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MINI-REVIEW

Shuttling happens: soluble flavin mediators of extracellular electron transfer in *Shewanella*

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Abstract The genus *Shewanella* contains Gram negative γ-proteobacteria capable of reducing a wide range of substrates, including insoluble metals and carbon electrodes. The utilization of insoluble respiratory substrates by bacteria requires a strategy that is quite different from a traditional respiratory strategy because the cell cannot take up the substrate. Electrons generated by cellular metabolism instead must be transported outside the cell, and perhaps beyond, in order to reduce an insoluble substrate. The primary focus of research in model organisms such as *Shewanella* has been the mechanisms underlying respiration of insoluble substrates. Electrons travel from the menaquinone pool in the cytoplasmic membrane to the surface of the bacterial cell through a series of proteins collectively described as the Mtr pathway. This review will focus on respiratory electron transfer from the surface of the bacterial cell to extracellular substrates. *Shewanella* sp. secrete redox-active flavin compounds able to transfer electrons between the cell surface and substrate in a cyclic fashion—a process termed electron shuttling. The production and secretion of flavins as well as the mechanisms of cell-mediated reduction will be discussed with emphasis on the experimental evidence for a shuttle-based mechanism. The ability to reduce extracellular substrates has sparked interest in using *Shewanella* sp. for applications in bioremediation, bioenergy, and synthetic biology.

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Introduction

Members of the genus *Shewanella* comprise a diverse group of facultative anaerobes capable of respiring a wide array of substrates including organic compounds, toxic metals, and electrodes (Nealson and Scott 2006, Hau and Gralnick 2007). A number of metal oxides such as Fe(III) and Mn(IV) are insoluble at near neutral pH, presenting a unique challenge to organisms that respire them (Lovley et al. 2004). Dissimilatory metal reducing bacteria (DMRB), which includes most of the members of the *Shewanella* genus, are able to couple the oxidation of organic and/or inorganic compounds to the reduction of insoluble metals under anaerobic conditions (Lovley et al. 2004). This activity is thought to play a significant role in biogeochemical cycling of metals and has generated considerable interest in using *Shewanella* sp. for bioremediation of contaminated groundwater sites (Hau and Gralnick 2007). *Shewanella* sp. are also able to generate electrical current from anaerobic respiration of electrodes (Lovley 2008), or conversely, oxidize electrodes to potentially drive metabolic reactions (Rabaey and Rozendal 2010; Ross et al. 2011). A more in-depth understanding of the molecular mechanisms underlying the ability of *Shewanella* sp. to respire extracellular substrates will aid in the optimization and engineering of these bacteria and reactors for applied bioenergy, electrosynthesis, and bioremediation.

Under anaerobic conditions, *Shewanella* sp. are able to couple the oxidation of organic compounds such as lactate to the reduction of extracellular electron acceptors (Shi et al. 2007). Of great interest is how DMRB are able to transfer electrons to a substrate that cannot freely diffuse to the

cytoplasmic membrane/cytoplasm. In *Shewanella oneidensis*, the Mtr pathway, composed of three multiheme cytochromes (CymA, MtrA, and MtrC [formerly OmcB]) and an integral outer-membrane protein (MtrB), form an electron conduit through which electrons are able to move from the menaquinone pool across the outer-membrane (Fig. 1) (Shi et al. 2007). Once the electrons have reached the cell surface, they must be transferred from MtrC to an oxidized substrate. Four distinct mechanisms, which are not mutually exclusive, have been conceptualized for how an organism could transfer electrons from outer membrane cytochromes to an insoluble extracellular substrate (Hernandez and Newman 2001; Gralnick and Newman 2007): (1) direct contact between redox-active compounds/proteins present on the cell surface or cell-associated extracellular matrix and the insoluble substrate; (2) protein "nanowires" able to conduct electrons between the cell and substrate, facilitating respiration at a distance; (3) metal chelators/siderophores which solubilize the oxidized mineral and allow it to diffuse to the bacterial cell surface; (4) small molecule electron shuttles produced by the organism are cycled between the cell and extracellular substrate. Evidence for each of the above mechanisms has been presented for *Shewanella* sp.; however, a growing body of literature supports a critical role for soluble electron shuttles, and this review will focus mainly on that work.

Evidence for a soluble shuttle in *Shewanella*

The concept of an extracellular electron shuttle is not a new one. In fact, a number of exogenous electron shuttles, such as humic substances and sulfur compounds, have been identified (Straub and Schink 2004, Lovley et al. 2004, Stams et al. 2006). Humic substances, produced by the biodegradation of organic matter and reduced sulfur compounds, are able to serve as an intermediary of extracellular metal respiration by DMRB (Lovley et al. 1996, Straub and Schink 2004). Endogenous electron shuttles perform the same function but are produced and secreted by the organism. *Pseudomonas aeruginosa* produces and secretes redox-active phenazine compounds (Hernandez et al. 2004), which have recently been shown

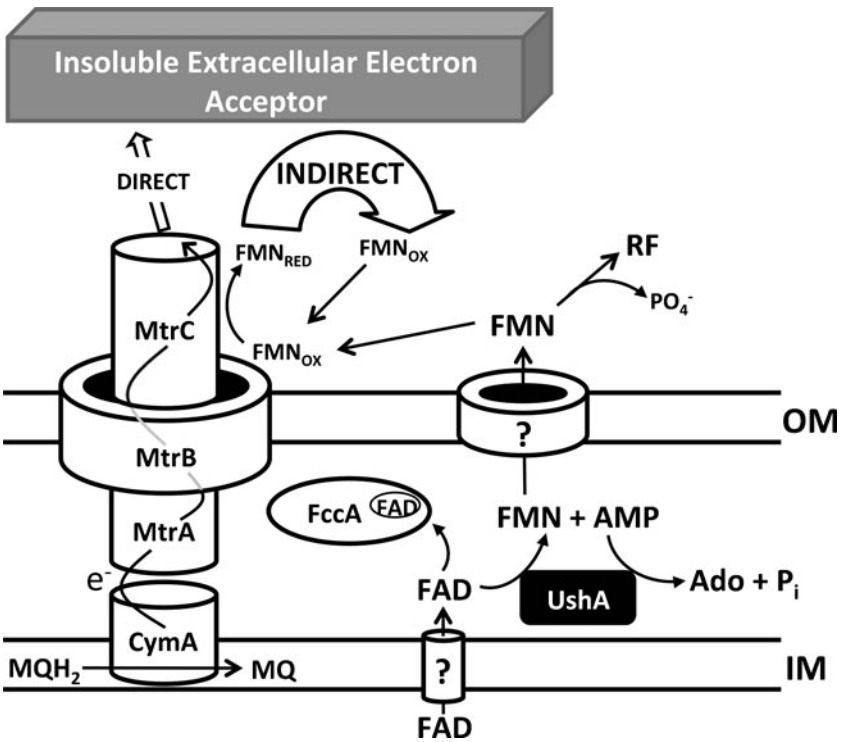

Fig. 1 Extracellular respiratory pathway in *Shewanella oneidensis*. CymA, a tetraheme *c*-type cytochrome anchored in the inner membrane, accepts electrons from the menaquinone pool and transfers them to MtrA, a periplasmic decaheme *c*-type cytochrome. Transfer of electrons from MtrA to MtrC, an outer-membrane decaheme *c*-type cytochrome is facilitated by MtrB, a non-heme containing integral outer-membrane protein (Shi et al. 2007). Flavin adenine dinucleotide (FAD) crosses the inner membrane by an unknown mechanism and is incorporated as a cofactor into periplasmic proteins (e.g., FccA) or hydrolyzed into flavin mononucleotide (FMN) and adenosine monophosphate (AMP) by UshA. FMN then diffuses through the outer membrane. UshA further hydrolyzes AMP into adenosine (Ado) and inorganic phosphate (P_i) which is recycled by the cell (Covington et al. 2010). Extracellular flavins are reduced by MtrC at the cell surface and move to an oxidized substrate by diffusion. The reduced flavin oxidizes the substrate and returns to the cell surface to be re-reduced (Coursolle et al. 2010). In addition, MtrC can directly transfer electrons via the solvent exposed apical heme group (Clarke et al. 2011). FMN is spontaneously hydrolyzed into riboflavin (RF) over time

to promote the survival of electrode-grown *P. aeruginosa* under anaerobic conditions (Wang et al. 2010). Endogenous extracellular electron shuttles represent a powerful strategy for bacteria that must reduce a substrate that may not be physically accessible to the cytoplasmic membrane of the cell. The best studied example of extracellular respiration mediated by soluble electron shuttles is that of *S. oneidensis*. It should be noted that when grown under anaerobic conditions, the primary source of ATP for *S. oneidensis* comes from substrate-level phosphorylation (Hunt et al. 2010), with little or no contribution from oxidative phosphorylation. Regardless, a terminal electron acceptor is still required to regenerate reducing equivalents within the cell used to oxidize the carbon and energy source, justifying the use of the term "respiration".

S. oneidensis strain MR-1 was isolated in 1987 with the ability to couple the oxidation of organic carbon to the reduction of insoluble manganese (IV) (Myers and Nealson 1988). Subsequently, the utilization of a wide range of extracellular electron acceptors, such as Fe(III), U(VI), and carbon electrodes has been demonstrated (Nealson and Scott 2006, Hau and Gralnick 2007). In the following years, numerous studies focused on both the kinetics of extracellular electron transfer and the genes/proteins involved; however, it was not until much later that evidence was presented regarding reduction of extracellular substrates in the absence of direct contact by *Shewanella* sp. In two separate studies, researchers precipitated Fe(III) oxide within either alginate beads or nano-porous glass beads (50 nM average pore diameter) and determined that *Shewanella alga* or *S. oneidensis* respectively, was able to reduce the Fe(III) oxide, despite the fact that the cells were too large to access the interior of the beads (Nevin and Lovley 2002, Lies et al. 2005). In the later study, the Mtr pathway (Fig. 1) was found to be required for reduction of Fe(III) oxide within the nano-porous glass beads. Both groups concluded that a soluble shuttle or chelator must be present; however, the identity of this molecule was not determined. In another study, a physical barrier was used to determine if direct cell contact is required for extracellular respiration of electrodes by *S. oneidensis*. Researchers fabricated an insulating layer over nanoelectrodes to control access of bacteria to the electrode surface (Jiang et al. 2010). The insulating layer had either (1) a window which allowed direct contact between bacteria and electrode (12 μm^2) or (2) nanoholes which precluded the bacteria but allowed diffusion of soluble compounds to the electrode surface (~280 nm^2). The current produced by *S. oneidensis* was similar regardless of the configuration of the electrode, suggesting that direct contact plays a relatively minor role under these conditions. The ability of *S. oneidensis* to reduce the electrode in the absence of direct contact is consistent with a shuttle-based extracellular respiratory mechanism.

The first study which attempted to identify secreted electron shuttles in *S. oneidensis* reported a quinone-like molecule that could rescue a menaquinone biosynthesis mutant (Newman and Kolter 2000); however, this compound was later determined to be an intermediate of quinone biosynthesis released by lysed cells (Myers and Myers 2004, Hernandez 2004). Subsequently, the identification of redox-active flavins secreted by *S. oneidensis* occurred almost simultaneously by two different groups. von Canstein and coworkers fractionated *S. oneidensis* culture supernatants and found that riboflavin and riboflavin mononucleotide (FMN) were present in micromolar quantities in both aerobically and anaerobically grown cultures (von Canstein et al. 2008). Marsili and coworkers came to the same conclusion stemming from the following observation. The current produced by *S. oneidensis* strains from the reduction of a carbon electrode dramatically declined when the surrounding medium was exchanged with fresh medium. However, if the original medium was put back after centrifugation to remove planktonic cells, the original level of current immediately returned (Marsili et al. 2008). In contrast, media exchange had little or no effect on current production from bacteria that are not thought to use a shuttling mechanism, such as *Geobacter* sp. (Bond et al. 2002, Bond and Lovley 2003, Srikanth et al. 2008). It appeared then that a significant portion of the current (73%) produced by *S. oneidensis* was dependent on a soluble factor, identified as riboflavin by mass spectrometry (Marsili et al. 2008). The dramatic increase in current following the replacement of cell-free culture supernatants also demonstrated that cells attached to the carbon electrode were responsible for the majority of measured current, dependent on the presence of flavins. The media exchange phenomenon was also observed by a different group using nanoelectrodes where 95% of the current measured was dependent upon a soluble factor (Jiang et al. 2010). While *S. oneidensis* has been the most intensively studied member the *Shewanella* genus, the secretion of flavins has been measured for a number of *Shewanella* sp. In every case, flavins are present in culture supernatants, suggesting a role for flavins across the *Shewanella* genus (von Canstein et al. 2008, Coursolle et al. 2010).

In addition to being redox active, flavins also weakly chelate metals (Albert 1950, Furia 1972). As mentioned in the introduction, a chelator could facilitate extracellular respiration by binding to, and solubilizing, metal oxides. Solubilization does not appear to be the mechanism, however, as addition of concentrations of a known Fe(III) chelator, comparable to those reached by flavins, has little effect on the rates of Fe(III) oxide reduction by *S. oneidensis* (Coursolle et al. 2010). Moreover, a chelator-based mechanism cannot explain the current generated by *Shewanella* sp. respiring electrodes, which are not solubilized.

The presence of flavins in *Shewanella* sp. culture media begs the question; are flavins involved in extracellular electron transfer under physiological conditions? In support of the respiratory role of flavins, the addition of purified riboflavin or FMN (1) reduces poorly soluble Fe(III) oxide, (2) enhances the reduction of Fe(III) oxide by *S. oneidensis*, (3) enhances the growth yield of *S. oneidensis* using Fe(III) oxide as the sole electron acceptor, and (4) increases current production from electrode-grown cultures (von Canstein et al. 2008, Marsili et al. 2008, Ross et al. 2009, Coursolle et al. 2010). Cyclic voltammetry (CV) has also been used to demonstrate the ability of flavins to reduce carbon electrodes. Oxidation potentials observed for *S. oneidensis* using the electrode as the sole electron acceptor are similar to those observed for sterile electrodes incubated with riboflavin (Marsili et al. 2008). The similarity between oxidation potentials strongly suggested that even though bacteria were attached to the electrode, electron transfer was mediated by flavins. Measurements of direct electron transfer from outer-membrane cytochromes to the electrode surface in the absence of flavins requires a higher potential (Baron et al. 2009), in agreement with experiments using purified outer-membrane cytochromes and Fe(III) oxide (Ross et al. 2009), lending further weight to the argument that flavins serve as a soluble redox shuttle.

Flavin biosynthesis

Shewanella sp. must first synthesize flavins before they can be secreted and utilized as extracellular electron shuttles. The basic biochemical pathway for flavin biosynthesis in bacteria, yeast, and plants has been extensively studied, and a thorough review of the field has been published (Abbas and Sibirny 2011). Briefly, one molecule of guanosine triphosphate and one molecule ribulose-5-phoshpate are converted into riboflavin by the proteins encoded by the *ribBA*, *ribD*, *ribH*, and *ribE* genes (Fig. 2). For simplicity, the *Escherichia coli* nomenclature will be used to describe riboflavin biosynthesis genes. Riboflavin is then converted first into FMN, and then flavin adenine dinucleotide (FAD), by the protein encoded by the *ribF* gene. In *Escherichia* sp. the same pathway is used; however, the bi-functional *ribBA* gene is encoded by two separate genes; *ribA* and *ribB* (Fig. 2). While the organization of riboflavin biosynthetic genes differs between *Bacillus subtilis* and *E. coli*, only one copy of each gene is found in the genome. A phylogenetically diverse set of bacterial species appear to have multiple copies of one or more riboflavin biosynthetic genes, namely *ribA*, *ribB*, *ribBA*, or *ribE* (Vitreschak et al. 2002). *S. oneidensis* has multiple copies of a number of riboflavin biosynthesis genes, and the genetic organization has features similar to both *Bacillus* and *Escherichia* (Fig. 2). In addition, RibE1 and RibE2 are only 55% similar at the amino acid level, suggesting functionality may have diverged significantly. The apparent redundancy of flavin biosynthetic genes is present in the 23 sequenced *Shewanella* sp. genomes with the exception of *Shewanella denitrificans*, which lacks the *ribB* gene. Interestingly, *S. denitrificans*, while still secreting flavins, is the only sequenced isolate that also lacks the Mtr respiratory pathway genes, and is therefore unable to reduce metal oxides or electrodes.

Most microorganisms are able to synthesize flavins de novo to fulfill nutritional requirements for the redox cofactor (Abbas and Sibirny 2011). *Shewanella* sp. must do this in addition to secreting flavins into the surrounding media (von Canstein et al. 2008, Marsili et al. 2008). The redundancy of flavin biosynthetic genes may represent a mechanism to satisfy both requirements, with the ability to regulate biosynthesis as two different processes are siphoning off riboflavin. A well-studied regulator of flavin biosynthesis gene expression is the evolutionarily conserved cis-acting riboflavin (RFN) element present in the untranslated leader region upstream of *ribD* and the riboflavin transporter gene *ribU* in *Bacillus* sp. (RFN, Fig. 2; *ypdA* not shown). When bound to FMN, the RFN element is thought to cause premature transcriptional termination and mask the ribosome binding site of the *ribD* led operon and *ypaA*, respectively (Winkler et al. 2002). Because both *Bacillus* and *Escherichia* sp. have only a single copy of each biosynthetic gene, negative regulation of any gene should decrease riboflavin biosynthesis. In

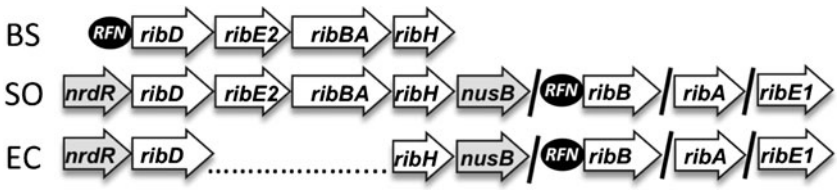

Fig. 2 Riboflavin biosynthetic genes of the genus *Shewanella*, *Bacillus*, and *Escherichia*. For simplicity, the *E. coli* gene nomenclature is used in this figure and throughout the text. The known (*Bacillus* (Winkler et al. 2002)) and predicted (*Shewanella* and *Escherichia* (Vitreschak et al. 2002)) RFN elements are indicated by *black circles*. Genes colored *gray* (*nrdR* and *nusB*) are not riboflavin biosynthetic genes but are included here to show that the genetic context of *rib* genes is conserved between *E. coli* and *S. oneidensis*. The genetic organization depicted for *Shewanella* sp. is the same for all publically available sequenced genomes with the exception of *S. denitrificans* which lacks *ribB*

Shewanella sp., there is a predicted RFN element upstream of the *ribB* gene but not upstream of the operon which contains the *ribBA* gene (Vitreschak et al. 2002), effectively creating two expression pathways. Expression of the *ribB* gene could be controlled by the intracellular concentration of FMN and expression of the *ribBA* gene may not. It is possible that individual flavin biosynthetic genes encode proteins that function specifically for electron shuttle production, distinct from nutritional flavin production.

Energetics of flavin biosynthesis

Implicit in the term "shuttle" is the repeated oxidation and reduction of a flavin molecule. It is thought that in natural systems, only a catalytic amount of an electron shuttle would be produced and undergo multiple reduction–oxidation cycles (Lovley et al. 2004). Anaerobically grown *S. oneidensis* cultures are able to couple the oxidation of lactate to the reduction of riboflavin, and if the riboflavin is re-oxidized, *S. oneidensis* is able to re-reduce the riboflavin (Coursolle et al. 2010). Extracellular riboflavin is not consumed or destroyed by *S. oneidensis*, it is simply reduced. Consistent with this result *Shewanella* sp. accumulate flavins to micromolar concentrations when grown on Fe(III) oxide or electrodes, an amount insufficient to account for observed electron transfer if used only once (von Canstein et al. 2008, Marsili et al. 2008).

The synthesis/secretion of flavins represents an energetic cost, which presumably, is outweighed by the benefits, i.e., the ability to more rapidly respire an alternative terminal electron acceptor inaccessible to other bacteria. The biosynthesis of a single molecule of riboflavin consumes as much as 25 molecules of ATP, and using this figure, it is estimated that <0.1% of the cell's ATP budget is needed to account for the concentrations of secreted flavin observed in electrode-grown *S. oneidensis* cultures (Marsili et al. 2008). Importantly, the observed concentration of riboflavin increased the rate of electron transfer by 370%. Flavins also accelerate the reduction of Fe(III) oxide, a more environmentally relevant substrate (von Canstein et al. 2008, Coursolle et al. 2010). These data together strongly suggest that production/secretion of flavins confers an advantage when *S. oneidensis* is grown on an insoluble electron acceptor. Cell density may also play a significant role in the efficiency of shuttle secretion. In environments with low cell density, diffusion may limit the usefulness of secreted shuttles; however, relatively high cell densities might make shuttle secretion mutually beneficial to the community (Lovley et al. 2004). *Shewanella* sp. often grow in biofilms on mineral surfaces (Lower et al. 2001) and electrodes (Marsili et al. 2008), resulting in relatively high local cell densities. The biofilm growth mode may be a strategy to increase the efficiency of shuttle secretion.

Secretion of flavins

Flavins accumulate in *Shewanella* culture supernatants and, depending on the species and type of culture assayed, yields of up to 5.5 μmole flavins per gram protein have been observed (von Canstein et al. 2008, Coursolle et al. 2010). This is in contrast to *E. coli* JM109 cultures where concentrations of only 0.7 μmole flavins per gram of protein are observed (von Canstein et al. 2008). The flavin profile of *S. oneidensis* culture supernatants has been examined and is dominated by FMN with a smaller amount of riboflavin present. The idea that FMN is being actively secreted by *S. oneidensis* is also supported by the fact that FMN secretion correlated with cell growth, and ceased when cultures reached stationary phase (von Canstein et al. 2008). This result is the opposite of what one would expect if FMN was being released by lysis.

The mechanism by which flavins traverse the inner membrane of *Shewanella* sp. is unknown. Three classes of bacterial riboflavin transport genes have been identified, but homologs of these proteins are absent from the genome of *S. oneidensis* (Vitreschak et al. 2002, Abbas and Sibirny 2011). FMN and riboflavin are the primary flavin species measured in *S. oneidensis* culture supernatants; however, FAD is the form that initially enters the periplasm and is subsequently processed into FMN and riboflavin (Fig. 1) (Covington et al. 2010). UshA, a predicted periplasmic 5′ nucleotidase, hydrolyzes FAD to FMN and adenosine monophosphate (AMP). UshA further hydrolyzes AMP to adenosine and inorganic phosphate which the cell can recycle (Covington et al. 2010). The secretion of FAD followed by hydrolysis may serve to fulfill two purposes. *S. oneidensis* must incorporate FAD into the periplasmic fumarate reductase FccA which requires a non-covalently bound FAD cofactor (Leys et al. 1999). FAD molecules not bound by FccA could then be hydrolyzed by UshA and used as electron shuttles.

The Mtr pathway and flavin reduction

The Mtr pathway is composed of a number of muti-heme cytochromes which effectively form an electron conduit from the inner membrane to the cell surface (Fig. 1) (Shi et al. 2007). The deletion of genes in this pathway severely inhibits the ability of *S. oneidensis* to respire extracellular substrates including Fe(III), Mn(IV), and carbon electrodes (Beliaev and Saffarini 1998, Beliaev et al. 2001, Bretschger et al. 2007). The MtrABC complex has been reconstituted in proteoliposomes and shown to transfer electrons across the membrane in vitro (Hartshorne et al. 2009). Heterologous expression in *E. coli* demonstrates that expressing a portion the Mtr pathway (*mtrCBA*) is both necessary and sufficient for reduction of Fe(III) oxide, albeit at rates far

below those measured for *Shewanella* sp. (Jensen et al. 2010). The first evidence that the Mtr pathway was responsible for the reduction of extracellular shuttles came from a study that examined the ability of *S. oneidensis* to reduce 2,6-anthraquinone disulfonate (AQDS), an analog of redox-active moieties of humic acids. Strains harboring mutations of Mtr genes were deficient in AQDS reduction (Lies et al. 2005). Subsequently, evidence that the Mtr pathway was responsible for the reduction of flavins, and that the reduction of flavins accelerated the reduction Fe (III) oxide, came from kinetic studies of purified proteins. Ross and coworkers purified the outer-membrane cytochromes MtrC and OmcA and examined the rate at which they reduced soluble and insoluble Fe(III) compared to whole cells (Ross et al. 2009). In the purified system, reduction of insoluble Fe(III) oxide was an order of magnitude slower than with whole cells containing a comparable concentration of cytochromes. Only upon addition of exogenous flavins was the rate of Fe(III) oxide reduction by purified proteins comparable to that of whole cells (Ross et al. 2009).

Genetic analysis confirmed the role of the Mtr pathway in the reduction of extracellular flavins. Wild-type *S. oneidensis* is able to reduce oxidized flavins under anaerobic conditions (Coursolle et al. 2010). Strains with gene deletion mutations in *cymA, mtrA*, *mtrB*, or *mtrC* were impaired for reduction of flavins. The aforementioned mutants were also impaired for reduction of carbon electrodes despite the fact that each culture was provided with an identical concentration of flavins (Coursolle et al. 2010).

While there is no doubt that the Mtr pathway plays an essential role in the respiration of extracellular substrates and the reduction of extracellular flavins, a great deal of debate has circled around the contribution of direct contact vs. electron shuttling to extracellular respiration. The recently solved crystal structure of MtrF, a paralog of MtrC in *S. oneidensis*, illustrates the capacity for both (Clarke et al. 2011). MtrF, like MtrC, is a decaheme *c*-type cytochrome predicted to localize on the surface of the cell (Fig. 1). Heterologous expression of MtrF functionally complements *S. oneidensis* strains lacking MtrF, MtrC, and OmcA, another decaheme *c*-type cytochrome displayed on the surface of the cell, and thus can carry out MtrC-like iron reduction (Bücking et al. 2010, Coursolle and Gralnick 2010). The MtrF crystal structure clearly shows the arrangement of the heme groups within the protein, from which a path for electron flow through the protein can be derived. The ten heme groups of MtrF form what has been termed a "staggered cross" that transects the length of the protein with adjacent hemes positioned close enough to allow electron transfer (Fig. 3). Hemes 10 and 5 are located at opposing poles of the protein and are solvent exposed;

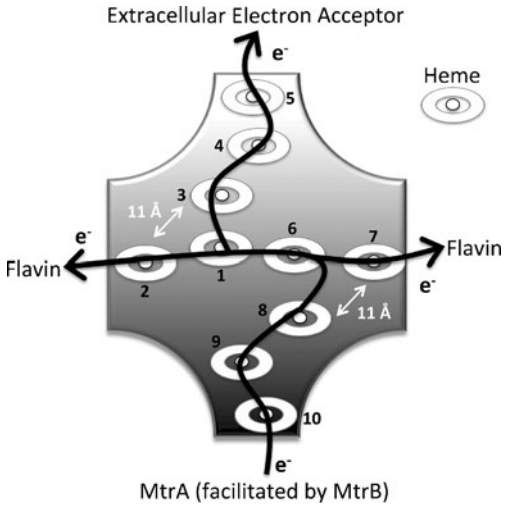

Fig. 3 Cartoon depicting the organization of heme cofactors in MtrF. Heme groups are shown as *white circles* and are numbered as in Clarke et al. 2011. Adjacent heme groups are between 4 and 6 Å apart with the exception of hemes 2–3 and 7–8 which are 11 Å apart. The orientation of MtrF with respect to electron flow was predicted using software designed to determine sites of protein–protein interaction (with MtrB) (Clarke et al. 2011)

positioned in such a way to facilitate electron transfer from MtrA (or another periplasmic electron carrier) and to an extracellular electron acceptor, respectively. Hemes 2 and 7 are located at the sides of the protein near a β-barrel of extended Greek-key split barrel domains (Clarke et al. 2011), common in flavin binding proteins (Hubbard et al. 1999). This layout effectively results in three avenues for electron egress. Electrons enter the chain at heme 10, move through the "staggered cross", and are eventually transferred to one of three terminal hemes (2, 5, or 7). Given location and adjacent protein domains, it is likely that heme 5 directly reduces extracellular substrates and hemes 2 and 7 reduce flavins. Despite repeated attempts, the researchers that solved the MtrF crystal structure were unable to resolve a flavin in either of the proposed binding sites, despite growing crystals in the presence of FMN (Clarke et al. 2011). This is perhaps not surprising, though as a shuttling mechanism, would require the rapid reduction and release of flavins, suggesting that the affinity of MtrF for flavins may be relatively low. Incubation of completely reduced MtrF with FMN or Fe(III) citrate resulted in the oxidation of 40% or 100% of the heme groups, respectively (Clarke et al. 2011). Only a subgroup of hemes appear to facilitate reduction of FMN, consistent with the proposed orientation of MtrF and the path electrons must take to reach hemes 2 and/or 7. Additionally, it was observed that the reduction of FMN by purified MtrF occurred much faster than the reduction of insoluble Fe(III) oxide (ferrihydrite) or Fe(III) citrate (Clarke et al. 2011), a result also seen with MtrC (Ross et al. 2009). These results are in agreement with the previous finding that addition of flavins enhance the

reduction of Fe(III) oxide (von Canstein et al. 2008, Ross et al. 2009, Coursolle et al. 2010) and electrodes (Marsili et al. 2008) by *S. oneidensis*. The faster reaction kinetics and the FMN binding domains together suggest that flavins are the primary substrate of MtrF and MtrC. This hypothesis can be tested using the knowledge of the spatial arrangement of the heme cofactors of MtrF/C to facilitate construction of site directed mutants where electron flow through the protein is constrained.

Concluding remarks

It is clear from the available literature that (1) *S. oneidensis* respires extracellular substrates dependent on the Mtr pathway (Beliaev and Saffarini 1998, Beliaev et al. 2001, Bretschger et al. 2007), (2) *Shewanella* sp. accumulate flavins in culture supernatants (von Canstein et al. 2008, Marsili et al. 2008, Coursolle et al. 2010), and (3) the Mtr pathway from *S. oneidensis* catalyzes the reduction of flavins (Coursolle et al. 2010). Furthermore, the addition of flavins accelerates the rate at which *S. oneidensis* is able to reduce Fe(III) oxide (von Canstein et al. 2008) and electrodes (Marsili et al. 2008, Baron et al. 2009). One of the strongest pieces of evidence supporting a soluble electron shuttle continues to be the reduction in current measured from electrode-grown biofilms when the surrounding medium is exchanged. If direct contact is the primary mechanism of electron transfer to extracellular substrates, it is difficult to imagine how exchange of culture media could so drastically effect the current produced on electrodes; i.e., an immediate 50–95% reduction in current, depending on the system used (Ringeisen et al. 2006, Biffinger et al. 2007, Marsili et al. 2008, Jiang et al. 2010). It is possible that the exchange of culture media could disrupt cell–electrode contacts or bacterial "nanowires"; however, the dramatic increase in current seen immediately after the re-introduction of cell-free culture supernatants, and not with fresh medium, makes this argument unlikely.

Contrasting shuttle-mediated electron transfer observed in *Shewanella* sp., direct electron transfer from a reduced molecule associated with the cell surface/extracellular matrix appears to be the mechanism used by *Geobacter* sp. to respire extracellular substrates. A study examining the ability of *Geobacter metallireducens* to reduce Fe(III) oxide trapped within alginate beads failed to detect reduction of Fe(III) in the absence of direct contact (Nevin and Lovley 2000). Additionally, current produced by electrode-grown *Geobacter sulfurreducens* is unaffected by exchange of the surrounding medium (Bond and Lovley 2003, Srikanth et al. 2008), unlike *S. oneidensis*. The evolutionary origins of direct contact vs. shuttling are difficult to speculate on; however, the ecological niche occupied by each DMRB no doubt plays a large role in which mechanism dominates.

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