

## Chapter 2

# *Shewanella oneidensis* and Extracellular Electron Transfer to Metal Oxides

**Daad Saffarini, Ken Brockman, Alex Beliaev, Rachida Bouhenni and Sheetal Shirodkar**

**Abstract** Anaerobic metal reduction by bacteria plays an important role in biogeochemical cycles, bioremediation, and in biotechnological applications such as electricity generation. *Shewanella oneidensis* is one of the best-studied metal reducing bacteria and its analysis led to the identification of the mechanisms this bacterium uses for respiratory metal reduction. The major proteins involved in metal reduction in *S. oneidensis* consist of an outer membrane decaheme *c*-type cytochrome (MtrC), an outer membrane porin (MtrB) and a periplasmic decaheme *c*-type cytochrome (MtrA). These proteins form a complex that is located on the outer cell surface and transfers electrons extracellularly to the metal oxides. Although other proteins, such as the outer membrane decaheme *c*-type cytochrome OmcA, are thought to be involved in metal reduction, their role in this process appears to be minor. Several mechanisms to explain the extracellular electron transfer to metal oxides have been proposed. These include direct contact of cells with metal oxides, the use of flavins or electron shuttles, and the use of conductive appendages or nanowires. Flavins, which are thought to allow metal reduction at a

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D. Saffarini (✉)

Department of Biological Sciences, University of Wisconsin-Milwaukee,  
Milwaukee, WI 53211, USA  
e-mail: daads@uwm.edu

K. Brockman

The Research Institute, Nationwide Children's Hospital, The Ohio State  
University College of Medicine, Columbus, OH 43205, USA  
e-mail: kenneth.brockman@nationwidechildrens.org

A. Beliaev

Pacific Northwest National Laboratory, Richland, WA, USA  
e-mail: Alex.Beliaev@pnnl.gov

R. Bouhenni

Summa Health System, Division of Ophthalmology Research,  
Akron, OH 44309, USA  
e-mail: bouhennir@summa-health.org

S. Shirodkar

Amity Institute of Biotechnology, Uttar Pradesh, India  
e-mail: sshirodkar@amity.edu

distance from the cells, have been shown to function as cofactors that bind to the outer membrane cytochromes and mediate electron transfer. Conductive appendages or pili, also known as nanowires, have been implicated in mediating electron transfer at a distance. However, *S. oneidensis* mutants that lack pili are able to reduce metals similar to the wild type. Recently, these appendages have been shown to consist of membrane extensions and membrane vesicles. Thus, metal reduction by *S. oneidensis* appears to be mostly the result of direct contact of cell's outer membrane cytochromes with the insoluble metal oxides.

**Keywords** Metal reduction • *Shewanella oneidensis* • Extracellular electron transfer • Electron shuttles • Nanowires • MtrC • MtrA • MtrB

## 2.1 Introduction

Iron is an essential micronutrient for almost all living organisms and is one of the most abundant elements on earth. In nature, iron exists in either reduced ( $\text{Fe}^{+2}$ ) or oxidized ( $\text{Fe}^{+3}$ ) forms with speciation determined by key environmental variables such as dissolved oxygen tension and pH. Microorganisms are major contributors to cycling of iron between the oxidized and reduced forms, a process that has become known as the microbial “ferrous wheel” (see [31, 49, 103] and references within). Bacteria and Archaea can use reduced iron as an electron source in aerobic, anaerobic, and acidic environments. As a result,  $\text{Fe(II)}$  is oxidized to  $\text{Fe(III)}$ , which can then be used by metal reducing bacteria as a terminal electron acceptor for anaerobic respiration. The two best-studied metal reducing bacteria, *Shewanella oneidensis* and *Geobacter metallireducens*, were almost simultaneously isolated in pure culture in 1988 [54, 70]. Since then, many other metal reducing bacterial and archaeal species capable of respiratory metal reduction have been isolated and identified. These include facultative anaerobic bacteria such *S. putrefaciens*, *S. loihica*, *Pantoea agglomerans*, and *Thermus* strain SA-01 [35, 36, 48, 76], anaerobic bacteria such as *G. metallireducens* (reviewed in [53]) and *Ferribacterium limneticum* [22, 23], and anaerobic archaea such as *Geoglobus ahangari* [47]. Since the isolation of these organisms, our understanding of the molecular mechanisms of metal reduction, its involvement in biogeochemical cycles, and its potential use in bioremediation and electricity production, has increased exponentially. In this chapter, we focus on *S. oneidensis* MR-1 and discuss the molecular mechanisms this bacterium uses to transfer electrons extracellularly to metal oxides.

## 2.2 The *Shewanella* Genus

Members of the genus *Shewanella* are Gram-negative  $\gamma$ -Proteobacteria. They are widespread in diverse environments that include freshwater and marine sediments and water columns, crude oil pipelines, hydrothermal vents, iron-rich microbial mats, activated sludge, and marine fish ([36, 93, 113] for review). Although some *Shewanella* species were recovered from freshwater environments, these isolates are thought to be of marine origin and their presence in freshwater systems is predicted to be recent [43]. The *Shewanella* genus is best known for extracellular electron transfer and, with the exception of *S. denitrificans*, all species sequenced to date have the genes required for this process. The DOE Joint Genome Institute site (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>) includes the genome sequences of 36 *Shewanella* species, 23 of which are complete. Analysis of these genomes provided insight into the environmental adaptation and evolution of the *Shewanella* species and revealed diverse metabolic abilities among its members [36].

### 2.2.1 *Shewanella oneidensis* MR-1

*S. oneidensis* MR-1 is one of the best characterized members of the *Shewanella* genus and the first to have its genome sequenced [44]. It was initially isolated as a Mn(IV) reducer from Oneida Lake sediments and identified as *Altermonas putrefaciens* [70] before being classified as *Shewanella oneidensis* [106]. *S. oneidensis* MR-1 uses fermentation products as carbon and/or energy sources and has a well-developed chitin utilization system [76, 114]. Metabolically, it is the most diverse of the *Shewanella* species with regard to the electron acceptors it can use for respiration which include  $O_2$ , fumarate,  $NO_3^-$ ,  $NO_2^-$ , trimethylamine N-oxide (TMAO), dimethylsulfoxide (DMSO), iron and manganese oxides, and sulfur species such as elemental sulfur and sulfite [25, 36, 67, 70, 76, 102]. Radionuclides and toxic metals such as Tc, U, Cr, can also serve as electron acceptors [6, 52, 59, 60, 73, 75, 76]. Forty-one *c* cytochromes are encoded by the *S. oneidensis* MR-1 genome [44, 89, 104], reflecting its ability to use a wide array of electron acceptors.

The central metal reductase complex in *S. oneidensis* MR-1 is composed of three subunits, MtrB, MtrC, and MtrA. These proteins are encoded by the *mtrCAB* operon that is expressed under microaerobic and anaerobic conditions, even in the absence of metal electron acceptors. Contrary to expectations, the expression of *mtrCAB* is highest in the presence of fumarate and not in the presence of metal oxides [4, 5]. Although these genes are required for metal reduction, their expression is decreased when fumarate-grown cells are transferred to media containing metal oxides [5]. Elevated gene expression under fumarate-growth conditions was also observed for *cymA* and *omcA* [4, 5] that have roles in metal reduction as described in more detail below. Expression of *mtrCAB* and *omcA* is controlled by the cAMP receptor protein CRP [17, 92]. This protein regulates the expression of

many anaerobic reductase genes in *S. oneidensis* MR-1, unlike its role in the regulation of carbon metabolism in other bacteria. The role of CRP in anaerobic respiration is not limited to *S. oneidensis* MR-1. A similar mode of regulation has been shown in *Shewanella* sp ANA-3 [68] suggesting that this may be a property of the *Shewanella* genus.

## 2.3 The *S. oneidensis* MR-1 Metal Reduction Electron Transport Chain

### 2.3.1 *CymA Links the Metal Reductase to the Menaquinol Pool*

CymA is a 21 kDa membrane-anchored *c*-type cytochrome that belongs to the NapC/NirT family of quinol dehydrogenases [74, 97]. In contrast to its family members, CymA lacks specificity and is involved in electron transfer to multiple terminal reductases [69, 74]. CymA-dependent reductases, which include the nitrate, nitrite, DMSO, Fe(III), and fumarate reductases, appear to be located in the periplasm or outer membrane of *S. oneidensis* MR-1 [21, 37, 40, 41, 51, 66, 72, 74, 96]. In contrast, CymA is not involved in electron transfer to inner membrane-anchored enzymes such as the TMAO, thiosulfate, and sulfite reductases [8, 16, 25, 38, 102].

CymA is tetraheme *c* cytochrome that binds one high-spin and three low-spin hemes [58]. It is a menaquinol-7 dehydrogenase and its activity is inhibited by the respiratory chain inhibitor 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO) and by site directed mutagenesis of the putative quinol-binding site [26, 63, 74, 116]. Interestingly, increased expression of SirCD, which is predicted to function as a quinol oxidoreductase during sulfite reduction in *S. oneidensis* [102], can complement an *S. oneidensis* MR-1 *cymA* mutant and allow reduction of Fe(III) and other electron acceptors [19].

Notably, the location of CymA in the inner membrane does not appear to be essential for its interaction with the menaquinol pool. A soluble CymA that lacks the membrane-spanning domain (the first 35 amino acids of the protein) can complement a *cymA* mutant [96]. Because CymA is thought to interact with menaquinones in the cytoplasmic membrane, it is not clear how it can still function in the absence of its membrane anchor. Zagar and Saltikov suggested that additional sites in CymA could interact with the inner membrane and allow further interactions with the quinol pool [116].

### 2.3.2 *MtrA: A Periplasmic Decaheme c Cytochrome*

MtrA is a 32 kDa decaheme *c*-type cytochrome that has been shown to be essential for metal reduction in *S. oneidensis* MR-1 and *Shewanella* sp. ANA-3 [3, 87]. The sequence of MtrA includes a leader peptide and its secretion into the periplasmic space was confirmed by heme staining and Western blot analysis using MtrA-specific antibodies [3, 87]. In cells lysed by osmotic shock, the protein is present mostly in the outer membrane and is associated with MtrCB with a 1:1:1 stoichiometry [13, 91]. MtrA binds 10 low-spin hemes, and has a low amino acid to heme ratio compared to other heme-containing proteins [32, 84]. Based on small-angle X-ray scattering and analytical centrifugation data, MtrA is estimated to be a monomeric protein of 104 Å in length [32]. Using a bacterial two-hybrid system, Borloo et al. [9] determined that MtrA interacts with CymA supporting the hypothesis that CymA transfers electrons directly to the terminal metal reductase. This interaction, however, appears to be transient and CymA is able to reduce MtrA in vitro without the formation of a CymA-MtrC complex [33]. In addition to its role in electron transfer, MtrA appears to be required for stability of the outer membrane porin MtrB [94].

### 2.3.3 *MtrB: An Outer Membrane Porin*

MtrB is an outer membrane protein that is essential for metal reduction [2, 20] but is the least studied of the metal reductase components. Computer analysis using PRED-TMBB and proteinase K digestion of MtrB-containing proteoliposomes predicted MtrB to have 28 β-strands that form the transmembrane β-barrel, periplasmic N-terminus and short loops, and 14 long loops exposed on the exterior cell surface [109]. Based on this model, MtrB forms a pore of 30–40 Å in diameter that can easily fit MtrA [32, 109]. The N-terminus of MtrB from *S. oneidensis* MR-1 and metal reducing *Shewanella* and *Ferrimonas* species contains a conserved CXXC motif that appears to be important for metal reduction [108]. Substitution of the first cysteine in the *S. oneidensis* MR-1 CXXC motif, C42, with an alanine, results in complete loss of metal reduction [108]. Substitution of both cysteine residues in this motif with serines also led to complete loss of metal reduction, and the mutagenized MtrB was not detected in Western blots, likely due to degradation. These results suggest that the N-terminus CXXC motif is important for stability of MtrB (Saffarini and Beliaev, unpublished results).

### 2.3.4 *MtrC: An Outer Membrane Decaheme c-type Cytochrome*

MtrC was first identified in 2001 as a major contributor to metal reduction [3] and it is the most studied component of the metal reductase. It is a decaheme *c*-type cytochrome located on the outer surface of *S. oneidensis* MR-1 cells and it transfers electrons directly to metal oxides and electrodes of microbial fuel cells [11, 12, 91, 110]. The external location of MtrC on the cell surface was determined by proteinase K treatment of whole cells or MtrC-containing liposomes and by atomic force microscopy [10, 56, 109]. MtrC is a lipoprotein [71] with a conserved N-terminal sequence (CGGS) found in MtrC proteins from other *Shewanella* species. The cysteine acts as the lipid attachment site and its replacement with a serine leads to accumulation of soluble MtrC in the culture supernatant [100] (Shirodkar and Saffarini, unpublished). Targeting MtrC to the outer cell surface requires the Type II secretion system, and mutants deficient in this system completely lose the ability to reduce metals [10]. MtrC is predicted to be a monomeric protein [42] with a uniform distribution on the surface of *S. oneidensis* MR-1 cells [56]. It also appears to have a slow turnover rate and is relatively insensitive to oxygen [111]. Biochemical analyses of MtrC indicate that it binds 10 low-spin hemes that are reduced within a potential window of +100 to −400 mV [42]. The crystal structure of MtrC has not been resolved, but a model was generated based on the structure of MtrF, an outer membrane decaheme *c*-type cytochrome described in more detail below. Based on this model, MtrC is predicted to have two domains with the hemes arranged in a staggered cross motif and in close proximity to each other thus facilitating electron transfer [27].

### 2.3.5 *The Outer Membrane Cytochromes OmcA and MtrF*

In addition to MtrC, the *S. oneidensis* MR-1 genome encodes two decaheme *c*-type cytochromes designated OmcA and MtrF. These proteins exhibit similarity to MtrC and their genes are located upstream of the *mtrCAB* operon. The participation of MtrF and OmcA in metal reduction has been extensively investigated and the results indicate that although both proteins are capable of metal reduction, their contribution to respiratory growth with Fe(III) appears to be minor. Mutants that lack MtrC exhibit a 75 % decrease in Fe(III) reduction compared to the wild type, suggesting that the residual activity is due to other outer membrane cytochromes. The contribution of OmcA and MtrF to metal reduction was determined using mutants that lack these genes. Deletion of *mtrF* or *omcA* did not result in a significant reduction in the mutants' ability to use metal oxides as electron acceptors. The role of MtrF and OmcA, however, became more evident in mutants that also lacked *mtrC*. Double ( $\Delta mtrC\Delta omcA$ ) and triple ( $\Delta mtrC\Delta omcA\Delta mtrF$ ) were completely deficient in metal reduction [20] indicating that the residual metal reductase

activity observed in *mtrC* mutants is due to the activity of OmcA, MtrF, or both. Interestingly, deletion of either *omcA* or *omcA* and *mtrF* in a  $\Delta mtrC$  background gave comparable results with regard to metal reduction [20]. If MtrF contributes to metal reduction in vivo, one would expect the triple mutant to be more deficient in metal reduction than the  $\Delta mtrC\Delta omcA$  mutant. These results suggest that OmcA plays a bigger role than MtrF in metal reduction, perhaps accounting for the majority of the residual reductase activity observed in the  $\Delta mtrC$  mutants. To further investigate the roles of OmcA and MtrF in metal reduction, mutants that lack all outer membrane *c*-type cytochromes were generated and transformed with medium to high copy-number plasmids carrying either *omcA* or *mtrF*. Introduction of *omcA* into these mutants did not restore metal reduction, in contrast to complementation with *mtrF* that allowed the mutant to reduce iron oxides to wild type levels [15, 20]. These results are surprising because MtrF is not known to play a significant role in metal reduction compared to OmcA. The inability of OmcA to restore metal reduction in the mutant was attributed to the absence of MtrC that is thought to transfer electrons to OmcA [15].

Purified OmcA has one high-spin and 9 low-spin hemes and can strongly bind to and reduce hematite and ferrihydrite [7, 46, 55, 64, 112]. It attaches to hematite in a conformation that allows direct electron transfer through maximum contact with the metal [46]. OmcA also strongly interacts with MtrC to form a tight complex, and this interaction is thought to enhance metal reduction [64, 91, 99]. MtrF is predicted to be a component of the MtrDEF complex that is similar to MtrCAB, but is postulated to have a function distinct from other outer membrane *c*-type cytochromes [82]. The *mtrDEF* genes are highly expressed in cell aggregates in response to calcium and it is suggested that MtrDEF play a role in detoxification or reduction of radionuclides under aerobic conditions [62]. The structures of OmcA and MtrF were recently determined at 2.7 and 3.2 Å respectively [18, 29]. Although these proteins share low sequence identity (25 %), their basic structure and heme arrangement appear to be similar. In both proteins, hemes are arranged in two chains that intersect and form a staggered cross motif. Each heme is within 7 Å from its nearest neighbor thus allowing rapid electron transfer between hemes [18, 29].

Although the genomes of several *Shewanella* species contain *omcA* and *mtrF*, others lack these genes despite the fact that these species are able to reduce metals similar to *S. oneidensis* MR-1 [36]. Examples include *S. putrefaciens* W-3-18-1 and *Shewanella* sp. strain HRCR-6 that express, in addition to MtrC, an outer membrane *c*-type cytochrome designated UndA. This protein is an 11-heme *c*-type cytochrome that appears to play a role in metal reduction, can partially restore Fe (III) reductase activity to an *S. oneidensis* MR-1  $\Delta mtrC\Delta omcA$  mutant, and appears to have uranium (VI) reductase activity [98, 115]. The crystal structure of UndA from strain HRCR-6 was recently determined at 1.67 Å [28]. Despite the differences in the number of heme *c* groups that each protein binds, a comparison of UndA and MtrF structures indicated they share a conserved 10 heme staggered cross motif [28], similar to OmcA and possibly MtrC.

## 2.4 Electron Shuttles and Microbial Nanowires

There has been much debate as to whether *S. oneidensis* MR-1 reduces metals directly through contact of the reductase with the metal oxides or through intermediates such as electron shuttles and nanowires. Electron shuttles are soluble redox-active molecules that can mediate electron transfer between the cell surface and metal oxides or electrodes. The role of electron shuttles in metal reduction gained interest following the report by Newman and Kolter [77] who documented the potential involvement of excreted quinones in extracellular electron transfer [77]. In 2008, two groups identified riboflavin and flavin mononucleotide (FMN) as the electron shuttling molecules in *S. oneidensis* MR-1 and other *Shewanella* species [61, 107]. Although flavin secretion is thought to be important for metal reduction, which occurs under anaerobic conditions, flavin concentrations were similar in supernatants of aerobic and anaerobic cultures [107]. All 23 sequenced *Shewanella* species have the genes for riboflavin biosynthesis, including *S. denitrificans* that secretes flavins but does not reduce metals [14]. Flavins have been shown to accelerate electron transfer to metals oxides and are thought to allow greater access to these electron acceptors [1, 18, 50, 61, 107]. Kotloski and Gralnick isolated a mutant that lacks the bacterial FAD exporter (Bfe; SO\_0702) and determined that this protein is involved in flavin secretion [50]. The *bfe* mutant was severely impaired in ferrihydrite reduction but was able to reduce ferric citrate similar to the wild type. Based on their results, the authors suggested that flavins account for 75 % of insoluble metal reduction activity under their laboratory conditions, while the rest is due to direct contact of cells with metal oxides [50]. The slower rate of metal reduction in the absence of flavins led to the hypothesis that the activity of MtrC and OmcA results in a bottleneck in the electron transfer pathway that is relieved by redox active molecules such as flavins [1, 90]. Recently however, Okamoto and colleagues demonstrated that acceleration of electron transfer by free flavins is not energetically favorable [78]. Rather, flavins associate directly with outer membrane *c* cytochromes as semiquinone cofactors that mediate one-electron transfer reactions [78, 79]. A similar mechanism appears to operate in the anaerobic metal reducer *Geobacter sulfurreducens* [80, 81]. Flavin binding to MtrC and OmcA exhibits specificity where FMN binds to MtrC and riboflavin associates with OmcA [78, 79].

In addition to flavins, metal reducing bacteria are thought to use appendages, called nanowires, to transfer electrons to metal oxides and electrodes of microbial fuel cells at a distance. In *S. oneidensis* MR-1 cells grown under limited oxygen conditions, electrically conductive appendages, or nanowires, were detected using scanning tunneling microscopy and tunneling spectroscopy [39]. Mutants that lack outer membrane *c*-type cytochromes, and therefore are deficient in metal reduction, produced non-conductive nanowires [30, 39]. Electrically conductive nanowires were also identified in the metal reducer *G. sulfurreducens* [86]. In this bacterium, PilA, the major component of type IV pili, was found to be electrically conductive, and its loss resulted in loss of iron oxide reduction [86]. Aromatic amino acids in



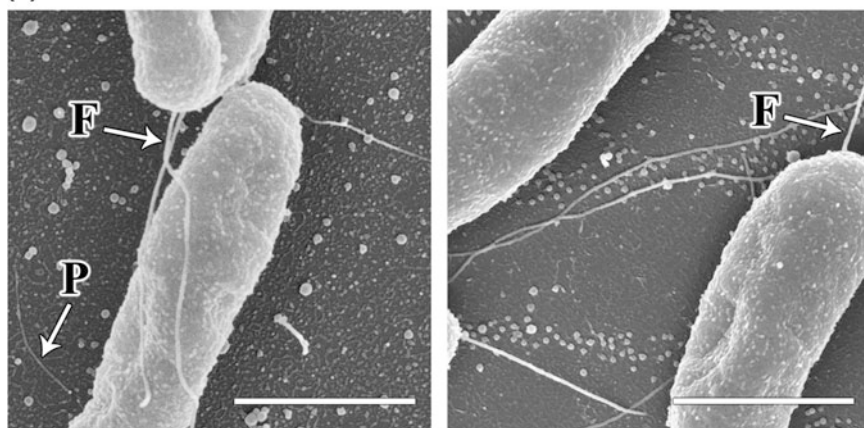
the *G. sulfurreducens* PilA C-terminus appear to be important for PilA function, and their substitution with other amino acids decreases conductivity and metal reduction [105]. *S. oneidensis* MR-1 expresses two types of pili, Type IV and Msh, on its cell surface [11]. To determine the role of these appendages in metal reduction and electricity production, we generated mutants that are deficient in type IV and Msh pili biogenesis (Fig. 2.1a). The mutants were able to reduce metal oxides similar to the wild type (Fig. 2.1b) and generate electricity in microbial fuel cells [11], suggesting that unlike *G. sulfurreducens* pili, the *S. oneidensis* MR-1 pili are not involved in long-range extracellular electron transfer. Recently, the nature of the conductive appendages or nanowires in *S. oneidensis* MR-1 was revealed [83]. Pirbadian and colleagues were able to show in real time the formation of *S. oneidensis* MR-1 nanowires. Using immuno-fluorescence imaging, the authors were able to monitor the formation of the nanowires and show that they are extensions, or vesicles, of the outer membrane and periplasm [83]. These vesicles would be expected, therefore, to contain outer membrane *c*-type cytochromes that are capable of extracellular electron transfer.

## 2.5 Extracellular Electron Transfer by *S. oneidensis* MR-1

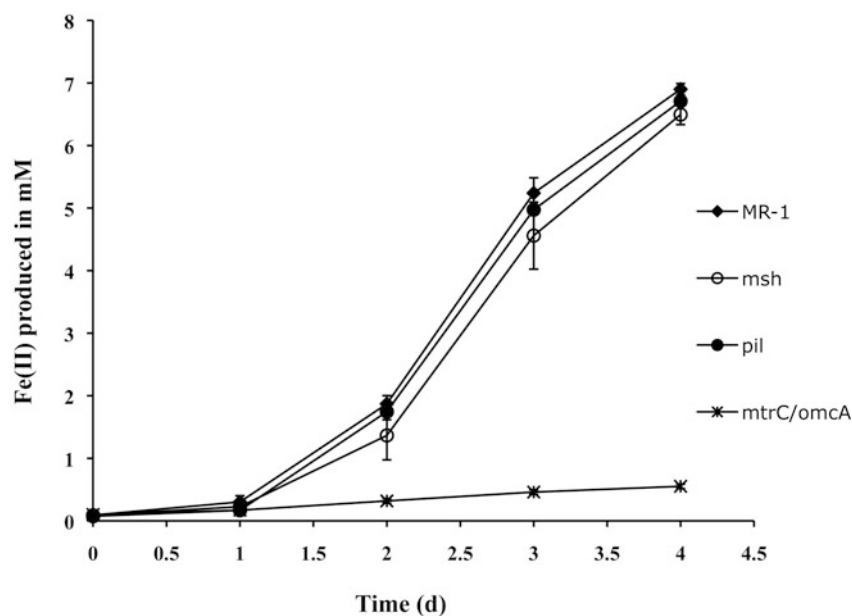
The localization of the metal reductase in the outer membrane of *S. oneidensis* MR-1 cells with exposure to the extracellular environment requires electron transfer from the inner membrane, through the periplasmic space and outer membrane, and finally to the extracellular electron acceptor. Following the identification of the MtrCAB proteins, a simple model that describes this electron transport chain was proposed [3]. Since then, a wealth of data elucidated protein-protein interactions, protein localization and structure, and provided a more detailed and refined model of the mechanism by which *S. oneidensis* MR-1 transfers electrons extracellularly to metal oxides. Five proteins have been confirmed through biochemical and genetic analyses to be involved in metal reduction. These consist of MtrB, MtrC, OmcA, MtrA, and CymA (Fig. 2.2). The core metal reductase components consist of MtrCAB. These proteins confer metal reductase activity on *E. coli* [45] and are sufficient to account for physiological levels of metal reduction in *S. oneidensis* MR-1 [109]. MtrA is a periplasmic protein that forms tight interactions with MtrB and MtrC [91] and is embedded within MtrB forming a “porin cytochrome” electron transfer module [88]. MtrC is exposed on the outer cell surface, and presumably interacts with MtrA within the MtrB pore. Our understanding of the interactions between MtrA and MtrC within the porin cytochrome model is sufficient to explain electron transfer from MtrA to MtrC and subsequently to extracellular electron acceptors.

OmcA, similar to MtrC, is a decaheme *c*-type cytochrome that is exposed on the cell surface and requires the type II secretion system to reach its final destination. Analysis of OmcA crystals suggests that the protein forms a dimer in the outer membrane [29], and cross-linking experiments identified an MtrC/OmcA complex

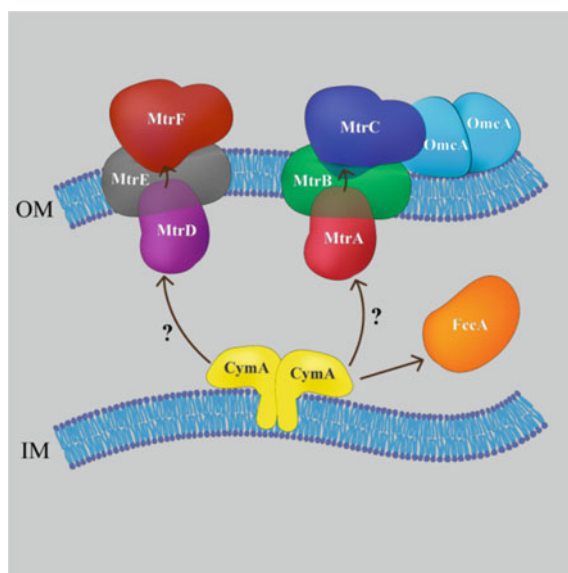
(a)



(b)



**Fig. 2.1** Role of *S. oneidensis* MR-1 pili in metal reduction. **a** Scanning electron micrograph of *S. oneidensis* MR-1 (left panel) and  $\Delta pil\Delta msh$  mutant that lacks the type IV and Msh pili biogenesis systems (right panel). Flagella and pili are indicated. White bar = 1  $\mu m$ . **b** Iron oxide reduction by *S. oneidensis* MR-1 and mutants strains. In contrast to the mutant that lacks MtrC and OmcA, mutants deficient in type IV or Msh pili biogenesis ( $\Delta pil$  and  $\Delta msh$ ) reduced iron oxide similar to the wild type (see [11] for more details)



**Fig. 2.2** Model of the *S. oneidensis* MR-1 electron transport chain that leads to extracellular metal reduction. CymA is predicted to be a dimer and a quinol oxidoreductase that transfers electrons from the inner membrane to MtrA. It is not clear at present if an intermediate electron carrier links CymA with the decaheme periplasmic proteins MtrA. The core metal reductase complex consists of MtrABC, where MtrC is exposed on the cell surface and MtrA is embedded in the MtrB pore and transfers electrons from the periplasm to MtrC. OmcA participates in metal reduction and is predicted to be a dimer. MtrDEF is a second outer membrane complex that is similar to MtrCAB, but its function in metal reduction is not clear (see text for more detail)

in a 2:1 ratio [29, 112, 117] in support of the oligomeric state of OmcA. Although OmcA reduces metal oxides, this reduction does not appear to contribute to bacterial growth in contrast to metal reduction by MtrC [65]. OmcA is thought to receive its electrons from reduced MtrC [99], yet *mtrC* mutants can still carry out metal reduction. This discrepancy can be explained by two possibilities. OmcA may be reduced by MtrA and not MtrC in vivo, or a yet to be identified electron carrier is responsible for reducing OmcA in the absence of MtrC. Although the mechanisms that lead to OmcA reduction warrant further investigation, it is clear that MtrC and OmcA participate in extracellular electron transfer to metal oxides and electrodes of microbial fuel cells.

CymA, a membrane anchored *c*-type cytochrome, is the only confirmed link to date between the menaquinol pool and the metal reductase MtrCAB. CymA, as mentioned above, is a menaquinol oxidase predicted to form a homodimer [9, 58] and is anchored to the inner membrane facing the periplasm (Fig. 2.2). Use of a bacterial two-hybrid system provided evidence for the interaction of CymA with MtrA [9]. This interaction appears to be transient and leads to MtrA reduction [33]. Direct electron transfer, however, from CymA in the inner membrane to MtrA that

is part of an outer membrane-embedded complex has been debated given the dimensions of proteins involved and the distance that separates them. Small-angle X-ray scattering data estimated MtrA to be an elongated monomer of 10.4 nm in length that fits within the MtrB pore [32]. The distance between the periplasmic side of the inner membrane and the outer surface of the outer membrane is estimated to be roughly 28–30 nm [24, 101]. Given that reduction of insoluble electron acceptors (i.e., metal oxides) occurs on the outer cell surface, electron carriers must traverse the periplasmic space and the outer membrane (28 nm) to deliver electrons to MtrC and/or OmcA. If MtrA protrudes into the periplasmic space but forms a tight complex with MtrCB, then the gap between CymA and MtrA is too wide to allow direct electron transfer between the two proteins. The organization of the MtrCAB complex in the membrane is crucial to our understanding of how electrons are transferred from CymA to MtrA. Is MtrA completely embedded in the MtrB pore, or does it protrude enough into the periplasm to allow interactions with CymA? Does the confirmation of the MtrCAB complex change when it interacts with electron acceptors allowing better CymA/MtrA interactions? Is there an unidentified electron carrier that can bridge the gap between CymA and MtrA? We currently do not have answers to these questions, but the possibility of an intermediate electron carrier exists and two *c*-type cytochromes, FccA and STC, have been proposed to serve as intermediates that transfer electron from CymA to MtrC. Strong evidence, however, to support the involvement of these proteins in metal reduction is lacking. FccA is a flavocytochrome *c* with confirmed fumarate reductase activity [51, 57, 92], whereas STC is a small tetraheme *c*-type cytochrome that appears to bind chelated Fe(III) in vitro but its function in vivo has not been determined [85, 104]. Experiments with bacterial two-hybrid systems suggested that FccA and STC interact with MtrA, but interactions between STC and CymA were not detected [9]. In contrast, using NMR spectroscopy, Fonseca et al. [34] suggested that FccA and STC transiently interact with CymA and MtrA thus acting as the bridge in the periplasmic electron transfer to the OM. This finding would predict that mutants deficient in STC or FccA are impaired in metal reduction. In contrast to this notion, deletion of *cctA* that encodes STC does not affect the ability of *S. oneidensis* MR-1 cells to reduce metals [12]. Furthermore, deletion of *fccA* leads to increased metal reductase activity [95]. These findings led Fonseca et al. [34] to predict that STC and FccA have redundant functions. In the absence of double mutants that lack metal reductase activity, we cannot conclude that either STC or FccA participate in electron transfer to MtrA.

## 2.6 Concluding Remarks

Since the isolation of *S. oneidensis* and *G. metallireducens* in pure culture in 1988, intensive investigation and a wealth of data provided in depth insight into the physiology, biochemistry, and genetics of dissimilatory metal reducing bacteria. *S. oneidensis* is a model organism for studying metal reduction, and in this chapter we

focused mainly on the key components used by this organism to enable extracellular respiration. Unlike other respiratory pathways, the metal reducing electron transport chain extends from the inner membrane to the outer cell surface in a process that has become known as extracellular electron transfer. Several mechanisms have been proposed to explain this process in *S. oneidensis* and other bacteria. These include (i) conductive nanowires, (ii) production of soluble extracellular electron shuttles, and (iii) direct contact of bacterial cells with the insoluble metals. Conductive extracellular appendages, or nanowires, have been shown recently to be outer membrane vesicles that would contain metal reductase components. The commonly used term of nanowires to describe these vesicles does not accurately reflect the nature of these structures. Once these vesicles separate from the cell, and without a continuous source of electrons from the cytoplasm, they will be incapable of extracellular electron transfer. Flavin electron shuttles, that were thought to allow access to metal oxides at a distance, function as cofactors that bind to the outer membrane cytochromes and mediate electron transfer. Thus it appears that *S. oneidensis* reduces metal oxides and electrodes of microbial fuel cells mostly by direct contact. In spite of several major breakthroughs described in this review, gaps in our understanding of the metal reduction pathway still exist. The precise molecular structures of the periplasmic and outer membrane electron transport complexes as well as the biogenesis of the metal reductase complex will be crucial to further our understanding of extracellular electron transport. It is noteworthy that extracellular electron transfer is not unique to *S. oneidensis* and is prevalent in bacterial and archaeal species. Understanding the mechanisms that underlie this process not only sheds light on an unusual yet widespread and environmentally significant microbial activity, it also allows us to better design and use these microorganisms in a variety of applications that range from bioremediation of contaminated subsurface environments to electricity and biofuel production.

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