹s⁻¹). Points are experimental data from experiments measuring Fe²⁺ formation in whole cell incubations with goethite (10 mg/ml). Reaction conditions are as described in Materials and Methods. Lines represent simulated rates of Fe²⁺ formation at various flavin concentrations (μM). The concentration of OmcA used in simulation is the same as the concentration in the actual experiment (8.6 x 10⁻⁸ M). See Fig. 8 B for the mechanism used in the simulation.
Figure 3-8:  (A) Mechanism used for simulation of single turnover experiments for OmcA/flavin goethite reduction shown in Fig. 6. The rate constants were experimentally determined. Flavin reduction by OmcA occurs very fast followed by a slower reduction of goethite by flavin. (B) Mechanism used for simulation of whole cell-mediated goethite reduction shown in Fig. 7. To simulate multiple turnover (steady state) data at the linear part of the Michaelis-Menten curve (where iron is limiting and not saturating), OmcA was kept reduced thereby assuring that the rate of OmcA reduction was not rate limiting.
DISCUSSION

Of the three proposed mechanisms for DIR of metal oxides, i) direct contact, ii) redox shuttles and iii) metal chelators, the first two have received the most attention. Experimental evidence for direct contact as the route of electron transfer first came from AFM studies by Lower et al. (Lower et al., 2001). These workers were able to show preferential binding of goethite to *Shewanella* whole cells that were anaerobically- as opposed to aerobically-grown. The AFM force curves suggested the involvement of a 150 kilodalton protein which they referred to as the “putative reductase” critical for such binding. More recently, Lower et al. (Lower et al., 2007) again used AFM to study the interaction of OmcA and MtrC with hematite. They showed that the OmcA-hematite interaction is twice the strength of the MtrC-hematite interaction and proposed that such strong binding interactions allowed for direct electron transfer to the iron oxide TEA. The direct contact mechanism was also supported by a study by Xiong et al. (Xiong et al., 2006) using dynamic light scattering and fluorescence correlation spectroscopy to measure the binding affinity between OmcA and hematite. Also, using NADH as the electron donor, they measured a maximal activity of 60 nmol Fe\(^{2+}\) mg\(^{-1}\) OmcA min\(^{-1}\) for the reduction of hematite by OmcA. Recent work in our laboratory using an *in vitro* model system of purified TM fractions (Ruebush et al., 2006) is also consistent with the direct contact mechanism. We proposed that the most likely mechanism for insoluble iron reduction would be through direct contact since we assumed that small molecules would be removed from the TM sample after centrifugation and dialysis. Another study in support of a direct contact mechanism for *Shewanella* involves nanowires (Gorby et al., 2006). While the identity of the terminal reductase in the nanowires has yet to be identified, implicit in the arguments for this mechanism is direct contact between the nanowire and the metal oxide.
The possible involvement of secreted electron shuttles in poorly soluble minerals was first proposed by Newman and Kolter (Newman and Kolter, 2000). Experimental evidence, although indirect, for participation of electron shuttles in DIR by dissimilatory Fe(III)-reducing microorganisms was further examined by the work of Nevin and Lovley (Nevin and Lovley, 2002). They were able to show reduction of iron oxide trapped in porous alginate beads. In a similar experiment, Lies et al. (Lies et al., 2005) demonstrated reduction of iron (hydr)oxides precipitated in nanoporous glass beads by *S. oneidensis* MR-1. These studies demonstrated the phenomenology of electron transfer without direct contact, but more substantive evidence was not provided until very recently. Two groups independently provided chemical evidence for production of extracellular flavins as redox mediators by *S. oneidensis* (Marsili et al., 2008; von Canstein et al., 2008). They showed that addition of flavin increased the rate of goethite reduction but had no effect on soluble iron reduction. One concern over the use of electron shuttles is the energetic efficiency: unless used for many cycles of electron transfer, the energy expended to synthesize a flavin molecular may be greater than that returned. Marsili et al. (Marsili et al., 2008) argued that accumulation of flavins in *Shewanella* biofilms increased transfer of electrons to an electrode by 370% while the energy requirement to produce the observed levels of flavins was <0.1% of the total cellular ATP produced at that oxidation current. In order to produce 250 nM flavin over 72 hrs, it would potentially cost the cell $6.7 \times 10^{-3}$ μmol ATP/mg protein/hr (Marsili et al., 2008).

Lacking in all of the above studies are supportive kinetic studies. In expounding the virtue of kinetic analysis, Cleland (Cleland, 1975) stated that kinetics should be the final arbiter of mechanistic studies. The scale-up kinetic studies described here were performed to identify the physiologically-relevant mechanisms by using the criteria of kinetic competence. Such scale-up studies, while difficult, can also identify relevant factors contributing to catalysis at the different scales (pure enzymes, cell fractions, whole cells) and should lead toward better understanding of
how to make predictions for natural systems. The limitations for such studies (Brantley et al., 2006) are briefly described below.

To scale from steady-state kinetics, one must use the Michaelis-Menten equation,

$$v = \frac{V_{\text{max}} [S]}{K_m + [S]}$$

while for whole cells, data are generally fit to the Monod equation:

$$\frac{d[S]}{dt} = \frac{-(\mu_{\text{max}}/Y)X[S]}{K + [S]}$$

Here $[S]$ is the concentration of the rate-limiting substrate, $\mu_{\text{max}}$ is the maximum specific growth rate, $Y$ is the growth yield coefficient, $X$ is the biomass concentration and $K$ is the half-saturation constant for growth. Scale-up from purified enzymes to membrane fractions to whole cells requires comparison of these two equations, both of which are only valid under selected conditions. For example, cell viability is important in whole-cell studies because substrate toxicity, cell growth and cell death will impact the Monod fits. If the kinetics can be performed in a short time span, under non-growth conditions (where no cell growth occurs and $X_a = \text{constant}$), the Monod equation has the same form as the Michaelis-Menten equation. In that case, $V_{\text{max}}$ is equal to $(\mu_{\text{max}}/Y)X_a$ and $V_{\text{max}}/K_m$ is equal to the slope of the Monod plot (non-growth conditions with whole cells). At low substrate concentrations, the slope of both curves is an indicator of the kinetic response of the system to substrate concentration. Also, such a response is only valid in whole cells when the substrate of interest can be manipulated. Such is the case with extracellular enzymes such as OmcA and MtrC described in this study. To determine rate constants, the concentration of the enzyme must be known (yielding $k_{\text{cat}}$ and the second-order rate constant). Hence our determination of OmcA and MtrC concentration by Western blots.
If involved in physiologically-relevant catalysis, kinetic constants obtained from transient-state studies should be as fast or faster than overall steady-state rates of membrane fractions and these rates, in turn, must be as fast or faster than overall whole culture rates (Cleland, 1975; Lorimer et al., 1976). When rates cannot scale up to account for catalysis in membrane factions or whole cells (i.e. the transient-state rates with purified enzymes are too slow to account for rates at steady-state), the enzyme of interest is either not involved in catalysis or its involvement is not in accordance with the proposed mechanism (Brantley et al., 2006).

**Reduction of soluble ferric species**

While our present study was designed mainly to address the mechanism of electron transfer to insoluble metal oxides, we also studied the soluble iron chelates as a control for comparison and to validate our studies with the metal oxides. Past research from our laboratory and others has shown that soluble TEAs can be reduced in the periplasm at the CM (Ruebush et al., 2006). Nevertheless, OM hemeproteins are capable of efficiently reducing chelated ferric species (Wang et al., 2008). The rate constants obtained here with OmcA and MtrC and EDTA-Fe$^{3+}$, NTA-Fe$^{3+}$ and citrate-Fe$^{3+}$ are comparable to values obtained by Wang et al. (Wang et al., 2008) with reactivities on the order of EDTA-Fe$^{3+}$ > NTA-Fe$^{3+}$ > citrate-Fe$^{3+}$. The rate constants obtained from the stop-flow experiments are faster than those obtained from the TM or whole cells. Furthermore, the rate constant with each ferric chelate is within one order of magnitude for each scale, an acceptable error given the reproducibility of our experiments.

It is noteworthy that our rates with whole cells and with the transient-state analysis of NTA-Fe$^{3+}$ yielded rate constants lower by 2-3 orders of magnitude than those calculated by Borloo et al. (Borloo et al., 2007). In their study, they quantified OmcA and MtrC using heme staining of SDS-PAGE gels. This difference may be due to differences in incubation and assay conditions. Borloo et al. (Borloo et al., 2007) used fumarate as the TEA while we used ferric
citrate. We have shown that ferric citrate-grown cells produce up to 19 times more heme than fumarate-grown cells. Also, in contrast to our study, Borloo and coworkers included ferrozine in the reaction mixture while we added ferrozine after the reaction had been quenched with acid. This may impact the rate of iron reduction. While it may be difficult to compare our whole cell kinetic constants with those of Borloo et al. (Borloo et al., 2007), our rate constants obtained from stop flow are very similar to those determined by Wang et al. (Wang et al., 2008). Both results are consistent with OmcA and MtrC being kinetically competent to catalyze soluble iron reduction by direct contact.

The ability to extrapolate steady-state rates from transient-state rate constants is difficult even with purified enzymes (Fierke et al., 1987). Extrapolation from pure enzymes to whole cells is even more problematic. However, as described below, such an exercise, in which data are simulated with a mechanism and measured rate constants, can be valuable in eliminating proposed mechanisms.

**Reduction of insoluble iron oxide**

Scale-up kinetics of soluble iron yielded rate constants within approximately one order of magnitude from purified enzymes to TM and WC. As discussed above, the rate constants obtained at all three scales for the soluble electron acceptors are therefore consistent with the conclusion that OmcA and MtrC are kinetically competent. In contrast, scale-up kinetics with goethite clearly show that OmcA and MtrC cannot participate in iron oxide reduction through direct contact alone: the rate constants for OmcA and MtrC with goethite are two to three orders of magnitude lower than rates obtained with whole cells or TM fractions. The inability to simulate or account for steady-state results unequivocally eliminates the mechanism related to direct contact between the hemeproteins and the iron oxide for this organism. This result reaffirms what biochemists have long known: formal binding of a substrate (formation of a
ternary complex) is not a prerequisite for catalysis (e.g. Rubisco has no formal binding site for CO$_2$ or O$_2$ (Bowes and Ogren, 1972; Gutteridge et al., 1984); also, the Theorell-Chance mechanism (Gates and Northrop, 1988) should be considered). In other words, while OmcA or MtrC may bind tightly to the iron oxide substrate (Lower et al., 2007; Xiong et al., 2006), this does not necessarily imply anything about the kinetics of particulate iron oxide reduction.

The other hypothesized mechanism invoking direct contact is electron transfer through putative nanowires (Gorby et al., 2006). Our results do not allow us to comment on the involvement of nanowires. Nanowires have been reported to be produced by *Shewanella* under micro-anaerobic conditions (Gorby et al., 2006). The present study is not under such conditions and in fact, is under electron acceptor-rich conditions. Therefore, nanowires are not involved in the experimental results discussed here.

**Reduction of flavins**

The inability to kinetically account for goethite reduction by OmcA or MtrC could be resolved by inclusion of flavin in the reaction mechanism. Consistent with published results (von Canstein et al., 2008), we were able to stimulate goethite reduction with flavins. Addition of Chelex$^\text{®}$-treated spent culture medium stimulated goethite reduction by whole cells. If indeed electron transfer by direct contact is too slow to account for catalysis, it would follow that the TM fraction and whole cells (which had been centrifuged and washed) should not have yielded faster rate constants than those obtained with the purified OmcA and MtrC. However, we found that despite multiple centrifugation steps, the TM still contained flavins. This residual flavin can then participate in electron transfer and account for the higher rate constants obtained with the TM. While we also found flavins in the washed whole cells, our data do not allow us to distinguish between extracellular and intracellular flavins. The whole cells, resuspended in fresh growth medium, would presumably be actively synthesizing and secreting flavins which would stimulate
iron oxide reduction. There remains the possibility that flavins are found in the extracellular medium not due to active secretion but by inadvertent loss from a flavoprotein. While this possibility cannot be eliminated from the results of our study, we argue that this is unlikely simply due to the high concentration of flavin found in the extracellular medium. Concentrations of extracellular FMN and riboflavin were found to be 30 times higher than intracellular concentrations (von Canstein et al., 2008). It is difficult to envision Shewanella being so energetically inefficient through the loss of essential prosthetic groups.

If flavins are part of the kinetic mechanism, then the $k_{cat}/K_m$ values determined from our steady-state experiments with the TM and the whole cells are not true second-order rate constants between goethite and OmcA or MtrC. They are apparent rate constants obtained with flavin acting as a shuttle. This is substantiated by our kinetic simulations of steady-state data from whole cells. Using a mechanism involving flavin mediation, the experimentally-determined rate constants and the experimentally-determined concentrations of OmcA and flavin, we were able to simulate the steady-state data. Our proposed mechanism for OmcA and MtrC reduction of goethite is shown as a simulation in Fig. 3-8. The validity of the mechanism shown along with the concentration of heme protein and flavin and with the rate constants is supported by the kinetic simulation shown in Fig. 3-6 and Fig. 3-7.

**Conclusions**

Using the concept of kinetic competence and scale-up kinetic analysis, we have attempted to determine the mechanism of OM electron transfer for Shewanella. Scale-up kinetics dictates that the rates of individual steps in a mechanism must be just as fast as or faster than the overall steps in a pathway. Accurate scale-up is difficult; the number of variables contributing to rates of a reaction increases significantly as one goes up in scale on the kinetic ladder. Our scale-up kinetic studies showed for the first time that:
1) within experimental error, reduction of soluble iron forms can be scaled from isolated enzyme to whole cell experiments by scaling using the concentration of enzyme in each experiment: this implies that OmcA and MtrC can account for catalysis of soluble iron in whole cells (i.e. kinetic competence has been demonstrated);

2) OmcA and MtrC are not kinetically competent to account for physiological goethite reduction via direct contact because the relevant rate constants are 3 orders of magnitude too slow;

3) when flavins are added into the kinetic mechanism, the reaction rates are greatly increased and can therefore account for the reduction of insoluble iron oxides in vivo.

Thus, of the three possible mechanisms used by *Shewanella* to transfer electrons to insoluble Fe oxides -- direct contact (Lower et al., 2007; Xiong et al., 2006), electron shuttle (Marsili et al., 2008; von Canstein et al., 2008), and chelation (Taillefert et al., 2007) -- our data has eliminated direct contact as a plausible mechanism of electron transfer from OmcA or MtrC to iron oxides and is consistent with the involvement of electron shuttles in DIR. Flavins are particularly implicated.
INTRODUCTION

Microbial processes play a central role in the cycling of organic and inorganic compounds on Earth. A select number of these compounds are assimilated by organisms and used as building material for the cell. In addition, a large number of organic and inorganic compounds are used as electron donors and acceptors for respiratory metabolism. Since several respiratory metabolisms evolved prior to oxygenic photosynthesis, microorganisms developed strategies to utilize organic and inorganic terminal electron acceptors (TEAs) before using molecular oxygen (Madigan, 2000). Due to their favorable redox chemistry and their prevalence in sediments, metals such as iron and manganese represent primary terminal electron acceptors. The use of metals in respiratory metabolism is known as dissimilatory metal reduction (DMR) (for extensive review, see (Lovley, 1991)) and indirectly influences many elemental cycles (e.g. C, N, O) (Banfield, 1997). Interest in microbial DMR has intensified upon discovery of the ability of DMR bacteria to affect the fate and transport of environmentally-relevant contaminants, including radionuclides such as U(VI) (Anderson et al., 2003; Liu et al., 2002; Liu et al., 2006; Lovley and Phillips, 1992; Marshall et al., 2006; Sanfilippo et al., 2004; Shelobolina et al., 2007).

Two model organisms capable of DMR are Shewanella and Geobacter species. Shewanella oneidensis MR-1 is a facultative anaerobe with a diverse respiratory capacity capable of linking anaerobic manganese reduction to growth (Burdige et al., 1992; Myers and Nealson,
In *Shewanella* the majority of c-type cytochromes are localized to the outer membrane (OM) where they play a direct role in the reduction of extracellular substrates (Myers and Myers, 1992; Myers and Myers, 1997). Much effort has focused on identification of the terminal metal reductase (Beliaev et al., 2001; Hartshorne et al., 2007; Myers and Myers, 2002; Ross et al., 2007; Shi et al., 2006) and two extracellularly exposed OM hemeproteins, OmcA and MtrC, have been implicated. OmcA and MtrC have both iron and manganese reductase activity and while it is likely that identical pathways of what are utilized (e.g. *mtr* genes), it remains to be determined how manganese oxides are reduced (Myers and Myers, 2003). Three potential mechanisms for reduction of extracellular electron acceptors have been proposed: reduction through direct contact between OM cytochromes and the mineral surface (Lower et al., 2007; Lower et al., 2001; Xiong et al., 2006), reduction at a distance with electron shuttles (Marsili et al., 2008; von Canstein et al., 2008) and reduction of solubilized metal-chelate complexes (Taillefert et al., 2007).

Many lines of evidence have been cited to support reduction by direct contact and reduction of endogenous electron shuttles as mechanisms for solid-phase metal reduction by *Shewanella* sp. For example, using atomic force microscopy (AFM), Lower et al. (2007) measured binding between both OM hemeproteins OmcA and MtrC with the iron oxide hematite and reported that OmcA shows a greater affinity toward hematite than does MtrC. These workers suggested that this tight binding is a pre-requisite for direct electron transfer. Although their work does not prove the mechanism, they have only implied that such binding may be necessary if the direct contact mechanism can be utilized by this organism. Xiong et al. (2006) also showed tight binding between OmcA and hematite by using dynamic light scattering and fluorescence correlation spectroscopy. Previous work from our laboratory (Ruebush et al., 2006a; 2006b) demonstrating the rates of metal oxide reduction by membrane fractions from *Shewanella* also supported a direct contact mechanism in that solid-phase metal oxides were reduced by TM.
fractions without the addition of soluble components. Direct contact between cellular components and the iron oxides is also consistent with the nanowire mechanism proposed by Gorby et al. (Gorby et al., 2006).

In support of the ability of *S. oneidensis* to reduce solid-phase metal oxides through electron shuttles, past research from our laboratory and others has shown that flavins can accelerate electron transfer (ET) to iron oxides (Marsili et al., 2008; Ross et al., 2009; von Canstein et al., 2008). Rates obtained with flavin addition could kinetically account for *in vivo* goethite reduction rates. The involvement of flavins as an electron shuttle could be consistent with previous reports that *Shewanella* secretes a small quinone-like compound involved in ET (Newman and Kolter, 2000) and can utilize sterically sequestered iron located in alginate beads (Nevin and Lovley, 2002).

While extracellular electron transfer in DMR has been extensively studied with iron oxides, data for mechanisms of ET to manganese oxides is lacking. Manganese oxides are one of the strongest naturally occurring oxidizing agents in the environment and differ from iron oxides in mineralogy, reactivity and stability (Post, 1999). Initial studies of manganese oxide reduction with *S. oneidensis* indicated a requirement of direct contact for growth despite qualitative evidence suggesting significant manganese reduction can occur without permanent attachment to the mineral as observed by continuous light microscopic observation (Myers and Nealson, 1988; Nealson et al., 2002). It has been proposed that the Mn oxide interacts with the cells without the need for permanent attachment (Nealson et al., 2002).

In this study, we employ transient- and steady-state kinetic analyses on two structurally distinct manganese oxides, birnessite and bixbyite, to characterize the role of OM cytochromes OmcA and MtrC. Our studies are performed at three different kinetic scales: 1) transient-state manganese reduction using purified OmcA and MtrC, 2) steady-state kinetics with purified total membrane (TM) fractions, and 3) steady-state whole cell kinetics.
MATERIALS AND METHODS

Materials

Xanthine oxidase from buttermilk, Triton X-100 SigmaUltra, ferric citrate, NTA (N,N-bis[carboxymethyl]glycine) and riboflavin were purchased from Sigma. CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) was purchased from Soltec Ventures, Inc. EDTA ((ethylenedinitrilo)tetaacetic acid disodium salt, dihydrate) was purchased from EMD Chemicals Inc.

Organism and growth conditions

Shewanella oneidensis MR-1 (ATCC 700550) was maintained on Luria-Bertani (LB) agar. Single colonies were used to inoculate 5 ml aerobic LB media and then allowed to grow at 30°C to an optical density of 0.6 at 600 nm. The culture was centrifuged at 10,000×g for 15 minutes and the pellet was washed with HEPES buffered saline (HBS, 0.7% NaCl in 10 mM HEPES, pH 7.4) and then re-suspended in one tenth the volume in the same solution. This cell suspension (1 ml) was added to a 4.5 L of defined argon-purged M1 minimal medium as per Myers (Myers and Nealson, 1988) and harvested when the ferrous ion concentration reached 35 mM (Ruebush et al., 2006).

Total membrane isolation

The TM fraction was isolated according to Ruebush et al. (2006) modified from (Myers and Myers, 1992) using lysozyme, EDTA and 5% Brij.
**OmcA, MtrC and MtrC/A/B purification**

OmcA, MtrC, and MtrC/A/B were purified as described previously (Ross et al., 2007; Ross et al., 2009). Briefly, TM (10 mg/mL) fractions were solubilized with 50 mM HEPES pH 7.5 and 5% Triton X-100 and subjected to anion exchange chromatography (Q-sepharose fast flow, Amersham) using a linear gradient of 0-100% B at 1 ml/min over 160 min (Buffer A= 50 mM HEPES pH 7.5, 5% Triton X-100, Buffer B = Buffer A + 0.5 M NaCl). The OmcA fraction, as determined by Western blot analysis was isolated and subjected to MonoQ chromatography. OmcA was loaded onto a MonoQ column (Amersham) and eluted with a linear gradient of 0-100% buffer B at 1 ml/min over 160 min (Buffer A= 50 mM HEPES pH 7.5, 0.2% Triton X-100, Buffer B = Buffer A + 0.5 M NaCl).

While the first heme peak (as detected by 409 nm absorbance) contained OmcA, the second heme peak that eluted from the Q-sepharose column contained the MtrC/A/B complex (Ross et al., 2007) as visualized by heme staining. MtrC/A/B was purified further using a Sephacryl s-300 gel filtration column (Amersham). To isolate MtrC from the purified complex, we first exchanged detergents by binding the complex to a Q-Sepharose column (Q-sepharose fast flow, Amersham) and then washed with three column volumes of buffer A (50 mM HEPES pH 7.5, 0.5% CHAPS). MtrC was eluted with a linear gradient of 0-100% buffer B at 1 ml/min over 160 min (Buffer B = Buffer A + 0.5 M NaCl).

Extinction coefficients for OmcA and MtrC were determined with the pyridine hemochromogen assay (Berry and Trumpower, 1987). The extinction coefficient for OmcA at 409 nm is equal to 1,670,000 M\(^{-1}\) cm\(^{-1}\) and for MtrC, \(\varepsilon_{409} = 1,440,000 M^{-1} cm^{-1}\).

For standards on Western blots, recombinant MtrC was purified as inclusion bodies as previously described by Ross et al. (2007). MtrC content in the purified inclusion bodies was determined by its UV absorbance associated with its aromatic amino acids. The extinction coefficient at 280 nm (\(\varepsilon_{276} = 48,800 \text{ M}^{-1}\text{cm}^{-1}\)) was determined as per Edelhoch (1967).
Transient-state kinetics

OmcA and MtrC were reduced enzymatically with xanthine oxidase (Cheng et al., 2007). Stock solutions of xanthine oxidase (2 units/mL), xanthine (0.5 mM) and benzyl viologen (1 mM) were purged with oxygen-free argon and transferred to a Coy anaerobic chamber (3% hydrogen, \(N_2\) atmosphere). Reaction mixtures contained purified OmcA (or MtrC) (0.5 \(\mu\)M), xanthine (0.15 mM), benzyl viologen (1.5 \(\mu\)M), and xanthine oxidase (0.01 units/ml).

Reduction was monitored by UV/Vis spectroscopy and was complete after approximately 2 hours. Fully reduced protein was then loaded into an Applied Photosystems (Surrey, U.K.) SX.18MV stopped flow apparatus. Heme oxidation was monitored with a diode array detector and rates were obtained by monitoring the decrease in 550 nm absorbance associated with the ferroheme oxidation.

Western blot quantification of MtrC and OmcA

Quantification of MtrC and OmcA in TM and WC samples was performed as described previously (Chapter 3). Briefly, purified MtrC or OmcA was used as a protein standard for SDS-PAGE/Western blots and ranged from \(1.8 \times 10^{-11}\) to \(1.8 \times 10^{-12}\) moles per lane. OmcA and MtrC were quantified in TM fractions \((0.5 \mu\text{g per lane})\) and whole cell \((7.5 \mu\text{l of cell suspension (2.25} \times 10^7\text{ total cells)})\). After separation of the proteins using SDS-PAGE the samples were transferred to a nitrocellulose membrane (Whatman, Protran BA 83) and protein bands visualized with alkaline-conjugated anti-rabbit IgG and AP substrate (Immobilon™ Western, Millipore). Blots were then scanned and analyzed using ImageJ64 public software from the NIH (http://rsbweb.nih.gov/ij/).
Manganese reduction assays with whole cells and TM fractions

Reduction assays using TM fractions were performed as described previously (Ruebush et al., 2006a; 2006b). Reactions contained 0.1 mg/ml TM protein, 10 mM formate and Mn oxides as specified in the figure legends, in 100 mM HEPES pH 7.5. Reactions were initiated by addition of formate. At specified times, 700 μl aliquots were removed from a 3 ml reaction volume and immediately filtered through a 0.2 μm polytetrafluoroethylene (PTFE) membrane (VWR International). Soluble Mn²⁺ was determined using a formaldoxime colorimetric assay as described previously, with modifications (Armstrong et al., 1979; Brewer and Spencer, 1971). Briefly, formaldoxime reagent (50 μl) was added to 500 μl of filtered samples and pH was adjusted to 9.2 with 6 μl of ammonium hydroxide (28-30% v/v). After 10 min, 500 μl of 100 mM Tris-HCl, pH 9.2 was added and the absorbance at 450 nm was measured.

Whole cell experiments were identical to TM incubation except that whole cells replaced the TM fraction. Isolation of whole cells was performed anaerobically at 4°C. The cells were centrifuged at 10,000 × g for 15 minutes. The cells were then washed twice with M1 minimal medium to remove all trace iron and then suspended in 1/30 of the original volume in defined medium. From this stock, 300 μl (or 4.3 x 10⁸ cells) were added to the Mn-reduction reaction mixtures to initiate the experiment.

For Mn-oxide reduction with TM and WC in the presence of flavin, reactions were performed under saturating substrate conditions.

Miscellaneous methods

Protein content was determined using the method of Lowry (Lowry et al., 1951) with bovine serum albumin (BSA) as a standard. Mn₂O₃ (bixbyite) was purchased from Alfa Aesar and powdered birnessite [Na₀.₅₈(Mn(IV)₁.₄₂, Mn(III)₀.₅₈)O₄ • 1.₅H₂O] was synthesized by the
methods of Golden et al. (Golden et al., 1986). Bixbyite and birnessite were structurally defined using X-ray diffraction (XRD).

RESULTS

Transient-state kinetic studies of acetate-Mn\(^{3+}\) with OmcA, MtrC and MtrC/A/B

Kinetic constants between OmcA and MtrC and acetate-Mn\(^{3+}\) were obtained with stopped flow measurements as described previously for soluble Fe\(^{3+}\) chelates (Wang et al., 2008). Rates of manganese reduction were determined by monitoring the absorbance change (550 nm) associated with the oxidation of the ferroheme to ferriheme upon addition of acetate-Mn\(^{3+}\) (Fig. 4-1). While both OmcA and MtrC, purified as described previously (Ross et al., 2009), contain 10 hemes per molecule, the data did not indicate significant differences in heme reactivity since the traces could be fit with a single exponential. Rate constants were obtained from plots of observed rates versus Mn concentration: the slopes of linear fit lines are equal to forward rate constants (k\(_f\)) and the y intercepts are equal to the reverse rate (k\(_r\)).

We also performed transient-state kinetics for the protein complex MtrC/A/B. Our unpublished electrochemistry data reveal a change in reduction potential of MtrC when it is in the protein complex. Thus, transient state experiments of the reduced heme protein complex with acetate-Mn\(^{3+}\) were performed and compared to purified MtrC (Fig. 4-1, inset). MtrC/A/B data, showing no variation in heme reactivity despite containing 20 hemes per molecule, was fit with a single exponential. The second order rate constants (M\(^{-1}\)s\(^{-1}\)) for soluble manganese acetate were as follows: OmcA ≥ MtrC/A/B ≥ MtrC (Table 4-1).
Total membrane Mn oxide reductase activity

Total membrane (TM) fractions have been isolated from *S. oneidensis* MR-1 grown anaerobically with lactate as the carbon source and ferric citrate as the terminal electron acceptor (Ruebush et al., 2006). As previously observed, addition of formate to the TM fractions resulted in the reduction of Mn oxides (Ruebush et al., 2006a; 2006b). Rates of birnessite reduction by TM fractions were comparable to those reported by Ruebush et al. (2006) with Mn$^{2+}$ concentrations reaching 800 μM after 120 min (data not shown). We have utilized this *in vitro* formate-dependent system in the reduction experiments with bixbyite and in the absence of formate no reduction was observed (Fig. 4-2).

Having established formate-dependent reduction of birnessite and bixbyite by TM fractions, reduction assays were performed at increasing substrate concentrations. Increasing Mn-oxide concentrations resulted in increased rates of Mn$^{2+}$ formation. In this experiment, the rate of Mn reduction by the TM could be saturated by Mn$^{2+}$ yielding a maximal rate ($V_{max}$) (Fig. 4-3).
Figure 4-1. Reaction of ferro-OmcA, -MtrC, or -MtrCAB with acetate-Mn$^{3+}$. Plot of the observed rate ($k_{obs}$) for OmcA (●), MtrC (○), and MtrCAB (■) at various concentrations of acetate-Mn$^{3+}$. Inset: The reaction between reduced OmcA (0.35 μM) with 0.1 mM acetate-Mn$^{3+}$ was monitored by rapid scan. Spectra are representative of reduced (line) and oxidized (dotted) OmcA after addition of acetate-Mn$^{3+}$. 
Table 4-I: Second order rate constants for OmcA, MtrC and MtrC/A/B with manganese acetate.

<table>
<thead>
<tr>
<th>Acetate-Mn^{3+}</th>
<th>OmcA (M^{-1}s^{-1})</th>
<th>MtrC (M^{-1}s^{-1})</th>
<th>MtrC/A/B (M^{-1}s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.1 x 10^5 ± 3.9 x 10^4</td>
<td>1.4 x 10^5 ± 1.4 x 10^4</td>
<td>2.3 x 10^5 ± 1.3 x 10^4</td>
</tr>
</tbody>
</table>
Steady-state kinetic analysis of manganese reduction

Formate-dependent reduction of Mn oxides was studied with TM and whole cells grown in lactate. The reduction kinetics were fit to the Michaelis-Menten equation to determine $K_m$, $k_{cat}$ and $k_{cat}/K_m$ for manganese oxides with TM (Fig. 4-4) and whole cells (Fig. 4-5). $K_m$ is the half saturation constant, $k_{cat}$ is the maximal velocity ($V_{max}$) expressed as turnover number and $k_{cat}/K_m$ is the apparent second order rate constant, or catalytic efficiency of the enzyme which can be experimentally determined by the slope at low substrate concentration for a plot of rate versus substrate concentration. We quantified the amount of OmcA and MtrC in these preparations, and have expressed velocities as turnover number: moles of Mn$^{2+}$ produced per second per mole of either MtrC or OmcA (s$^{-1}$) (Ross et al., 2009). Fig. 4-4 and 4-5 show data only for OmcA (the MtrC-normalized velocity plots are similar and therefore not shown). The second-order rate constants ($k_{cat}/K_m$) are listed in Tables 2 and 3.

Transient-state kinetic analysis of manganese reduction

Single-turnover experiments were also performed with Mn oxides. OmcA and MtrC were slowly reduced enzymatically with xanthine and xanthine oxidase (and catalytic amount of methyl viologen) over a period of 2-4 hours. Reactions with birnessite or bixbyite were monitored by heme oxidation at 550 nm. These reactions were much slower than the reaction with the soluble Mn forms yielding much slower second-order rate constants. Furthermore, of significance, the rate constants ($k_{cat}/K_m$) are up to 3 orders of magnitude lower than the rate constants obtained from steady-state kinetics of Mn oxides with TM or whole cells (Table 4-2 and Table 4-3).
Figure 4-2. Enzymatic reduction of bixbyite. Mn(II) formation is plotted as a function of time for incubations of purified TM fraction and bixbyite. Anaerobic reaction mixtures contained 0.1 mg/ml TM protein with 80 mg/ml (507 mM) bixbyite plus (●) or minus (○) 10 mM formate in 100 mM HEPES pH 7.5.
Figure 4-3. Reduction of Mn oxides. Reaction mixtures contained 0.1 mg/ml TM protein, 10 mM formate and increasing amounts of (A) birnessite (0.46 mM (○), 0.93 mM (●), 1.85 mM (▼), 3.7 mM (▲), 7.4 mM (■), 14.8 mM (□), 23.2 mM (♦), and 29.7 mM (◊)) in 100 mM HEPES pH 7.5 or (B) bixbyite (2.1 mM (▲), 4.2 mM (♦), 10.6 mM (■), 21.1 mM (○), 31.7 mM (▲), 42.3 mM (○), 84.4 mM (●), and 169 mM (□)).
Figure 4-4. Steady-state kinetic analysis of birnessite (●) and bixbyite (○) reduction. TM-mediated Mn oxide reduction rates are expressed as moles Mn$^{2+}$ per mole of OmcA per second. TM fractions (0.1 mg/ml) were added to 3 ml reaction samples containing buffer and various concentrations of Mn oxide. The reaction was initiated upon addition of 300 μl of 100 mM formate and 700 μl aliquots were taken at 0, 20, and 40 min for Mn$^{2+}$ quantification.
Figure 4-5. Steady-state kinetic analysis of birnessite (●) and bixbyite (○) reduction. Whole cell-mediated Mn oxide reduction rates are expressed as moles Mn$^{2+}$ per mole of OmcA per second. Whole cells (300 μl) were added to 3 ml reaction samples containing buffer and various concentrations of Mn oxide. The reaction was initiated upon addition of 300 μl of whole cell suspensions and 700 μl aliquots were taken at 0, 20, and 40 min for Mn$^{2+}$ quantification.
Flavin-dependent stimulation of Mn oxide reduction

Flavins (flavin adenine dinucleotide, flavin mononucleotide and riboflavin) have been detected in the extracellular medium of *S. oneidensis* (Marsili et al., 2008) and are proposed to act as electron shuttling agent for iron oxide reduction (von Canstein et al., 2008). Our previous kinetic studies have demonstrated their importance in whole cell-mediated goethite reduction (Ross et al., 2009). Therefore, we investigated their role in Mn oxide reduction. Steady-state kinetic experiments were performed at saturating concentrations of birnessite (6.4 mg/ml) or bixbyite (15 mg/ml) and either TM (Fig. 4-6A) or whole cells (Fig. 4-6B). Again, velocities ($k_{cat}$), expressed as mole of Mn$^{2+}$ per mol of either OmcA or MtrC per second were calculated for TM and whole cells (Ross et al., 2009). The velocities increased approximately 5-fold upon addition of 20 μM riboflavin. The fold stimulation was greatest in whole cell experiments with bixbyite when compared to the flavin minus control experiments and the velocities ($k_{cat}$ values) exhibited a trend of: WC bixbyite > WC birnessite > TM bixbyite > TM birnessite.

Reaction of riboflavin with Mn oxides

The reaction of riboflavin with either birnessite or bixbyite was studied using UV/Vis spectroscopy. Riboflavin was reduced by the xanthine oxidase system and its reaction with birnessite or bixbyite was monitored by the increase in 450 nm absorbance associated with oxidized flavin. These single turnover experiments performed at different Mn oxide concentrations yielded a second-order rate constant of 4.5 M$^{-1}$s$^{-1}$ and 0.38 M$^{-1}$s$^{-1}$ for birnessite and bixbyite, respectively.
Simulation of steady-state results with experimentally-determined rate constants

To validate our experimentally-determined rate constants and the importance of flavins, we have simulated whole cell reaction rates using a flavin-dependent mechanism. Simulations were performed with Kinsim using rate constants for OmcA and flavin (Ross et al., 2009) and flavin and Mn oxide (this study). To further assess the role of flavins in whole cell catalyzed DMR, we simulated steady-state results from whole cell experiments using a flavin-dependent mechanism (Fig. 4-8). As shown in Fig. 4-7, using our experimentally determined rate constants for individual steps of electron transfer between OmcA and flavin and between flavin and Mn oxide, a concentration of 1.2 μM flavin successfully simulated the time course of manganese reduction by whole cells.
Table 4-2. Second order rate constants for OmcA with insoluble manganese oxides.

<table>
<thead>
<tr>
<th></th>
<th>Purified Enzyme Experiments</th>
<th>TM Experiments</th>
<th>Whole cell Experiments</th>
</tr>
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<tbody>
<tr>
<td>Birnessite</td>
<td>$1.4 \pm 0.17 \text{ M}^{-1}\text{s}^{-1}$</td>
<td>$1247 \pm 45 \text{ M}^{-1}\text{s}^{-1}$</td>
<td>$59 \pm 9 \text{ M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>Bixbyite</td>
<td>$0.25 \pm 0.022 \text{ M}^{-1}\text{s}^{-1}$</td>
<td>$320 \pm 58 \text{ M}^{-1}\text{s}^{-1}$</td>
<td>$50 \pm 12 \text{ M}^{-1}\text{s}^{-1}$</td>
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</table>
Table 4-3. Second order rate constants for MtrC with insoluble manganese oxides.

<table>
<thead>
<tr>
<th></th>
<th>Purified Enzyme Experiments</th>
<th>TM Experiments</th>
<th>Whole cell Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birnessite</strong></td>
<td>1.2 ± 0.32 M⁻¹s⁻¹</td>
<td>1504 ± 47 M⁻¹s⁻¹</td>
<td>62 ± 10 M⁻¹s⁻¹</td>
</tr>
<tr>
<td><strong>Bixbyite</strong></td>
<td>0.16 ± 0.029 M⁻¹s⁻¹</td>
<td>402 ± 66 M⁻¹s⁻¹</td>
<td>52 ± 14 M⁻¹s⁻¹</td>
</tr>
</tbody>
</table>
Figure 4-6. Stimulation of Mn oxide reduction with flavin. (A) TM and (B) whole cell-mediated birnessite and bixbyite reduction rates are expressed as moles Mn$^{2+}$ per mole of OmcA per second. TM fractions (0.1 mg/ml) or whole cell suspensions (300 μl) were added to 3 ml reaction samples containing buffer or defined media and various concentrations of manganese oxides. The reaction was initiated upon the addition of 300 μl of 100 mM formate or cell suspension and 700 μl aliquots were taken at 0, 20 and 40 min for Mn$^{2+}$ quantification. Velocities obtained for reactions containing 20 μM riboflavin (RF) are represented by white bars. Steady-state kinetic experiments were performed at saturating concentrations of birnessite (6.4 mg/ml) or bixbyite (15 mg/ml).
Figure 4-7. Simulation of steady-state whole cell-mediated (A) birnessite or (B) bixbyite reduction using experimentally-determined rate constants between OmcA and riboflavin (5.9 x $10^5$ M$^{-1}$s$^{-1}$), riboflavin and birnessite (4.5 M$^{-1}$s$^{-1}$) and riboflavin and bixbyite (0.38 M$^{-1}$s$^{-1}$). Points are experimental data from experiments measuring Mn$^{2+}$ formation in whole cell incubations with birnessite (3 mg/ml) or bixbyite (26.7 mg/ml). Reaction conditions are as described in Materials and Methods. Lines represent simulated rates of Mn$^{2+}$ formation at various flavin concentrations (μM). The concentration of OmcA used in simulation is the same as the concentration in the actual experiment (8.6 x $10^{-8}$ M). See Fig. 7B for the mechanism used in the simulation.
Figure 4-8. Mechanism used for simulation of whole cell-mediated (A) birnessite and (B) bixbyite reduction shown in Fig. 7. To simulate multiple turnover (steady-state) data at the linear part of the Michaelis-Menten curve (where manganese is limiting and not saturating), OmcA was kept reduced thereby assuring that the rate of OmcA reduction was not rate limiting.
DISCUSSION

The scale-up kinetic studies described here were performed to identify the physiologically-relevant mechanisms of DMR using the criteria of kinetic competence. The implications and limitations of such studies have been illustrated previously for iron oxides reduction and the study described here utilized the same concepts (Brantley et al., 2006; Ross et al., 2009). Such examination of kinetic scaling cannot prove a mechanism, but can be used to eliminate mechanisms of DMR (Ross et al., 2009). The limitations for such studies are briefly described below.

We described the kinetic equations used for comparison between partially purified enzymes and whole cells in our paper on scale up of iron reduction (Ross et al., 2009). Briefly, we compare the Michaelis-Menten equation for enzymes to the Monod equation for whole cultures. If whole cell kinetics are performed under non-growth conditions where the biomass is constant, the Monod equation takes the form of the Michaelis-Menten equation and $V_{\text{max}}$ is equal to $\left(\frac{\mu_{\text{max}}}{Y}\right)X$ and $V_{\text{max}}/K_{\text{m}}$ is equal to the slope of the Monod plot (non-growth conditions with whole cells). At low substrate concentrations, the slope of both curves is an indicator of the kinetic response of the system to substrate concentration. Also, such a response is only valid in whole cell studies when the substrate of interest can be manipulated. Such is the case with extracellular enzymes OmcA and MtrC described in this study.

An examination of the enzyme of interest can be made using the concept of kinetic competence. For example, if involved in physiologically-relevant catalysis, kinetic constants obtained from transient-state studies should be as fast or faster than overall steady-state rates of membrane fractions and these rates, in turn, must be as fast or faster than overall whole culture rates (Cleland, 1975; Lorimer et al., 1976). When this does not hold true (transient-state rates are
at least an order of magnitude slower than whole cell catalyzed rates) the enzyme of interest is either not involved in catalysis or its involvement is not in accordance with the proposed mechanism (Brantley et al., 2006).

Reactivity of OM cytochromes

To determine the role of OM cytochromes in manganese reduction, OmcA, MtrC, and MtrC/A/B were tested for their ability to directly reduce soluble acetate-Mn^{3+} (Fig. 4-1). Past research from our laboratory and others has shown that soluble TEAs can be reduced in the periplasm at the CM (Maier and Myers, 2004; Myers et al., 2000; Ruebush et al., 2006). Nevertheless, OM hemeproteins part of the extracellular ET pathway are capable of efficiently reducing a number of soluble electron acceptors extracellularly, including chelated ferric species and U(VI) (Marshall et al., 2006; Ross et al., 2009; Wang et al., 2008). The promiscuity of this pathway in extracellular ET was further examined for manganese reduction. Transient-state rate constants obtained here with OmcA, MtrC, and MtrC/A/B and acetate-Mn^{3+} had reactivities on the order of OmcA ≥ MtrC/A/B ≥ MtrC (Table 4-1) and were comparable to reduction rates with chelated ferric species (10^5 M^{-1}s^{-1}) (Ross et al., 2009; Wang et al., 2008). Also, while it has been determined that MtrC/A/B has both soluble and insoluble iron and manganese reductase activity (Ross et al., 2007), we are not aware of any previous studies where rate constant for manganese reduction were documented. Here we report for the first time a rate constant for MtrC/A/B and acetate-Mn^{3+} of 2.3 x 10^5 ± 1.3 x 10^4 M^{-1}s^{-1}, which is comparable to OmcA and MtrC reduction of acetate-Mn^{3+} (Table 4-1). These findings support the involvement of non-specific terminal reductase(s) in extracellular electron transfer (Ruebush et al., 2006). This ET cannot participate in the proton motive force generation at the CM and only serve as grounding wires (Ross et al., 2007).
Reduction of insoluble manganese oxides

Our previous work indicated the importance of flavins in iron oxide reduction mechanism for OM cytochromes OmcA and MtrC. Using scale up kinetic comparisons here, we found rates of manganese reduction with these purified heme proteins to be two to three orders of magnitude lower than rates obtained with whole cells or TM fractions. These results corroborate our earlier studies with iron oxides and clearly show that OmcA and MtrC cannot participate in extracellular electron transfer to manganese oxides through direct contact alone.

Rates of extracellular electron transfer to mineral oxides are controlled by mineral reactivity, which is dependent upon mineral structure, mineral composition and surface area (Post, 1999). In the present study we have examined two structurally different manganese oxides, birnessite and bixbyite. Upon comparison of kinetic data for these two minerals at the various kinetic scales, transient-state rate constants for birnessite were 5 to 7 times larger than bixbyite while rate constants for in vitro birnessite reduction were 3.5 times greater than bixbyite reduction (Table 4-2 and 4-3). Surprisingly, rate constants were almost identical for both minerals in whole cell experiments. Work by Burdige et al., (Burdige et al., 1992) examined whole cell reduction of various manganese oxides and found that S. putrefaciens reduces Mn oxides with an order of reactivity of birnessite > δ-MnO₂ > pyrolusite; δ-MnO₂ and pyrolusite have rates 74% and 62% of the birnessite reduction rates, respectively (Burdige et al., 1992). In contrast, our whole cell data suggest no difference between birnessite and bixbyite reduction.

Flavin-dependent stimulation of Mn oxide reduction

The inability to kinetically account for goethite reduction by OmcA or MtrC could be resolved by inclusion of flavin in the reaction mechanism. Recent work by von Canstein et al. (2008) showed that flavins enhance iron oxide reduction but not soluble iron reduction. These workers showed that specific activity increased 10-fold when 10 μM riboflavin, FAD or FMN
was added to whole-cells grown with ferric citrate (von Canstein et al., 2008). We observed a similar effect when riboflavin was added to whole cell reactions of manganese oxides with a 5-fold increase in reaction velocities (Fig. 4-6). Comparison of our whole cell manganese kinetics with iron reduction studies from our lab and others indicate a trend where the extent of flavin stimulation is dependent upon the mineral reactivity. While flavins stimulated goethite reduction rates 10-fold, bixbyite and birnessite reduction rates were only stimulated 5-fold and 4 fold, respectively. Thus, it seems flavins have a greater effect on structured, less reactive mineral species compared to poorly soluble, amorphous or even soluble species. Based on standard redox potential, manganese reduction is more thermodynamically favorable than iron reduction (e.g. Mn$^{4+}$/Mn$^{2+}$ redox couple is +550 mV compared to +200 mV for Fe$^{3+}$/Fe$^{2+}$ at neutral pH) (Madigan, 2000; Zehnder and Stumm, 1988). As such, it follows that rate constants for manganese oxides at all three kinetic scales (purified enzymes, TM fractions and whole cells) are an order of magnitude larger than rate constants for goethite reduction.

With flavins as part of the kinetic mechanism, the $k_{cat}/K_m$ values determined from our steady-state experiments with the TM and the whole cells are not true second-order rate constants between goethite and OmcA or MtrC. They are apparent rate constants obtained with flavin acting as a shuttle. This is substantiated by our kinetic simulations of steady-state data from whole cells. In order to determine relevance of transient-state rate constants in whole cell studies, simulations were performed using Kinsim with experimentally-determined transient-state rate constants and substrate concentrations from whole cell reactions. By monitoring product formation (Mn$^{2+}$) at initial velocity conditions, the simulation output yielded a straight-line (Fig. 4-7). Data points from whole-cell studies were plotted with the kinetic simulation data and whole cell data fit the simulation only when flavins were included in the reaction mechanism.
Conclusions

While accurate scale-up is difficult, especially with insoluble substrates, we have attempted to distinguish kinetic differences due to mineral reactivity and show the following conclusions for the first time:

1) As observed for iron oxides, OmcA and MtrC are not kinetically competent to account for physiological manganese oxide reduction via direct contact because the relevant rate constants are 3 orders of magnitude too slow;

2) In the presence of flavins, the reaction rates are greatly increased and can account for the reduction of insoluble manganese oxides in vivo;

3) The extent of flavin stimulation is dependent upon the mineral reactivity, with regards to mineral composition (Fe versus Mn) and mineral structure.

Therefore, of the possible mechanisms used by Shewanella of electron transfer to extracellular substrates -- direct contact (Lower et al., 2007; Xiong et al., 2006), electron shuttle (Marsili et al., 2008; von Canstein et al., 2008), and chelation (Taillefert et al., 2007) -- our data has eliminated direct contact as a plausible mechanism of electron transfer from OmcA or MtrC to manganese oxides and is consistent with the involvement of electron shuttles in DMR. Our work does not prove or disprove the viability of the metal-chelate mechanism; however, our research is consistent with the ability of Shewanella to reduce metal-ligand complexes. Flavins are particularly implicated by our work.
Chapter 5

CONCLUSIONS

Mineral transformations shape the geologic landscape on Earth and have been studied for many years. Not until recently though, has the impact of microorganisms on these processes been examined carefully. Since the first studies by Lovley and Phillips (1988) and Myers and Nealson (1988) showing a link between reduction of iron and manganese to microbial growth, much attention has been focused on mechanisms of dissimilatory metal reduction (DMR). Of the two model organisms of DMR, \textit{Geobacter sulfurreducens} and \textit{Shewanella oneidensis} MR-1, our laboratory has focused on the latter due to its respiratory versatility and fast growth rates. The \textit{S. oneidensis} genome has been sequenced, thus providing critical information for elucidation of electron transport systems and metal reduction capabilities. My work involving biochemical and kinetic characterization of the electron transfer pathway for DMR in \textit{S. oneidensis} has focused on the following:

1. Biochemical assays to determine protein-protein interactions and protein complexes responsible for electron transfer to extracellular substrates in DMR.

2. Kinetic analysis of iron reduction at multiple scales (transient-state and steady-state) to determine physiologically relevant electron transport mechanisms for DMR using the concept of kinetic competence.

Early biochemical and genetic studies of *S. oneidensis* revealed many multi-heme cytochromes important for DMR yet the complete pathway for electron transfer to extracellular substrate had not been elucidated. Previous work in our lab examined the sub-cellular location of DMR activity, determined appropriate assays for monitoring activity for soluble substrates, and initiated work to determine protein partners involved in DMR (Ruebush et al., 2006a; 2006b).

My work, presented in Chapter 2, revealed an OM protein complex MtrC/A/B consisting of MtrA, MtrB and MtrC. MtrC/A/B was purified using anion exchange and gel filtration chromatography and determined to have a molecular mass of ~198 kDa using analytical ultracentrifugation. To address the importance of this complex in metal reduction, preliminary kinetic studies using UV-visible spectroscopy revealed activity towards soluble and insoluble iron and manganese species. Reduced MtrC/A/B reacted fastest with soluble ferric (<15 sec) followed by the insoluble Mn oxide birnessite (4 min), and insoluble Fe oxides ferrihydrite (10 min) and goethite (35 min). This finding revealed a physiologically relevant OM protein complex with iron and manganese reductase activity capable of transferring electrons across the OM to insoluble substrates.

One important question in DMR is the mechanism of electron transfer at the microbe-mineral interface. Elucidation of the mechanism requires defining the terminal reductases involved and characterizing their interaction with mineral oxides. To date, three mechanisms have been proposed and include direct contact (Lower et al., 2007; Lower et al., 2001; Xiong et al., 2006), electron shuttles (Marsili et al., 2008; von Canstein et al., 2008) or metal chelators (Taillefert et al., 2007). While *Geobacter* requires direct contact, *Shewanella* may potentially utilize a combination of direct and indirect mechanisms. In order to determine a physiologically-relevant mechanism, I have utilized iron reduction kinetics at multiple scales and the concept of kinetic competence. This is described in Chapter 3. The first scale involved transient-state kinetic studies with purified OmcA and MtrC. With the exception of work by Wang et al. (Wang
et al., 2008), kinetic data for purified OM cytochromes involved in DMR with iron substrates is limited. The next scale examined iron reduction kinetics of an *in vitro* system with purified TM fractions or whole-cell suspensions. By quantifying the molar amounts of OmcA or MtrC for TM fractions and whole cells iron reduction activities were converted to a velocity, allowing for comparison to transient-state rate constants. Using scaling kinetics and kinetic competence, I have shown that OmcA and MtrC are able to account for catalysis of soluble ferric chelates in whole cell reactions. Kinetic competence was not upheld in reduction of the insoluble oxide goethite by purified OmcA or MtrC, with transient-state rates three orders of magnitude slower than steady-state rates. This finding is consistent with the conclusion that direct contact alone can not account for catalysis of what in whole cells. Addition of flavins stimulated iron oxide reduction. Furthermore, using Kinsim, whole cell goethite reduction was simulated and transient-state rates approached *in vivo* rates when flavins were included in the reaction mechanism, thus supporting the role of flavins as electron shuttles to insoluble substrates.

Another important geological process is the biogeochemical cycling of manganese. Like iron, manganese is predominant in the subsurface as solid Mn-oxide minerals and these minerals are more reactive than Fe-oxides, which makes Mn a primary factor in mineral transformations. To determine the role of OM hemeproteins OmcA and MtrC in manganese oxide reduction, the concept of kinetic competence (Cleland, 1975) and scaling kinetics (Brantley, et al., 2006) analysis were employed. The kinetic concepts and techniques from Chapter 3 for iron oxide reduction were utilized here for manganese. I have shown that physiologically relevant catalysis of manganese oxides by OmcA and MtrC requires the presence of an electron shuttle. Not only were whole cell manganese reduction rates stimulated upon addition of flavin, but the extent of stimulation was dependent upon the structure and surface area of the mineral. Whole cell manganese reduction could not be simulated with transient-state rate constants unless flavin was
included in the reaction mechanism. Again this eliminates the mechanism of direct contact as physiologically relevant and further supports the electron shuttle hypothesis.

**FUTURE RESEARCH**

Electron transfer to insoluble substrates such as Fe and Mn oxides requires the passage of reducing equivalents from the CM, through the periplasm and OM, to terminal reductases localized on the extracellular face of the OM. Understanding this process mechanistically will have an impact on bioremediation efforts and biotechnological advances in growth on electrodes for electricity. Future experiments involved in mechanisms of electron transfer may address the following issues (Figure 5-1):

1. Proteins directly responsible for electron transfer across the periplasm remain unknown. One major heme protein found in the periplasm, CctA may be involved in taking electrons from CymA at the CM and passing them along to MtrA at the OM (Gordon et al., 2000). While our protein cross-linking studies revealed no interaction between CymA and CctA, this does not rule out the possibility of these two proteins interacting *in vivo*. Further studies examining interactions of CymA or CctA with other proteins utilizing different methods may help to establish this missing link in the ET chain.

2. The interaction between CctA and MtrA has not been observed. If electrons are received at the inner leaflet of the OM by MtrA, and CctA transfers electrons across the periplasm, then other methods should be utilized to determine this interaction. We have the necessary tools to study this interaction using a nickel column and heterologously expressed proteins MtrA and CctA with cleavable His-tags. Preliminary data suggests a weak interaction between MtrA and CctA. Briefly, MtrA was bound to a nickel column and CctA was passed through. CctA eluted earlier when MtrA was absent from the column, suggesting
retention of CctA through specific interaction with MtrA. Future work is needed to
determine the specificity of this interaction by examining other proteins, such as OmcA,
that do not interact with MtrA in vivo.

3. While an OM MtrC/A/B complex has been purified and shown to have Fe and Mn reductase
activity, it is still unclear how electrons are transferred across the OM. MtrB has no heme
binding sites except for one CXXC domain and while MtrB has a redox-active cysteine
(Ross and Hartshorne, unpublished data) the mechanism of electron transfer from MtrA
through MtrB to MtrC remains unknown. Further work to understand how electrons are
transferred through the OM by this protein complex is important for understanding ET to
extracellular substrates and could possibly represent a basic model for an inter-membrane
ET pathway (Hartshorne). Elucidation of the crystal structure for MtrC/A/B is paramount
to defining this process.

4. One aspect of the ET pathway in DMR is the interaction of extracellularly exposed proteins
with insoluble substrates. Identification of the potential roles of OmcA and MtrC as
terminal reductase(s) has led to studies to examine direct binding between mineral surfaces
and OM proteins OmcA and MtrC using atomic force microscopy (AFM) (Lower et al.,
2007; Lower et al., 2001; Xiong et al., 2006). If indeed direct binding occurs in vivo, then
it would be beneficial to examine the potential metal binding sites on OmcA and MtrC. It
is possible to determine protein regions exposed to extracellular substrates, such as iron
oxides, through chemical modification of specific exposed residues. The modified residues
can be labeled and analyzed to determine which are most reactive and therefore most likely
to bind metals. This method can be extended to determine the potential flavin binding sites
of these proteins.

5. The kinetic mechanisms of flavins in extracellular ET should be examined in more detail.
Scale up kinetic studies with insoluble manganese oxides revealed the importance of
flavins for \textit{in vivo} manganese reduction. Of note, the extent of stimulation upon addition of micromolar amounts of riboflavin under conditions of saturating substrate concentration varied depending upon the structure and surface area of the mineral. Flavins had less of an effect on the more reactive mineral birnessite compared to bixbyite. Furthermore, the highly structured iron oxide goethite is less reactive than both manganese oxides yet exhibited the largest increase in rate when stimulated with flavin. It would be interesting to determine the controlling factors of this phenomenon and whether or not this could be extrapolated to predictions in natural systems. For example, using isothermal calorimetry (ITC) the affinity of flavin for various minerals could be determined.

6. Kinetic studies of ET to insoluble substrates are important for determining an overall reaction mechanism and identifying rate-limiting steps to be exploited for bioremediation efforts. Our group has determined the necessity of flavins in iron oxide reduction using kinetic scaling analysis which supports a mechanism of electron shuttles \textit{in vivo}. While transient-state kinetic studies of detergent-solubilized membrane proteins have provided valuable information about ET to insoluble substrates, reconstitution of individual enzymes or enzyme complexes into phospholipids micelles will provide a more physiologically-relevant environment to study these processes.
Figure 5-1: Schematic of electron transfer to extracellular substrates. Carbon sources, such as formate, are oxidized at the cytoplasmic membrane (CM) by primary dehydrogenases (formate dehydrogenase (Fdh) for formate). Reducing equivalents are passed through the lipid soluble quinone pool (MQ\(\rightarrow\)MQH\(_2\)) to CymA. Electrons are then transferred across the periplasm, possibly through CctA, to the inner leaflet of the outer membrane (OM). At this point, MtrA can accept electrons from either CymA or CctA and transfer them to MtrC through MtrB. Once reducing equivalents reach the outer leaflet of the OM, extracellular substrates can be reduced. 1) Protein cross-linking studies for CymA and CctA did not show any interaction. 2) Preliminary studies have shown a weak interaction between CctA and MtrA but more conclusive evidence is needed to corroborate this finding. 3) Work has begun to determine the mechanism of electron transfer across the OM and MtrA is hypothesized to interact with MtrC through MtrB. 4) Modification of exposed residues to determine the metal binding site of OM proteins OmcA and MtrC is underway. This work would help to identify specific residues involved in interaction with metal oxides. 5) A more detailed examination of flavin mediated metal oxide reduction is needed to determine specific mechanisms of electron shuttles in DMR and their importance in environmental settings.


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