Export and Role of Flavin Electron Shuttles in *Shewanella oneidensis* Strain MR-1

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Dedication

I dedicate this dissertation to S.B.S. We have traveled a long way together, but still have miles to go.

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1.1 Microbial Respiration

In general, respiration is the coupling of oxidation of reduced organic substrates to the flow of electrons through proteins and membranes to an electron acceptor. Respiration, in capable organisms, is the primary method used to generate biochemical energy needed for synthesis of compounds, macromolecules, and solute transport. Aerobic respiration is defined by the use of oxygen as the terminal electron acceptor. If oxygen is not the terminal electron acceptor, then it is anaerobic respiration and a select few examples of alternate electron acceptors are nitrate, fumarate, or metals such as iron, manganese, or uranium (Nealson & Scott, 2006). Respiration requires both electron donors and acceptors. Electrons will flow from a negative potential electron donor to a more positive potential electron acceptor. Some of the energy from this flow is harnessed by cells in the form of a proton motive force using electron carrying proteins with coupling sites and lipid-type molecules called quinones. The concentration difference of protons and the charge difference between the cytoplasm and either the periplasm in Gram-negative bacteria or the environment in Gram-positive bacteria are used to do work. Cells use this energy to generate adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate, drive locomotion, and facilitate transport. As electrons trek from donor to acceptor, there exists discrete carriers to move electrons either through membranes or across cellular distances. Typical electron carriers (flavoproteins, quinones, iron-sulfur proteins, and cytochromes) are important to understanding respiration or for the purpose of this thesis, extracellular electron transfer. For a review on bacterial respiration and its diversity see (Richardson, 2000).

Many electron transfer reactions are mediated by proteins with redox active co-factors. Flavoproteins are proteins that contain either a flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) co-factor. Flavin co-factors are made from riboflavin (vitamin B_2) precursor (Fischer & Bacher, 2005), and an isoalloxazine ring is shared among all three flavins. FMN requires the addition of a phosphate to riboflavin, and FAD requires the addition of an adenosine monophosphate (AMP) to FMN. The side chains of FMN and FAD are not involved in electron transfer, but are important securing the flavin within the protein (Ghisla & Massey, 1989). The flavin co-factor can be covalently or non-covalently bound to the flavoprotein, but is usually located in the active site (Massey, 2000). The isoalloxazine structure is able to accept one or two electrons and one or two protons and then donate electron(s) or proton(s) to an oxidized acceptor. Reduction potential of free flavin is approximately -200mV (versus Standard Hydrogen Electrode), whereas flavin co-factors vary from -495mV to +80mV depending on the protein structure (Ghisla & Massey, 1989).

Similar to flavoproteins, cytochromes transfer electrons and contain a prosthetic group. Instead of a flavin co-factor to carry electrons, cytochromes use hemes which are iron atoms coordinated by four nitrogen atoms in a porphyrin ring (Reedy & Gibney, 2004). The central iron atom is reduced to its ferrous form and then is oxidized back to its ferric form. Therefore, hemes of cytochromes are only able to transfer a single electron at a time. Cytochromes are divided into classes based on their heme groups. For instance, *c*-type cytochromes are covalently bound to two cysteine residues in a typical CXXCH motif (Reedy & Gibney, 2004). Other protein complexes can contain multiple classes of hemes or be associated with other electron transferring proteins such as iron-sulfur

proteins (Rieske, Maciennan, & Coleman, 1964). Iron-sulfur proteins are commonly associated with redox reactions and the electron transport chain. The redox active center of iron-sulfur proteins is coordinated by sulfur atoms, instead of porphyrin rings (Johnson, 1998). Similar to cytochromes, iron-sulfur proteins only transfer one electron at a time (Johnson, 1998).

Quinones are molecules that carry electrons and protons and like flavins, quinones are sometimes used as co-factors (McMillan, Marritt, Butt, & Jeuken, 2012). Quinones are hydrophobic, lipid-based, and found in the lipid phase of membranes. Furthermore, quinones are mobile within membranes and are able to carry protons and electrons from the coupling site of one protein to the coupling site of another. If the coupling sites are on the opposite sides of a membrane, protons can be transported from one side to the other. All quinones have isoprenoid side chains which are hydrophobic and contribute to their solubility in membranes. There are two common types of quinones, ubiquinone and menaquinone. Ubiquinone has a more positive reduction potential than menaquinone and is used when electropositive electron acceptors, like oxygen, are present. Conversely, menaquinone is commonly used during anaerobic respiration when low potential electron acceptors are present.

1.2 Metal-Respiring Microbes

With the ability of some metals to participate in redox reactions, occurrence in cytochromes and iron-sulfur proteins, and high abundance in the environment, it is not surprising that there are many metal-respiring microbes. These microbes are important for organic matter cycling, metal cycling, redox cycles, and freeing up other nutrients and

minerals bound to metals (Lovley, 1991). A common metal used by these microbes is iron. Iron is an essential element for many microbes and is the most common electron acceptor in many environments due to its high abundance in the Earth's crust. In anoxic zones, such as lakes, rivers, and oceans, Fe(III) oxides are reduced to Fe(II) which are water soluble and diffuse to oxygen rich zones to be re-oxidized to Fe(III) oxides. These oxides can then fall back down to complete an iron redox cycle.

Iron is commonly used in two different ways by microbes. As mentioned previously, iron is critical for many microbes and is required for cytochromes and iron-sulfur proteins. At non-acidic pHs, ferric iron is inaccessible to cells, because it commonly exists as insoluble oxides and hydroxides (Lovley, Holmes, & Nevin, 2004). To access this iron for cellular processes, the ferric iron is reduced to soluble ferrous iron which can be taken up by cells and incorporated. Alternatively or concurrently, siderophores are used to solubilize and bind ferric iron, and once bound to iron; siderophores are actively transported into the cell for assimilation (Sandy & Butler, 2009). The other type of iron respiration is dissimilatory iron reduction and is used for the generation of biochemical energy by using iron as an electron acceptor, instead of incorporating the iron into the cell.

Unlike oxygen, fumarate, or other soluble electron acceptors, iron oxides are generally insoluble at physiological pH. Since the electron acceptor cannot be brought into the cell, metabolic electrons must be transferred outside of the cell. To reduce insoluble iron, metabolic electrons must travel a relatively long distance and cross at least one insulating membrane. To accomplish this feat, specialized proteins and mediators exist at each step to transfer electrons from the plasma membrane to extracellular iron.

Much of the research on metal-respiring microbes has focused on two model organisms, *Geobacter* and *Shewanella*. These organisms are studied for their ability to produce current from metabolism and their genetic tractability. This thesis will focus on *Shewanella oneidensis* strain MR-1 (MR-1).

1.3 *Shewanella*

The *Shewanella* genus is a versatile collection of motile, facultative anaerobic, Gram-negative gamma-proteobacteria. *Shewanella*'s global aquatic presence, ability to proliferate in cold temperatures, and capacity to respire a variety of electron acceptors allows members of this genus to utilize diverse niches (Fredrickson et al., 2008; Hau & Gralnick, 2007; Venkateswaran et al., 1999). Furthermore, these bacteria serve as model organisms to study extracellular respiration because of their minimal growth requirements, existing tools for genetic manipulation, and sequenced genomes. The organism was first discovered in 1931 from spoiled butter and was named *Achromobacter putrefaciens* (Derby & Hammer, 1931). The genus then went through a series of name changes throughout the decades from *Pseudomonas* in 1960, *Alteromonas* in 1977, and finally to *Shewanella* in 1985 (Lee, Gibson, & Shewan, 1977; MacDonell & Colwell, 1985; Shewan, Hobbs, & Hodgkiss, 1960). The isolation of *Shewanella oneidensis* MR-1 (metal-reducing) was from Lake Oneida, New York (Venkateswaran et al., 1999).

In contrast to their ability to use a variety of electron acceptors, many members of the *Shewanella* species are unable to use complex carbon sources (Nealson & Scott, 2006). The carbon sources are mainly fermentation end products and many species live

syntrophically with fermenting organisms. When grown anaerobically on lactate, MR-1 produces acetate and carbon dioxide, further supporting association with fermenters and a role in removing fermentation end products from anaerobic environments.

Shewanella species are able to utilize a remarkable number of terminal electron acceptors including organics, inorganics, and insoluble metals (Hau & Gralnick, 2007). For a partial list see "Ecophysiology of the Genus *Shewanella*" by Nealson and Scott (Nealson & Scott, 2006). The number of cytochromes in the *Shewanella* genome likely accounts for the large number of usable electron acceptors. In the case of *Shewanella oneidensis* MR-1 (MR-1), the genome contains 42 putative *c*-type cytochromes, and the organism is able to export and reduce endogenous electron shuttles. (Coursolle, Baron, Bond, & Gralnick, 2010; Heidelberg et al., 2002; Kotloski & Gralnick, 2013; Meyer et al., 2004).

1.4 Extracellular Respiration in *Shewanella*

Since electrons cannot move long distances unaided, a specialized pathway of electron carriers is required to move electrons from the inner-membrane, across the periplasm, and past the outer-membrane to external electron acceptors (Page, Moser, & Dutton, 2003). In MR-1, soluble electron acceptors can diffuse to the cell and be reduced. Conversely, insoluble electron acceptors cannot diffuse to the cell. Therefore, electrons need to be transferred to the electron acceptor. Conceptually, four potential mechanisms exist for reduction of extracellular insoluble substrates (Gralnick & Newman, 2007). One mechanism is direct contact between the cell and the substrate, which allows for direct transfer of electrons from outer membrane proteins to the substrate. Another potential

mechanism is the use of electro-conductive structures from the outer membrane to the substrate. These structures bridge the gap between the cell and the substrate and allow electrons to flow from the cell to electron acceptor. A third mechanism is the use of substrate chelators. A substrate chelator binds oxidized substrate and facilitates contact with the cell through diffusion, enabling substrate reduction. A fourth mechanism is the use of electron shuttles. Electron shuttles are reduced by the cell then diffuse to and reduce insoluble electron acceptors. Oxidized shuttles are able to diffuse back to the cell and be reduced again.

MR-1 uses a combination of *c*-type cytochromes and flavin electron shuttles to transfer electrons to insoluble electron acceptors (Beliaev & Saffarini, 2001; Bücking, Popp, Kerzenmacher, & Gescher, 2010; Canstein, Ogawa, Shimizu, & Lloyd, 2008; Coursolle et al., 2010; Marsili et al., 2008). The extracellular electron transfer pathway in MR-1 consists of essential cytochromes and menaquinone biosynthetic proteins (Beliaev & Saffarini, 1998, 2001; Gescher, Cordova, & Spormann, 2008; Myers & Myers, 2004; C. R. Myers & Myers, 1997; Pitts et al., 2003). Metabolic electrons enter the quinone pool of the inner-membrane through a dehydrogenase. Reduced quinones are oxidized by a periplasmic facing, inner-membrane tetraheme cytochrome, CymA (Myers & Myers, 1997). Next, the electrons are transferred to a decaheme, soluble, periplasmic protein, MtrA. MtrA forms a stable complex with MtrB, a transmembrane outer-membrane protein (Ross et al., 2007). There are two proteins implicated as the terminal extracellular electron transfer protein, a transposon hit in *mtrC* created a strain deficient in Fe(III) reduction (Beliaev & Saffarini, 2001). Figure 1 shows a model of MR-1 extracellular electron transfer.

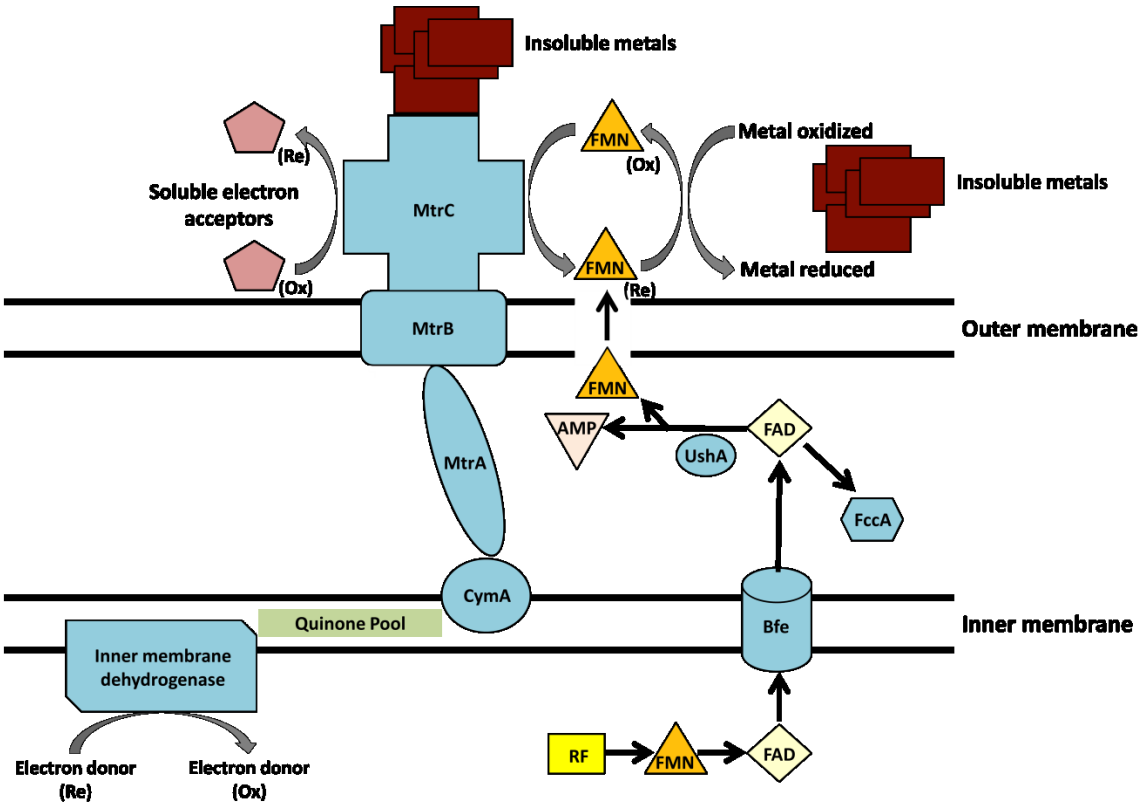

Figure 1.1
Model of extracellular respiration in *S. oneidensis* MR-1.

1.5 Flavins as Electron Shuttles

Flavins (FMN and FAD) play a role in one and two electron transfer reactions and are common cofactors used in enzymes such as dehydrogenases and monooxygenases (Walsh, 1980). For example, the periplasmic *Shewanella* protein, FccA, contains an FAD cofactor and catalyzes the reduction of fumarate to succinate (Maier, Myers, & Myers, 2003; Pealing et al., 1992). In addition to being periplasmic protein cofactors, flavins outside the cellular membrane can act as electron shuttles. Though reduction by direct contact occurs, it is not essential for electron transfer and another mechanism must exist in order to reduce substrates not accessible to cells (Lies et al., 2005). MR-1 utilizes soluble electron shuttles for the majority of extracellular respiration (Canstein et al.,

2008; Coursolle et al., 2010; Kotloski & Gralnick, 2013; Marsili et al., 2008). Shown in Figure 3.1 is the structure of relevant flavins and the similarities in structure between them. Flavins, mainly FMN and riboflavin (RF), accumulate in *Shewanella* supernatants (Canstein et al., 2008; Coursolle et al., 2010; Kotloski & Gralnick, 2013). Removal of flavins drastically reduces current in bioreactors and the rate of iron oxide reduction (Kotloski & Gralnick, 2013). Conversely, the addition of flavins increases the amount of current in bioreactors and the rates of iron oxide reduction (Canstein et al., 2008; Coursolle et al., 2010; Kotloski & Gralnick, 2013; Marsili et al., 2008).

When using flavins as electron shuttles, RF and its derivatives, FMN and FAD, are made in the cytoplasm and exported into the surrounding environment. Bacterial exporters are categorized into five families based on amino acid sequence and type of energy coupling. The multidrug and toxic compound extrusion (MATE) family plays a significant role in antimicrobial resistance in many bacteria (Kuroda & Tsuchiya, 2009). Based on sequence analysis, the transporter identified in *Shewanella* belongs to the MATE family. Approximately twenty MATE transporters have been studied and characterized, and there appear to be large numbers of MATE transporters in both prokaryotic and eukaryotic organisms (Kuroda & Tsuchiya, 2009). While there is no common structure that all MATE transporters recognize, most MATE transporters are able to recognize fluoroquinolones as substrates (Kuroda & Tsuchiya, 2009). The majority of MATE transporters use Na+ as the energy coupling ion; however, two MATE transporters, PmpM and AbeM, use H+ as the coupling ion (He et al., 2004; Su, Chen, Mizushima, Kuroda, & Tsuchiya, 2005). The environment that the organism occupies is likely to determine which coupling ion is used for transport (Kuroda & Tsuchiya, 2009).

Shewanella species are marine bacteria and would be predicted to use Na+ as the coupling ion, because salt water is abundant with Na+ and the pH is slightly alkaline.

1.6 Biotechnology Applications

The ability to transfer electrons outside of a cell has practical applications for biotechnology uses. At a basic level, microbes utilizing an electrode as a terminal electron accepter is a simple form of communication between computers and biology. The microbe can inform a computer about environmental factors by changing electrode reduction rates, and a computer operator can change cellular physiology by changing electrode potential. Other practical purposes for this type of respiration are harvesting electricity in remote locations, reducing the amount of carbon in wastewater by providing an unlimited electron acceptor, or pushing electrons into a cell to make commercially valuable products (Kim, Kim, Hyun, & Park, 1999; Liu & Logan, 2004; Rabaey & Rozendal, 2010).

Microbial electrochemical systems are a developing technology that combines microbiology, electrochemistry, and environmental and mechanical engineering. There are almost as many uses for microbial electrochemical systems as acronyms to describe them (Wang & Ren, 2013). At a minimalist level, microbial electrochemical systems consist of two chambers separated by a semi-permeable membrane. One chamber contains an anode and the other chamber contains the cathode. The anode and cathode are connected to each other by a wire and are immersed in an electrolyte solution. Oxidation of organic compounds occurs in the microbial electrochemical system and electrons are transferred to the anode. Then electrons are transferred to the cathode where reduction of

an electron acceptor occurs. In these types of systems, microbes can be used as self-regenerating catalysts on the anode in order to facilitate the breakdown of organic molecules, on the cathode to use electrons for biosynthesis, or on both the anode and cathode. Microbial electrochemical systems have the potential to use the vast diversity of microbial metabolism to break down or create a large diversity of compounds.

1.7 Goal of Thesis

The goal of this thesis project is to add to the understanding of why *Shewanella oneidensis* MR-1 uses both direct contact and soluble flavin electron shuttles to perform extracellular electron transfer. Preventing flavin export allows the study of direct contact without interference from electron shuttles. Moreover, it allows the investigation of why specifically FAD is exported and processed into FMN and AMP. Lastly, using data collected from experiments on extracellular electron transfer, the possibility of generating valuable commercial compounds or metabolic precursors in MR-1 from electrosynthesis is evaluated.

1.8 Summary of Thesis

Chapter 2 details the identification of the FAD transporter in MR-1. Bfe is the flavin transporter in MR-1 and is required for the export of electron shuttles in the form of FAD from the cytoplasm to the periplasm. This chapter compares strains that are able to export electron shuttles with strains unable to export electron shuttles with regards to iron oxide, iron citrate, and electrode respiration. In short, electron shuttles are crucial for efficient extracellular electron transfer of only insoluble electron acceptors such as iron

oxides and electrodes. The presence or absence of flavin electron shuttles has no effect on the respiration rate of soluble electron acceptors.

Chapter 3 addresses the fate of flavin adenine dinucleotide once it is exported from the cytoplasm. UshA, a periplasmic 5'-nucleotidase, cleaves adenosine monophosphate from flavin adenine dinucleotide to produce flavin mononucleotide. The presence or absence of this protein has no effect on total supernatant flavins, but does greatly affect supernatant fluorescence. The experiments in this chapter were essential to the identification of Bfe and allow the inquiry into why flavin adenine dinucleotide, instead of flavin mononucleotide is exported into the periplasm.

Chapter 4 explores the roles of flavin adenine dinucleotide and flavin mononucleotide in the periplasm. Using strains created from the research in chapters 2 and 3, the role of periplasmic flavoproteins with and without flavin co-factors are examined.

Chapter 5 deals with the possibility of producing adenosine triphosphate in MR-1 using electrons from an electrode. This chapter focuses on attempts to make adenosine triphosphate and other possible permutations that have potential to work.

Chapter 2: Flavin Electron Shuttles Dominate Extracellular Electron Transfer by *Shewanella oneidensis*

*This chapter is a reprint, with minor alterations, of a published manuscript.

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2.1 Summary

Shewanella oneidensis strain MR-1 is widely studied for its ability to respire a diverse array of soluble and insoluble electron acceptors. The ability to breathe insoluble substrates is defined as extracellular electron transfer and can occur via direct contact or by electron shuttling in *S. oneidensis*. To determine the contribution of flavin electron shuttles in extracellular electron transfer, a transposon mutagenesis screen was performed with *S. oneidensis* to identify mutants unable to secrete flavins. A multidrug and toxin efflux transporter encoded by SO_0702 was identified and renamed *bfe* (*b*acterial *f*lavin adenine dinucleotide [FAD] *e*xporter) based on phenotypic characterization. Deletion of *bfe* resulted in a severe decrease in extracellular flavins, while overexpression of *bfe* increased the concentration of extracellular flavins. Strains lacking *bfe* had no defect in reduction of soluble Fe(III), but these strains were deficient in the rate of insoluble Fe(III) oxide reduction, which was alleviated by the addition of exogenous flavins. To test a different insoluble electron acceptor, graphite electrode bioreactors were set up to measure current produced by wild-type *S. oneidensis* and the Δ*bfe* mutant. With the same concentration of supplemented flavins, the two strains produced similar amounts of current. However, when exogenous flavins were not supplemented to bioreactors, *bfe* mutant strains produced significantly less current than the wild type. We have demonstrated that flavin electron shuttling accounts for ~75% of extracellular electron transfer to insoluble substrates by *S. oneidensis* and have identified the first FAD transporter in bacteria.

2.2 Importance

Extracellular electron transfer by microbes is critical for the geochemical cycling of metals, bioremediation, and biocatalysis using electrodes. A controversy in the field was addressed by demonstrating that flavin electron shuttling, not direct electron transfer or nanowires, is the primary mechanism of extracellular electron transfer employed by the bacterium *Shewanella oneidensis*. We have identified a flavin adenine dinucleotide transporter conserved in all sequenced *Shewanella* species that facilitates export of flavin electron shuttles in *S. oneidensis*. Analysis of a strain that is unable to secrete flavins demonstrated that electron shuttling accounts for ~75% of the insoluble extracellular electron transfer capacity in *S. oneidensis*.

2.3 Observation

Extracellular electron transfer for respiration of insoluble oxide minerals by microbes is important for the biogeochemical cycling of metals, biotechnology, and bioremediation and may represent the earliest form of respiration on Earth (Lovley et al., 2004). In natural environments, microorganisms catalyze the breakdown of organic matter coupled to the reduction of a terminal electron acceptor. Some of the most abundant electron acceptors in soil and sediment environments are insoluble Fe(III) oxide minerals. Ferric iron can be mobilized from anaerobic environments through the activity of extracellular electron transfer by dissimilatory metal-reducing bacteria as Fe(II), which is soluble and can diffuse to the anoxic/oxic interface, where it may be assimilated or reoxidized. This metabolism can also be harnessed in devices called microbial fuel cells to harvest electrical current, where poised electrodes serve as the electron acceptor for

respiration (Lovley, 2012). Though we have studied these microbes in great detail, there are several mechanisms of extracellular electron transfer being debated.

To date, three strategies of extracellular electron transfer have been proposed to explain how dissimilatory metal-reducing bacteria are able to respire insoluble substrates: direct contact, nanowires, and electron shuttling. The two best-studied model systems for how bacteria respire insoluble substrates are *Geobacter sulfurreducens* strain GSU1501 and *Shewanella oneidensis* strain MR-1 (MR-1) (Lovley et al., 2011; L. Shi et al., 2012). While both organisms utilize a variety of multiheme *c*-type cytochromes, only *Shewanella* is able to respire insoluble substrates without direct contact (Lies et al., 2005; Nevin & Lovley, 2000). Both organisms are proposed to produce conductive "nanowires" that may facilitate respiration of insoluble substrates (Gorby et al., 2006; Reguera et al., 2005); however, these structures alone cannot explain the ability of *Shewanella* to reduce insoluble substrates at a distance. Unlike the case with *Geobacter*, all investigated *Shewanella* cultures accumulate riboflavin (B2) and flavin mononucleotide (FMN) in supernatants, which can act as electron shuttles to accelerate reduction of insoluble substrates (Canstein et al., 2008; Marsili et al., 2008), including multiple forms of Fe(III) oxide (Z. Shi et al., 2012), and facilitate sensing of redox gradients (R. Li, Tiedje, Chiu, & Worden, 2012). Secreted flavins are reduced by the Mtr respiratory pathway in MR-1 (Coursolle et al., 2010), and the crystal structure of a paralog of the outer-membrane-associated decaheme cytochrome MtrC reveals FMN binding domains near two solvent-exposed heme groups (Clarke et al., 2011), providing biochemical insight into how flavin electron shuttles facilitate respiration.

Without outer-membrane cytochromes, MR-1 is unable to respire insoluble electron acceptors by either electron shuttles or direct contact (Bücking et al., 2010; Coursolle et al., 2010). However, the contribution of electron shuttles versus direct contact to total extracellular electron transfer is unknown. A mutant unable to secrete electron shuttles is required to quantify the contribution of electron shuttling, especially since mutants defective in direct electron transfer are also impaired in reduction of flavin electron shuttles (Coursolle et al., 2010; Coursolle & Gralnick, 2010). *S. oneidensis* Δ*ushA* was mated with *Escherichia coli* WM3064 (Saltikov & Newman, 2003) containing Tn*phoA′*-1(Wilmes-Riesenberg & Wanner, 1992) to create transposon mutants. Transposon selection occurred under aerobic conditions on *Shewanella* basal medium (SBM) (Covington, Gelbmann, Kotloski, & Gralnick, 2010) plates containing 40 mM lactate (Sigma) and 20 μg ml^{-1} kanamycin. Isolated colonies were inoculated into 96-well plates containing liquid Luria-Bertani broth (LB) and 50 μg ml^{-1} kanamycin. The 96-well plates were incubated at 30°C for 16 h and then transferred to 96-well plates containing liquid SBM with 40 mM lactate and 10 μg ml^{-1} kanamycin. Plates were incubated at 22°C for 72 h before fluorescence was measured at 440-nm excitation and 525-nm emission in a Molecular Devices SpectraMax M2 plate reader. Cultures with two standard deviations less than the parent strain were selected, and sites of transposon insertions were determined by arbitrary PCR and sequencing. Out of ~8,000 mutants screened, two transposon insertions were found in a predicted transmembrane protein encoded by SO_0702. The transporter is a member of the MATE (multidrug and toxin efflux) family of Na^{+}-driven multidrug and toxin efflux pump proteins (COG0534) (Kuroda & Tsuchiya, 2009).

The electron shuttle production pathway in MR-1 requires the 5′-nucleotidase UshA, which processes flavin adenine dinucleotide (FAD) into FMN and AMP in the periplasm (Covington et al., 2010). Accumulation of FAD in Δ*ushA* culture supernatants indicates that FAD, not B2 or FMN, is the flavin transported across the cytoplasmic membrane of *S. oneidensis*. An in-frame deletion was generated, and the SO_0702 locus was renamed *bfe* (bacterial FAD exporter). Flavin profiles of supernatants from the MR-1, mutant, and complemented strains grown anaerobically in SBM with lactate and fumarate were analyzed by high-performance liquid chromatography (HPLC) (Figure 2.1). The major flavin detected in MR-1 cultures was FMN. The FMN detected in these supernatants resulted from the cleavage of FAD by UshA. While in Δ*ushA* cultures, the major flavin detected was FAD. Deletion of *bfe* resulted in a substantial decrease in flavin export in both backgrounds. When *bfe* was expressed in a multicopy plasmid in MR-1 or the Δ*ushA* strain, there was a 2-fold increase in total flavins compared to levels for vector controls without changing the primary supernatant flavin. All *Shewanella* strains tested grew at the same rate under anaerobic conditions in LB with 20 mM lactate and 40 mM fumarate, which indicated that no apparent deleterious effects from deletion or overexpression of *bfe* manifested under these conditions. Anaerobic doubling times for these strains were 61 ± 2 min (MR-1 with empty vector), 59 ± 3 min (MR-1 with *bfe* in multicopy), 60 ± 1 min (Δ*bfe* strain with empty vector), and 59 ± 2 min (Δ*bfe* strain with *bfe* in multicopy). Importantly, these strains range from background levels (MR-1 with empty vector) to twice the concentration (when *bfe* is in multicopy) of flavin electron shuttles in the culture supernatant, indicating that the metabolic burden of flavin electron

shuttle production is not significant enough to influence growth under the conditions tested.

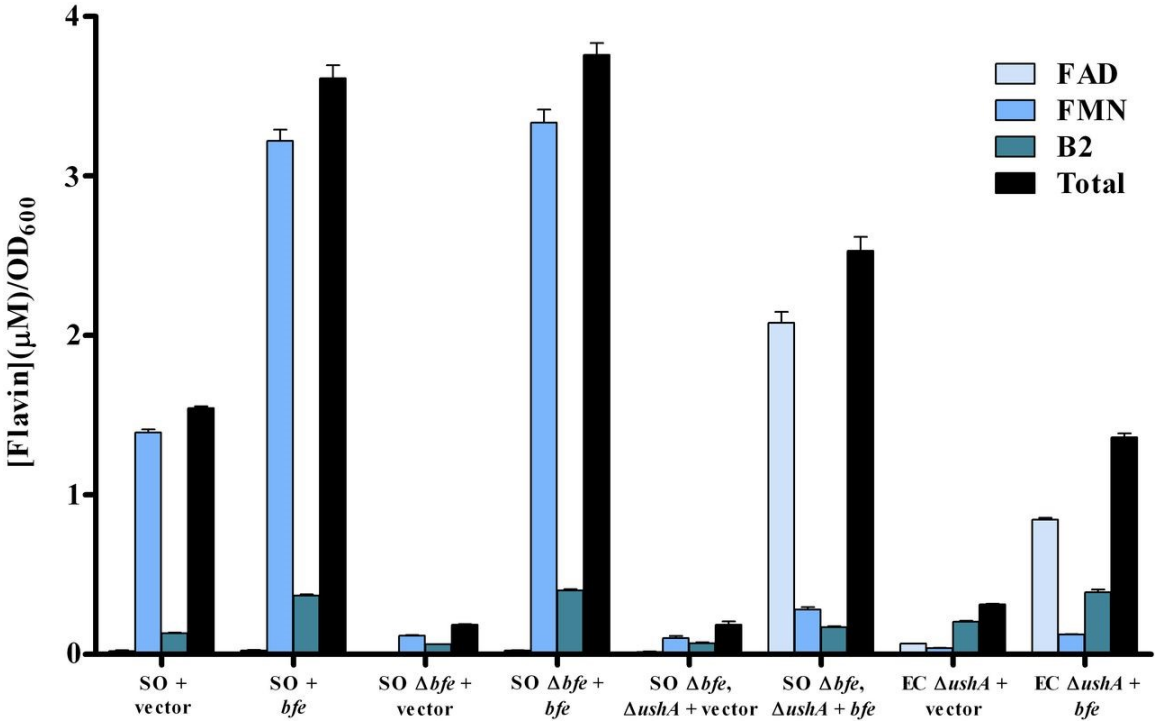

Figure 2.1

Flavin profile of *S. oneidensis* (SO) or *E. coli* (EC) strains quantified by HPLC. *S. oneidensis* cultures were anaerobically grown in SBM with 20 mM lactate and 40 mM fumarate at 30°C. Balch tubes were made anaerobic by flushing nitrogen gas through butyl rubber stoppers for 15 min. After 15 h of incubation, a sample was taken and cells were removed by centrifugation. HPLC was performed as previously described (Covington et al., 2010). The Δ*ushA E. coli* strain was grown in SBM with 20 mM lactate overnight at 37°C. Error bars indicate SEM (n = 3).

It is unlikely that expression of Bfe destabilized the cytoplasmic membrane to allow increased flavins in culture supernatants. If Bfe was destabilizing the cytoplasmic

membrane, an increase of all flavins should be observed. However, expression of *bfe* in Δ*bfe* Δ*ushA* double mutant culture supernatants resulted in a specific increase in FAD (Figure 2.1), consistent with Bfe specifically transporting FAD across the inner membrane. To provide further evidence for FAD transport, *bfe* was recombinantly expressed in *E. coli*. Supernatants from *E. coli ushA* mutant strains expressing *bfe* contained 12.5 times more FAD than empty vector controls (Figure 2.1).

Electron shuttles provide greater access for a cell to reduce insoluble electron acceptors by diffusing through biofilms or into areas too small for a cell to physically fit. In contrast, electron shuttles should have no bearing on the ability of the cell to respire soluble electron acceptors that are able to diffuse to the cell. If flavin electron shuttles are the primary mechanism for reduction of insoluble extracellular electron acceptors by MR-1, then the removal of flavins from medium should drastically reduce the reduction rates of insoluble electron acceptors but have no effect on reduction rates of soluble electron acceptors. To determine the contribution of flavin electron shuttles to Fe(III) reduction by MR-1, Fe(II) production over time was quantified with a ferrozine-based assay (Stookey, 1970) as previously described (Coursolle et al., 2010). Cells were provided 5 mM Fe(III) oxide (ferrihydrite) as the sole anaerobic electron acceptor (Figure 2.2 A). Strains lacking *bfe* reduced insoluble Fe(III) oxide at only ~25% of the rate of MR-1, demonstrating the importance of flavin electron shuttles under these conditions. A similar observation was made qualitatively using Mn(IV) oxide (birnessite) as the terminal electron acceptor (data not shown). We speculate that the residual Fe(III) oxide reduction capacity of the *bfe* mutant strain was mediated by direct contact. Rates of Fe(III) oxide reduction by MR-1 were known to increase with exogenous flavin addition

(Canstein et al., 2008; Coursolle et al., 2010). Overexpression of *bfe* increased the amount of supernatant flavins (Figure 2.1), resulting in strains that reduce Fe(III) oxide faster than MR-1 (Figure 2.2 A) Complementation (Figure 2.2 A) or addition of 10 μM FMN was able to alleviate the Fe(III) oxide reduction defect (see Figure 2.3 A in the supplemental material). As predicted, flavin electron shuttles were not necessary for reduction of soluble Fe(III) citrate (see Figure 2.3 B). Taken together, these experiments demonstrate the advantage of using flavin electron shuttles to reduce insoluble Fe(III) oxide under these conditions and provide evidence that the Mtr respiratory pathway itself is unimpaired in *bfe* mutant strains.

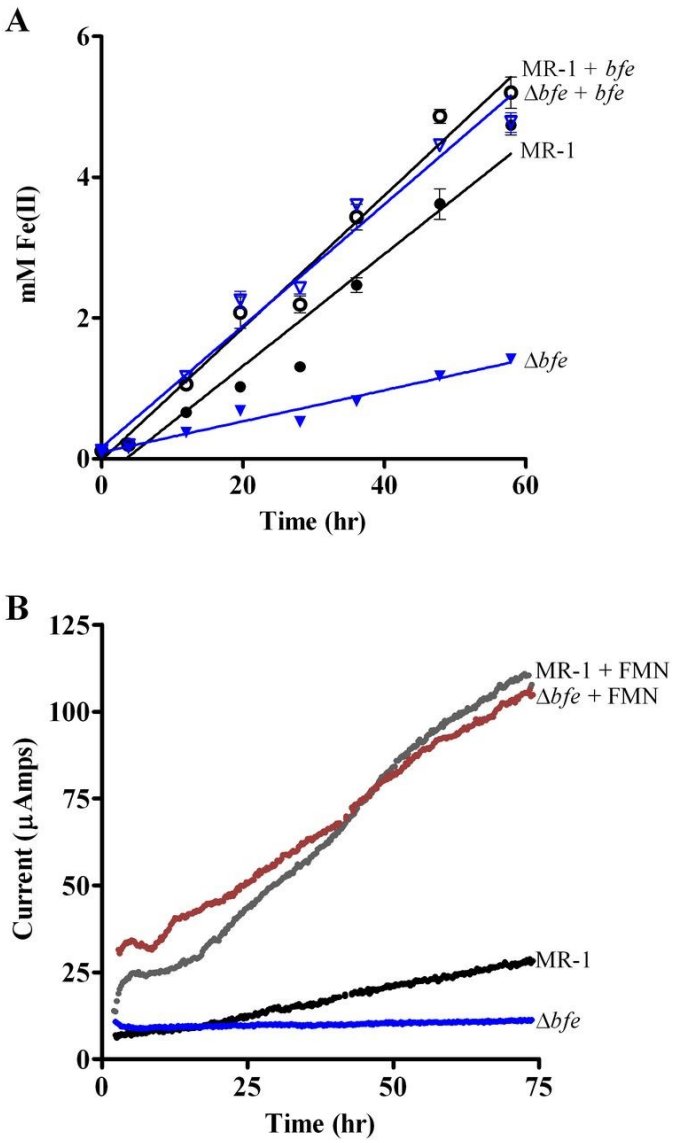

Figure 2.2

Electron shuttles accelerate reduction of insoluble extracellular electron acceptors. (A) Fe(III) oxide (ferrihydrite) reduction was quantified as previously described(Coursolle et al., 2010) for the following strains: MR-1 + vector (●), MR-1 + *bfe* (○), Δ*bfe* strain + vector (▾), and *Δbfe* strain + *bfe* (▽). Error bars indicate SEM (*n* = 3). (B) Bioreactors

were assembled as previously described (Marsili et al., 2008). One milliliter from an aerobic SBM culture with 20 mM lactate was added to 9 ml of an anaerobic SBM culture with 50 mM lactate and 40 mM fumarate. Cultures were grown at 30°C with shaking until an optical density at 600 nm of 0.4 was reached. The entire culture was added to the bioreactor. Bioreactors were continuously flushed with nitrogen gas, and electrodes were poised at a potential of +0.242 V versus a standard hydrogen electrode using a 16-channel VMP potentiostat (Bio-Logic SA). Current measurement of MR-1 (black), MR-1 + 10 μM FMN (flavin mononucleotide) (gray), the Δ*bfe* mutant (blue), and the Δ*bfe* mutant + 10 μM FMN (red) in bioreactors is shown. Data are representative of three replicates.

Analogous to Fe(III) oxides, graphite electrodes in three-electrode bioreactors are insoluble but do not become soluble once reduced and have different molecular surface features. Three-electrode bioreactors have a distinct advantage in that electrons transferred to the electrode are quantified and measured as current in real time (Marsili et al., 2008). The electrode acts as a proxy for various forms of Fe(III) oxides based on the set potential of the electrode. In bioreactors, strains with and without *bfe* were tested for their ability to reduce graphite electrodes set at a potential comparable to that of the ferrihydrate used previously. Without exogenous flavin electron shuttles, the current in bioreactors containing *bfe* mutants did not increase, unlike the case with bioreactors containing MR-1 (Fig. 2B). The stable current over 75 h for the *bfe* mutant suggests that there are no other electron shuttles accumulating to substantial quantities. When current production plateaus in bioreactors, that of the Δ*bfe* strain is ~75% lower than that of MR-1 without flavin supplementation, a difference similar in magnitude to the results

observed with Fe(III) oxide as an electron acceptor. The residual activity is likely due to a direct contact mechanism employed by *S. oneidensis* using the Mtr pathway. When bioreactors are supplemented with 10 μM FMN, the current of both MR-1 and *bfe* mutant strains is similar and higher levels of current are achieved (Figure 2.2 B) due to increased availability of flavin electron shuttles (Marsili et al., 2008). Addition of FAD to either Fe(III) oxide reduction assays or bioreactors also alleviated *bfe* mutant defects (data not shown), since UshA rapidly converts exogenous FAD to FMN (Covington et al., 2010).

2.4 Implications

Electron shuttling has been a controversial hypothesis for extracellular electron transfer since it was first suggested (Newman & Kolter, 2000). Quantifying the contribution of flavin electron shuttling to the ability of *S. oneidensis* to respire insoluble substrates required a mutant strain unable to accumulate flavins in the culture supernatant. Our results demonstrate that electron shuttling accounts for ~75% of the insoluble substrate respiratory capacity of *S. oneidensis* under laboratory conditions. Though we have specifically tested one form of Fe(III) oxide (ferrihydrite), graphite electrodes, and Mn(IV) oxide (birnessite), we believe flavin electron shuttles will be important for the ability of *S. oneidensis* to respire other insoluble substrates. Homologs of *bfe* exist in the genomes of closely related *Vibrio* species and in all sequenced *Shewanella* species, consistent with flavin accumulation in the culture supernatants of various *Shewanella* species (Canstein et al., 2008; Coursolle et al., 2010; Marsili et al., 2008). While *G. sulfurreducens* strain PCA has a MATE-like domain efflux pumphomolog of Bfe, the amino acid identity is below 30%, consistent with these

bacteria not secreting flavin electron shuttles. Characterization of *bfe* in *S. oneidensis* demonstrates the pivotal role of flavin electron shuttles in facilitating reduction of insoluble electron acceptors by these bacteria. Based on evidence presented here and on recent biochemical results (Clarke et al., 2011; Hartshorne et al., 2009), we propose that flavin electron shuttling and direct contact via outer-membrane-associated *c*-type cytochromes are sufficient to explain the extracellular electron transfer abilities of *S. oneidensis*. We are working to quantify the metabolic burden of flavin electron shuttle production and exploring the environmental relevance of this shuttle-based respiratory strategy.

2.5 Supplemental Information

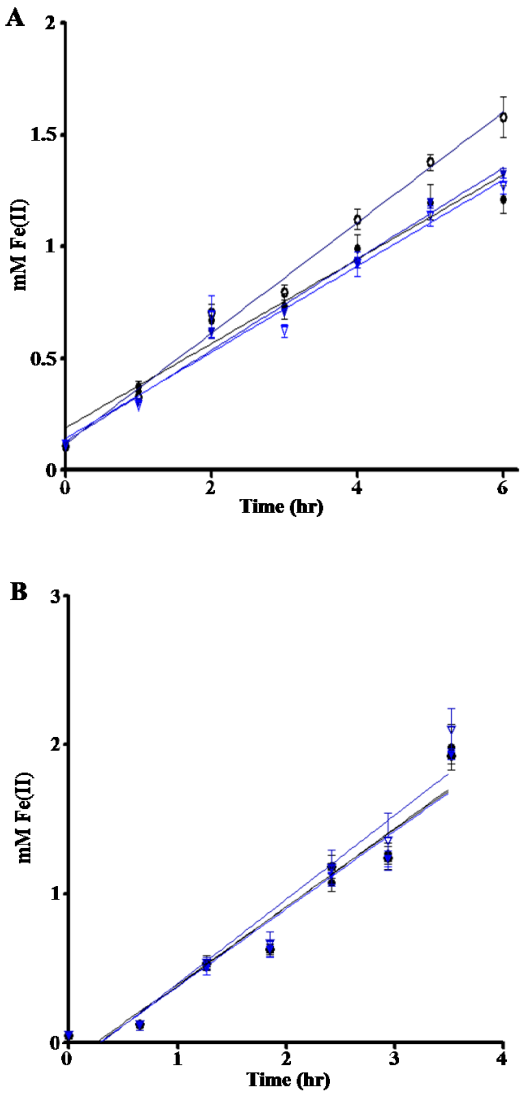

Figure 2.3

The Δ*bfe* strain reduces soluble Fe(III) at wild-type rates, and the insoluble Fe(III) reduction defect is rescued with the addition of exogenous flavins. The iron reduction assays were performed in an identical manner as Figure 2. (A) The reduction of insoluble Fe(III) with exogenous 10 μM FMN added by MR-1 + vector (●), MR-1 + *bfe* (O), Δ*bfe* + vector (▼), and Δbfe + bfe (▽). (B) The reduction of soluble Fe(III) by MR-1 + vector (●), MR-1 + *bfe* (O), Δ*bfe* + vector (▼), and Δ*bfe* + *bfe* (▽). Error bars indicate SEM (n=3).

2.6 Acknowledgements

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We thank members of the Gralnick lab for helpful comments and discussion and the Bond lab for electrochemical expertise.

2.7 Materials and Methods

Bacterial strains and growth conditions

MR-1 was described by Myers and Nealson (C. R. Myers & Nealson, 1988). A frozen freezer stock was struck for isolation on a Luria-Bertani (LB) agar plate and after incubation, a single colony was inoculated into 2 ml of LB medium and incubated aerobically while shaking at 200 r.p.m. for 16 hours at 30°C. For experiments with minimal medium, *Shewanella* basal medium (SBM) consisting of 0.225 g K_2HPO_4, 0.225 g KH_2PO_4, 0.46 g NaCl, 0.225 g $(NH_4)_2SO_4$, 0.117 g $MgSO_4$-$7H_2O$, and 10 mM HEPES adjusted to pH 7.2 per liter of double distilled water was used. One liter of SBM was supplemented with 5 ml of vitamins excluding riboflavin (Balch, Fox, Magrum, Woese, & Wolfe, 1979), 5 ml of trace minerals (Marsili et al., 2008), and 0.01% casamino acids (Difco). Other substances were added as indicated. When required, anaerobic cultures were made by flushing nitrogen through Balch tubes sealed with butyl rubber stoppers for 15 minutes.

Transposon mutagenesis

Transposon mutants were created by mating *S. oneidensis* Δ*ushA* (Covington et al., 2010) with *E. coli* WM3064 containing TnphoA′-1 (Wilmes-Riesenberg & Wanner, 1992). Transposon selection occurred under aerobic conditions on SBM plates containing 40mM DL-lactate (Sigma) and 50 μg ml^{-1} kanamycin (Fisher). Isolated colonies were inoculated into 96-well plates containing liquid LB and 50 μg ml^{-1} kanamycin. The 96-well plates were incubated at 30°C for 16 hours and then transferred to 96-well plates containing liquid SBM with 40 mM DL-lactate and 10 μg ml^{-1} kanamycin. Plates were incubated at 22°C for 72 hours before fluorescence was measured at 440 nm excitation and 525 nm emission in a Molecular Devices SpectraMax M2 plate reader. Cultures with two standard deviations less than the parent strain were selected and sites of transposon insertions were determined by arbitrary polymerase chain reactions (PCR) and sequencing.

Deletion and complementation

Shewanella oneidensis Δ*bfe* (strain JG1758) was generated by ligating the regions flanking the target gene into the suicide vector pSMV3. To amplify the flanking regions the following primers were used:

SO_0702 P1 Up For ApaI, ATTGGGCCCGCCGGGAAGCGTTTAAGGTAAAGT;

SO_0702 P2 Up Rev XhoI, GTTCTCGAGTATTCAGTACCGATCCGCAGGTGT;

SO_0702 P3 Down For XhoI, GTACTCGAGCGCCAATGGCAATGCTGGAAAT;

SO_0702 P4 Down Rev SpeI, GACCACTAGTTAACCGCTGCCACTTTCACGATAC.

The suicide vector was mated into MR-1 using *E. coli* WM3064 on LB agar plates with 100 µl of 60 mM diaminopimelic acid spread on top. The loss of *sacB* from the suicide vector was selected for on LB agar plates with 5% sucrose. PCR using primers flanking target genes confirmed that deletions occurred. Complementation of the deletion was performed using pBBR1MCS-2 and the following primers:
njk 20 SO_0702 comp Front, NNGGTACCTTCCCCTTTGATGTGTGTGA;
njk20.1 SO_0702 comp Re. SpeI, NNACTAGTCTAAAGGGTGTCGGCGGTT.

HPLC

High-performance liquid chromatography was performed with a 4.6 mm x 150 mm Eclipse XDB-C18 column with a 5 mm particle size (Agilent Technologies) maintained at 30°C. Samples (50 µl) were injected at a flow rate of 1 ml min-1 using an SIL-10AF auto-injector (Shimadzu) into a 20% methanol, 1% glacial acetic acid solution. Flavins were detected with an RF-10AXL fluorescence detector (Shimadzu) at excitation wavelength of 440 nm and emission wavelength of 525 nm. Standards were made with two-fold dilutions in SBM with concentrations ranging from 1 µM to 0.0625 µM. Flavin concentrations were calculated by comparing the area of each peak to the area of standard peaks.

Iron reduction assays

Strains were grown aerobically in LB medium for 16 hours, washed in SBM, and resuspended in SBM to an OD of 1.30. Thirty microliters was added to 270 µl of SBM

containing vitamins, minerals, 20 mM lactate, 5 mM of iron oxide or iron citrate in 96-well

plates. Plates were kept in a GasPak System anaerobic petri dish holder that was flushed with nitrogen gas for 15 min initially and between time points. At each time point, 50 µl of 5 M HCl was added to each well. Then, 30 µl was diluted into 270 µl of 0.5 M HCl, and 50 µl of this dilution was added to 300 µl of ferrozine reagent (Stookey, 1970) and read at 562 nm.

Three-electrode bioreactors

Bioreactors were assembled as described by Marsili *et al*. One milliliter from an aerobic SBM culture with vitamins, minerals, 0.01% casamino acids, and 20 mM DL-lactate was added to 9 ml of an anaerobic SBM culture with vitamins, minerals, 0.05% casamino acids, 50 mM DL-lactate, and 40 mM fumarate (Marsili et al., 2008). Cultures were grown at 30°C and shook at 200 r.p.m. until an OD of 0.4 was reached. The entire culture was added to the bioreactor. Bioreactors were continuously flushed with nitrogen gas and electrodes were poised at a potential of +0.242 V versus standard hydrogen electrode using a 16-channel VMP potentiostat (Bio-Logic SA).

Chapter 3: An Essential Role for UshA in Processing of Extracellular Flavin Electron Shuttles by *Shewanella oneidensis*

*This chapter is a reprint, with minor alterations, of a published manuscript.

Covington, E. D., Gelbmann, C. B., Kotloski, N. J., & Gralnick, J. A. (2010). An essential role for UshA in processing of extracellular flavin electron shuttles by *Shewanella oneidensis*. *Molecular Microbiology*, *78*(2), 519–532. doi:10.1111/j.1365-2958.2010.07353.x

3.1 Summary

The facultative anaerobe *Shewanella oneidensis* can reduce a number of insoluble extracellular metals. Direct adsorption of cells to the metal surface is not necessary, and it has been shown that *S. oneidensis* releases low concentrations flavins, including riboflavin and flavin mononucleotide (FMN), into the surrounding medium to act as extracellular electron shuttles. However, the mechanism of flavin release by *Shewanella* remains unknown. We have conducted a transposon mutagenesis screen to identify mutants deficient in extracellular flavin accumulation. Mutations in *ushA*, encoding a predicted 5′-nucleotidase, resulted in accumulation of flavin adenine dinucleotide (FAD) in culture supernatants, with a corresponding decrease in FMN and riboflavin. Cellular extracts of *S. oneidensis* convert FAD to FMN, whereas extracts of *ushA* mutants do not, and fractionation experiments show that UshA activity is periplasmic. We hypothesize that *S. oneidensis* secretes FAD into the periplasmic space, where it is hydrolysed by UshA to FMN and adenosine monophosphate (AMP). FMN diffuses through outer membrane porins where it accelerates extracellular electron transfer, and AMP is dephosphorylated by UshA and reassimilated by the cell. We predict that transport of FAD into the periplasm also satisfies the cofactor requirement of the unusual periplasmic fumarate reductase found in *Shewanella*.

3.2 Introduction

The shewanellae are a diverse genus of Gram-negative γ-proteobacteria that respire a wide array of organic and inorganic compounds (Hau & Gralnick, 2007; Nealson & Scott, 2006). Respiratory substrates include soluble organic compounds, toxic

metals such as uranium and technetium, and insoluble metallic solids such as Fe(III) and Mn(IV). Their influence on redox state and thereby on metal solubility has generated interest in using *Shewanella* for bioremediation of contaminated groundwater (Hau & Gralnick, 2007; Pinchuk et al., 2008). In addition, the ability of *Shewanella* to reduce electrodes has led to the development of microbial fuel cells in which the electrode serves as terminal electron acceptor for bacterial cultures (Gorby et al., 2006; Lovley, 2008; Marsili et al., 2008).

In many natural environments, the oxidized iron and manganese that can serve as electron acceptors for *Shewanella* are insoluble at neutral pH. Therefore, *Shewanella* face the problem of transferring electrons from the cell surface to the metal. Direct contact of *Shewanella* with metal surfaces can occur and does account for at least some of the metal reduction by *Shewanella* cultures (Baron, LaBelle, Coursolle, Gralnick, & Bond, 2009; Gorby et al., 2006; Lies et al., 2005). However, direct contact is not possible for every cell, particularly in multi-layer biofilms, and it is clear that other solutions must also play a role. The predominant mechanism used by *Shewanella* appears to be release of a diffusible mediator into the environment (Lies et al., 2005; Nevin & Lovley, 2002). Although several potential compounds have been suggested over the years, including quinones (Lovley, Coates, Blunt-Harris, Phillips, & Woodward, 1996; Newman & Kolter, 2000; Ward et al., 2004) and melanin (Turick, Tisa, & Jr, 2002), it has recently become apparent that the primary mediators used by *Shewanella* are flavins. Both riboflavin and flavin mononucleotide (FMN, Figure 3.1) are detected in low concentrations (between 250 nM and 1 μM, typically) in cultures of *Shewanella oneidensis* MR-1 and other *Shewanella* species (Canstein et al., 2008; Coursolle et al.,

2010; Marsili et al., 2008). Washout of flavins by replacement of media in bioreactors leads to approximately 80% decrease in *S. oneidensis*-induced current (Marsili et al., 2008), and addition of exogenous flavins to *Shewanella* cultures enhances the rate of Fe(III) reduction by those cultures (Canstein et al., 2008).

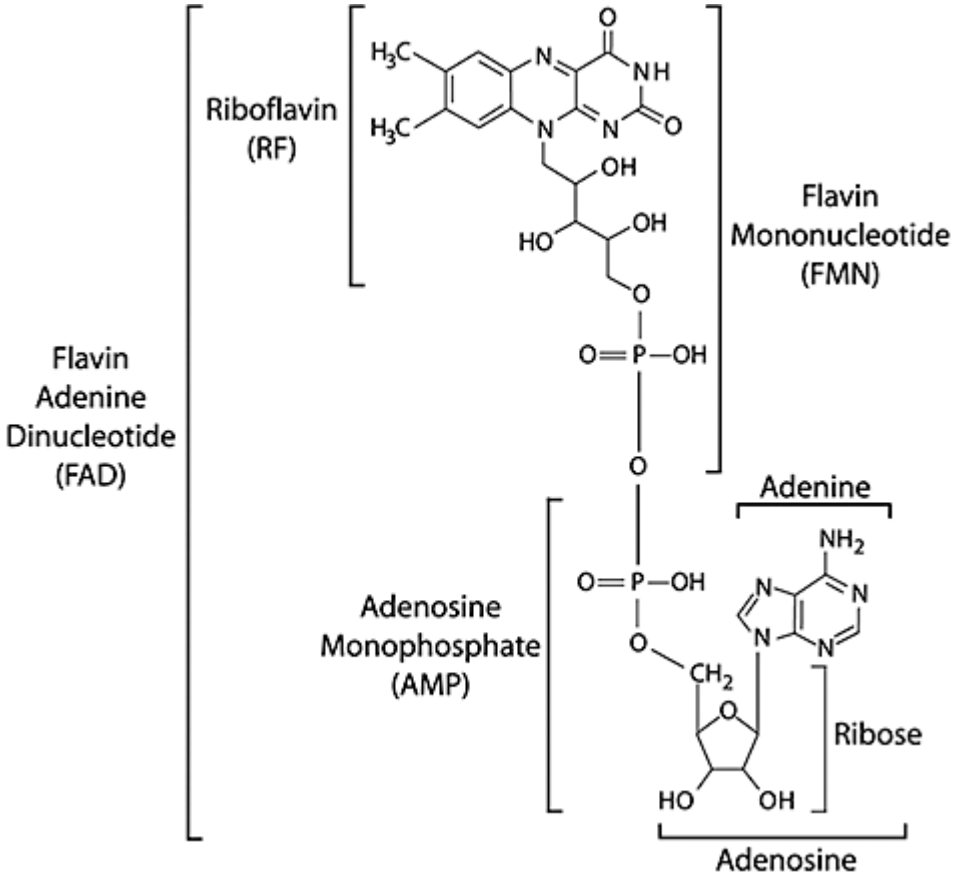

Figure 3.1
Structure of flavin adenine dinucleotide. Various moieties are indicated.

In theory, a diffusible mediator could enhance the rate of metal reduction either by chelating the metal or by shuttling electrons from the outer membrane (OM) to the metal surface. Although flavins can chelate iron through their isoalloxazine ring (Albert, 1950, 1953), evidence suggests that the shuttling activity is dominant. First, known

chelators have less effect on iron reduction rates than do flavins (Coursolle et al., 2010), and second, flavins enhance reduction of surfaces that cannot be chelated, such as carbon electrodes (Baron et al., 2009; Marsili et al., 2008; Velasquez-Orta et al., 2010). Current data indicate that electrons are passed from the menaquinone pool to the outer surface of the cell through the Mtr pathway, where the OM cytochromes MtrC and OmcA reduce extracellular substrates (L. Shi, Squier, Zachara, & Fredrickson, 2007), including flavins (Coursolle et al., 2010). Reduced flavins can transfer electrons to an insoluble electron acceptor and be re-reduced by the Mtr pathway. Though flavins may diffuse away from the cell, the most efficient mechanism could be through inter-molecular electron transfer between flavin molecules, the Mtr pathway and the insoluble terminal electron acceptor.

Although flavins are utilized by *Shewanella* as electron shuttles, they are also used ubiquitously as enzyme cofactors. The riboflavin derivatives FMN and flavin adenine dinucleotide (FAD, Figure 3.1) play a crucial role in biological redox reactions by catalyzing both one-electron and two-electron transfers in such diverse enzymes as dehydrogenases and monooxygenases (Walsh, 1980), and photoreceptors (Losi & Gärtner, 2008). One critical flavoenzyme in *Shewanella* is the fumarate reductase FccA, a periplasmic tetraheme *c*-type cytochrome containing a non-covalently bound FAD cofactor, required for utilization of fumarate as an anaerobic electron acceptor (T. M. Maier et al., 2003; Pealing et al., 1992; Turner et al., 1999).

Nothing is known of the mechanism by which *Shewanella* secrete flavins. Although several bacterial riboflavin importers have been identified (Burgess et al., 2006; Kreneva et al., 2000; Vitreschak, Rodionov, Mironov, & Gelfand, 2002; Vogl et al., 2007), none has been shown to export flavins. A potential exporter of FAD has been

identified in mitochondria of yeast (Bafunno et al., 2004), but this transporter has no apparent homologues in *Shewanella*. Because *Shewanella* relies on extracellular flavins for reduction of insoluble electron acceptors, the question of how *Shewanella* transports and processes flavins will be critical to understanding this mode of anaerobic respiration.

We have conducted a random mutagenesis screen designed to isolate mutants in the flavin secretion/release pathway. The mutant with the most severe phenotype was found to be disrupted in the open reading frame (ORF) *SO2001* encoding the gene *ushA*. We find that FAD is the predominant flavin species secreted by *S. oneidensis*, and that UshA processes periplasmic FAD to FMN. We also find that *Shewanella* cells are able to re-uptake and use the adenosine monophosphate (AMP) released from FAD hydrolysis as a carbon source and that this activity is also UshA-dependent. Our results have implications for the search for flavin transport mechanisms in *Shewanella* as well as for our understanding of the evolution of electron shuttling and applications of mediator-enhanced dissimilatory electrode reducing bacteria.

3.3 Results

Deletion of ushA *results in decreased flavin fluorescence in culture supernatants of* S. oneidensis

To identify genes involved in extracellular flavin production by *Shewanella*, we conducted a random mutagenesis screen, taking advantage of the native fluorescence profile of flavins (Sandro Ghisla, Massey, Lhoste, & Mayhew, 1974). *S. oneidensis* strain MR-1 was conjugated with a 6.5 kb transposable element, Tn*pho*A′-1, encoding a *lacZ* fusion and kanamycin resistance marker (Wilmes-Riesenberg & Wanner, 1992).

Kanamycin-resistant transductants were inoculated into 96-well plates, and mutants with potential flavin production defects were identified by decreased fluorescence in the culture medium. Out of approximately 10 000 mutants screened, six mutants were obtained with consistently less fluorescence than wild-type MR-1. Sequence analysis led to identification of the ORF *SO2001* as the transposon insertion site in five mutants.

SO2001 encodes a putative 61 kDa protein with 50% identity (amino acid alignment using NCBI blast) to the UDP-sugar hydrolase/5′-nucleotidase UshA of *Escherichia coli*. Given the significant similarity between the MR-1 and *E. coli* proteins we will refer to SO2001 as UshA. UshA is a conserved periplasmic metallophosphoesterase that hydrolyses a range of nucleotides, UDP-sugars and CDP-alcohols (Alves-Pereira et al., 2008; Glaser, Melo, & Paul, 1967; Neu, 1967). In *E. coli*, UshA is involved in nucleotide scavenging (Beacham, Kahana, Levy, & Yagil, 1973; Kakehi, Usuda, Tabira, & Sugimoto, 2007; Yagil & Beacham, 1975) and enhances intracellular survival of enteropathogenic *E. coli* by inhibiting host cell kinases (Berger, Rowan, Morrison, & Ziltener, 1996). In *Corynebacterium glutamicum*, UshA mediates phosphate acquisition from nucleotides in response to phosphate starvation (Ishige, Krause, Bott, Wendisch, & Sahm, 2003; Rittmann, Sorger-Herrmann, & Wendisch, 2005). The related protein ecto-5′-nucleotidase in mammals catalyzes degradation of purinergic signaling nucleotides (Sträter, 2006).

In order to characterize UshA involvement in flavin processing by *S. oneidensis*, we generated an *ushA* deletion strain referred to as Δ*ushA*. The Δ*ushA* mutant strain showed growth similar to wild-type strain MR-1 in minimal medium both under aerobic conditions and under anaerobic conditions with fumarate as an electron acceptor (Figure

3.2 A). Δ*ushA* also grew similarly to wild-type MR-1 either aerobically or anaerobically in Luria–Bertani (LB) medium (data not shown). At various time points during anaerobic growth on minimal medium, culture samples were removed and centrifuged. Fluorescence of the supernatants was measured in order to assess flavin accumulation (Figure 3.2 B). While fluorescence of medium from wild-type cultures increased roughly in parallel with cell growth, fluorescence of medium from Δ*ushA* cultures accumulated more slowly and to a level approximately twofold lower than the fluorescence of wild-type MR-1. Qualitatively similar results were observed when cells were grown aerobically or in rich medium (data not shown). Fluorescence of growth medium from Δ*ushA* cells with empty vector [101 ± 7 relative fluorescence units (RFU)] was restored when *ushA* was expressed on a complementation vector (214 ± 27 RFU), confirming that deletion of the *ushA* gene results in decreased accumulation of fluorescent material in the culture medium.

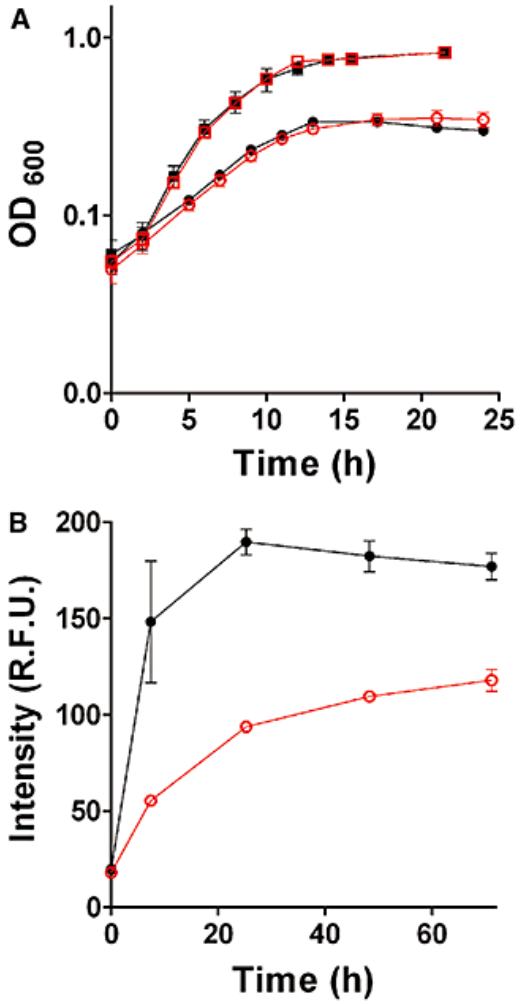

Figure 3.2

Decreased flavin fluorescence in cultures of Δ*ushA* mutant. A. Optical density of wild-type MR-1 (black, closed symbols) and Δ*ushA* (red, open symbols) grown aerobically in SBM with 20 mM lactate as carbon source (■) or anaerobically in SBM with 20 mM lactate and 40 mM fumarate as electron acceptor (●). Mean ± standard error of the mean (SEM) of triplicate cultures. B. Fluorescence intensity in RFU of supernatants from cultures of wild-type MR-1 (●) and Δ*ushA* (○) grown anaerobically as in (A). Time indicates the length of time cells were in culture before the sample was removed. Mean ± SEM of triplicate cultures.

ΔushA *strains secrete FAD instead of FMN or riboflavin*

The decrease in supernatant fluorescence observed in Figure 3.2 B may be explained not by a decrease in total flavins, but rather a shift in the flavins present. The quantum yield of FAD is lower than that of FMN or riboflavin (Rhee et al., 2009; Weber, 1950). In order to accurately measure the concentrations of various flavins, we analyzed supernatants from wild-type and Δ*ushA* mutant cultures by high-performance liquid chromatography (HPLC) to quantify riboflavin, FMN and FAD (Table 3.1). Wild-type cultures grown anaerobically for 24 h in minimal medium accumulated approximately 0.3 μM total flavins, with approximately 65% as FMN and 35% as riboflavin. Δ*ushA* culture supernatants accumulated similar total concentrations of flavins; however, approximately 75% was in the form of FAD (Table 3.1). Complementation of the *ushA* deletion strain with a wild-type copy of *ushA* returned the flavin production profile to match the wild-type strain (Table 3.1). The increased proportion of FAD in supernatants of mutant cultures suggests that Δ*ushA* mutants primarily secrete FAD rather than FMN or riboflavin.

Table 3.1

Flavin quantitation in supernatants of Δ*ushA* strain and complemented strains.

	Concentration (μM) in culture supernatant [a]			Percentage of total flavins in culture supernatant		
Strain	FAD	FMN	RF	FAD	FMN	RF
MR-1	0 ± 0	0.20 ± 0.01	0.11 ± 0.006	0 ± 0	64.3 ± 0.8	35.7 ± 0.8
Δ*ushA*	0.27 ± 0.01	0.07 ± 0.001	0.025 ± 0.001	73.1 ± 1.1	20.2 ± 0.7	6.7 ± 0.4
MR-1 + pBBR1MCS-3	0 ± 0	0.21 ± 0.006	0.05 ± 0.002	0 ± 0	81.8 ± 0.6	18.2 ± 0.6
Δ*ushA* + *pBBR1MCS-3*	0.28 ± 0.004	0.06 ± 0.001	0.01 ± 0.000	80.1 ± 0.5	16.4 ± 0.5	3.6 ± 0.03
MR-1 + pSO2001MCS-3	0 ± 0	0.19 ± 0.015	0.07 ± 0.004	0 ± 0	73.1 ± 0.4	26.9 ± 0.4
Δ*ushA* + *pSO2001MCS-3*	0 ± 0	0.20 ± 0.006	0.07 ± 0.002	0 ± 0	72.9 ± 0.2	27.1 ± 0.2

[a] Mean ± SEM concentration measured from culture supernatants of the indicated *S. oneidensis* strains. Data were obtained from triplicate cultures and are representative of three experiments.

FAD release could indicate a deleterious effect of *ushA* mutation on cell membrane integrity. However, wild-type and Δ*ushA* cultures grew at similar rates, as measured by OD_{600} (Figure 3.2 A). Additionally, the number of colony-forming units from mid-log cultures was not significantly different for wild-type ($1.06 \times 10^8 \pm 1.1 \times 10^7$) versus Δ*ushA* ($1.67 \times 10^8 \pm 4.2 \times 10^7$), indicating that the *ushA* mutation does not decrease cell viability. Membrane integrity of the Δ*ushA* strain was also assessed using a live/dead stain in which the membrane-permeable green dye SYTO9 stains all cells, while the membrane-impermeable red stain propidium iodide stains only cells with compromised membranes. The ratios of green to red fluorescence were identical (12.9 ± 0.4 vs. 12.7 ± 0.2) in samples from exponentially growing wild-type and Δ*ushA* cultures. This result indicates that the accumulation of FAD in *S. oneidensis*Δ*ushA* cultures cannot be attributed to membrane damage in cells lacking UshA, consistent with a specific mechanism of FAD secretion.

Shewanella oneidensis *UshA hydrolyses FAD to FMN*

The structure of FAD, consisting of an ADP nucleotide with 5′-linkage to a riboflavin moiety (Figure 3.1), is reminiscent of UDP-sugars and CDP-alcohols that have been reported to be substrates of *E. coli* UshA (Alves-Pereira et al., 2008; Glaser et al., 1967; Neu, 1967). However, despite this similarity in substrate structure, *E. coli* UshA exhibits very low FAD hydrolysis activity (Alves-Pereira et al., 2008). In order to test whether *S. oneidensis* UshA has the ability to catalyze hydrolysis of FAD, we added

sonicated extracts of wild-type and Δ*ushA* cells to solutions of FAD. Because FAD is less fluorescent than FMN or riboflavin (Weber, 1950), hydrolysis of FAD into FMN or riboflavin results in more intense fluorescence. Increases in fluorescence intensity were monitored over time as an indication of FAD hydrolysis (Figure 3.3 A). When wild-type cell extracts were added to solutions of FAD, fluorescence increased in a protein-dependent manner, consistent with hydrolysis of the FAD into a more highly fluorescent product. In contrast, Δ*ushA* cell extracts had no effect on FAD fluorescence intensity. HPLC analysis indicated that a majority of the reaction product from wild-type cell extracts was FMN, with a small percentage riboflavin (Figure 3.3 B). The small amounts of FMN and riboflavin detected in reactions with Δ*ushA* cell extracts were consistent with the percentage of impurities in the added FAD substrate and did not change over the course of 140 min (data not shown). The initial linear rate of FAD hydrolysis by wild-type cell extracts was 26.8 ± 0.8 nmol min^{-1} mg $protein^{-1}$, while the rate of FAD hydrolysis by Δ*ushA* cell extracts was only 0.088 ± 0.003 nmol min^{-1} mg $protein^{-1}$. These data strongly suggest that *S. oneidensis* UshA is capable of hydrolyzing FAD and releasing FMN.

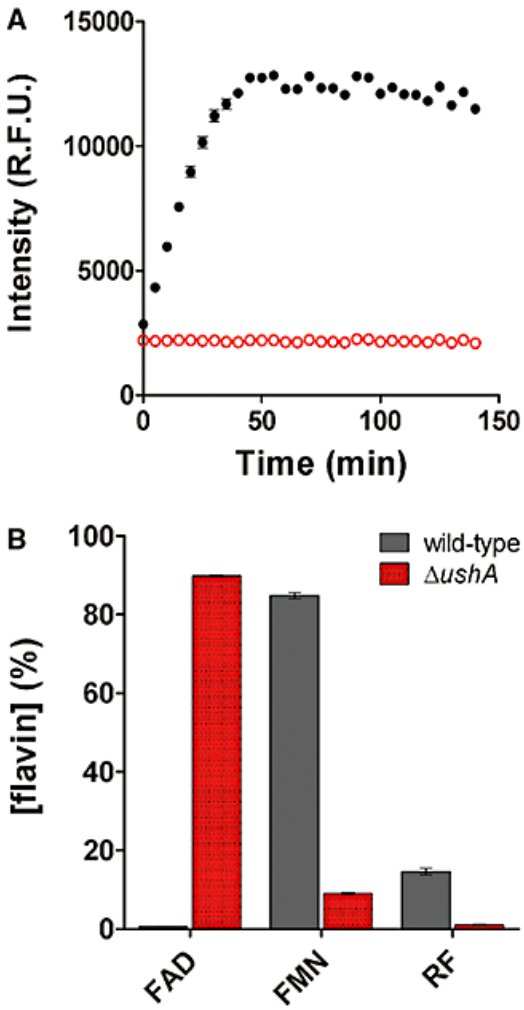

Figure 3.3

S. oneidensis UshA hydrolyses FAD to FMN in cell extracts. A. Sonicated cell extracts (0.06 mg ml^{-1} total protein) of wild-type MR-1 (●) and Δ*ushA* (○) were added to solutions of FAD. Mean fluorescence intensity (± SEM) is plotted versus time as FAD is hydrolysed to FMN. Data were obtained from triplicate cultures and are representative of four experiments. B. HPLC measurements of the per cent concentration of each flavin species after the final time point in (A). Samples treated with wild-type cell extracts are in solid; samples treated with Δ*ushA* cell extracts are patterned.

UshA activity is localized in the periplasm

Escherichia coli UshA is a soluble protein targeted to the periplasm by an N-terminal signal peptide of 25 amino acids (Burns & Beacham, 1986; Glaser et al., 1967; Neu & Heppel, 1965). A periplasmic localization is also predicted for the *S. oneidensis* UshA protein by using PSORTb 3.0 (Nancy et al., 2010). In order to test the localization of *S. oneidensis* UshA, we monitored FAD hydrolysis by intact cells. An FAD solution was added to washed cultures of *S. oneidensis*, and the fluorescence increase was measured over time as an indication of FAD hydrolysis (Figure 3.4 A). Similarly to cell extracts, intact wild-type *S. oneidensis* were able to hydrolyze FAD, while Δ*ushA* cells were not. The rate of FAD hydrolysis by intact wild-type cells was approximately 6.5 nmol min^{-1} mg $protein^{-1}$, somewhat slower than the rate of catalysis by cell extracts, consistent with FAD needing to cross through OM porins before hydrolysis.

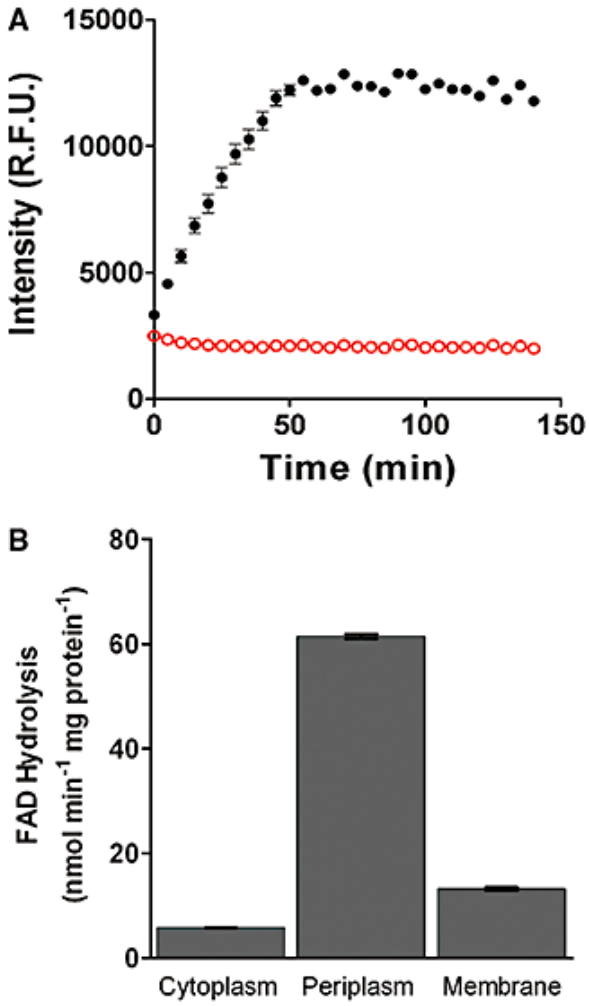

Figure 3.4

UshA activity is localized in the periplasm. A. FAD hydrolysis by intact wild-type MR-1 cells (●) and intact Δ*ushA* cells (○). Mean fluorescence intensity (±SEM) versus time. B. FAD hydrolysis (mean ± SEM) by cell fractions from wild-type MR-1. Cells were fractionated as in *Experimental procedures*, and equal amounts of protein from each fraction were added to solutions of FAD. The rate of hydrolysis was calculated from the slope of the initial linear increase in fluorescence intensity, as described in *Experimental procedures*. Triplicate samples were run from each fraction.

In order to further assess *S. oneidensis* UshA localization, cells were fractionated into periplasmic, cytoplasmic and total membrane fractions. After normalization of the bulk protein concentration, equal amounts of each fraction were added to FAD, and hydrolysis was monitored by fluorescence increase. The majority of UshA activity was found in the periplasmic fraction (Figure 3.4 B), consistent with the known periplasmic localization of the *E. coli* UshA homologue.

Previously, UshA has been detected in supernatants of *S. oneidensis* cultures, leading to speculation that UshA is secreted (Pinchuk et al., 2008). However, in the same study, cytosolic proteins were also detected in culture supernatants, suggesting that cell lysis may account for detection of UshA in the culture medium. In order to test whether UshA is secreted, we incubated supernatants of mid-log phase *S. oneidensis* MR-1 cultures with FAD in order to detect UshA activity (Figure S1). Although supernatants from wild-type MR-1 cultures slowly hydrolysed FAD, supernatants that had been passed through a 0.2 μm filter to remove cells remaining in the supernatant after centrifugation had no activity. From these data we infer that the UshA activity detected in culture supernatants is associated with intact cells and that UshA is not secreted from *S. oneidensis* at appreciable levels under the conditions tested.

The periplasmic hydrolysis of FAD by UshA explains the repeated failure by us and others to observe FAD accumulation in wild-type *Shewanella* cultures (Table 3.1) (Canstein et al., 2008; Marsili et al., 2008). The accumulation of FAD in Δ*ushA* culture supernatants, the ability of *S. oneidensis* UshA to hydrolyze FAD to FMN, and the periplasmic localization of UshA strongly suggest that FAD, rather than FMN or

riboflavin, is primarily secreted into the periplasm and that FMN is released into the culture medium via diffusion through OM pores.

UshA is required for growth of S. oneidensis *on mononucleotides*

Our results provide evidence that *S. oneidensis* UshA hydrolyses FAD, producing FMN. Based on known biochemistry of UshA, the AMP resulting from this reaction is likely to be hydrolysed immediately by UshA into adenosine and inorganic phosphate (Glaser et al., 1967). We tested whether *Shewanella* UshA performs this activity by taking advantage of the ability of *E. coli* to grow on AMP as a carbon source. It is known that in *E. coli* UshA is the only periplasmic enzyme capable of AMP hydrolysis (Kakehi et al., 2007), and therefore *E. coli ushA* mutants will not grow on AMP because the phosphate prohibits transport of this compound. We complemented an *E. coli*Δ*ushA* mutant with *S. oneidensis ushA* and saw that growth on AMP was restored (Figure S2), confirming that *S. oneidensis* UshA is capable of AMP hydrolysis. The ability of UshA to hydrolyze AMP suggests that *Shewanella* might be able to couple FAD hydrolysis to growth on AMP as a substrate, through UshA.

We first tested whether *S. oneidensis* can, like *E. coli*, grow with nucleotides as carbon sources. We measured aerobic growth of *S. oneidensis* cultures on AMP, GMP or CMP as the sole carbon source (Figure 3.5 A). Wild-type *S. oneidensis* is capable of growth on all three nucleotides, with growth on GMP and CMP slower than growth on AMP. This result contrasts with growth of *E. coli*, which is faster on GMP than on AMP (Kakehi et al., 2007), and is consistent with the ability of *S. oneidensis* to grow on DNA as sole carbon source (Pinchuk et al., 2008). The *S. oneidensis* mutant Δ*ushA* was unable

to grow using AMP, GMP or CMP (Figure 3.5 A), suggesting that UshA is the only periplasmic enzyme in *S. oneidensis* capable of hydrolyzing these nucleotides.

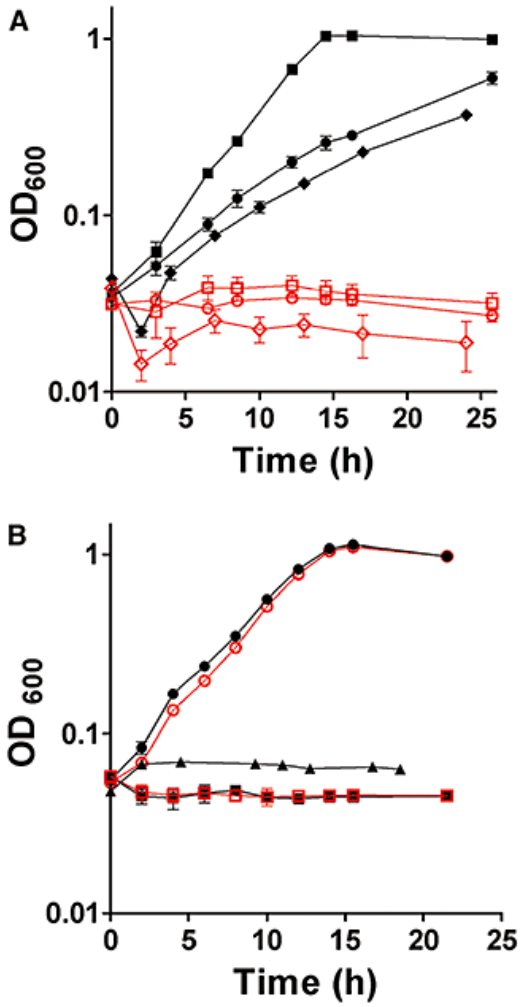

Figure 3.5

UshA is required for growth of *S. oneidensis* on AMP, GMP and CMP. A. Optical density of wild-type MR-1 (closed symbols) and Δ*ushA* (open symbols) grown aerobically in SBM with 10 mM of AMP (■), GMP (●) or CMP (◆) as sole carbon source. Mean ± SEM of triplicate cultures. B. Optical density of wild-type MR-1 (closed symbols) and Δ*ushA* (open symbols) grown aerobically in SBM with 10 mM of

adenosine (●), adenine (■) or ribose (▲) as carbon source. Mean ± SEM of triplicate cultures.

In *E. coli*, UshA hydrolyses AMP into adenosine and inorganic phosphate. Adenosine served as a carbon source for *S. oneidensis* (Figure 3.5 B), consistent with unpublished observations referenced in (Driscoll et al., 2007; Serres & Riley, 2006). Although unable to grow on AMP, Δ*ushA* mutants grew when provided with adenosine, confirming that adenosine utilization is downstream of UshA activity. However, neither they nor wild-type *S. oneidensis* were capable of growth on either adenine base (Serres & Riley, 2006) or ribose (Figure 3.5 B) as sole carbon source, leaving open the question of how adenosine is transported and utilized by *S. oneidensis*. Complemented Δ*ushA* mutants grew on AMP and adenosine, as expected (Figure S3). These results confirm that following hydrolysis of secreted FAD, the AMP moiety can be further hydrolysed and the adenosine base salvaged by *S. oneidensis*.

Processing of FAD by UshA not only makes adenosine available for reuse but also releases inorganic phosphate. We tested whether this phosphate could be used by *S. oneidensis* and in addition tested the proposal by Pinchuk and colleagues that UshA mediates the ability of *S. oneidensis* to use DNA as a phosphate source (Pinchuk et al., 2008). Wild-type and Δ*ushA* cultures were grown with DNA or AMP as the sole phosphate source. When tested, wild-type and Δ*ushA* cells grew equally well with DNA as phosphate source (Figure S4A). Δ*ushA* cultures were also capable of growth with AMP as a phosphate source, at a rate very similar to their growth with inorganic phosphate (Figure S4B). The ability of Δ*ushA* cultures to use AMP and DNA as a

phosphate source indicates that UshA is not required for use of nucleotides as phosphate sources by *S. oneidensis*. Other nucleotidases expressed under phosphate-limiting conditions may be sufficient to supply the phosphorus needs of Δ*ushA* cells (see *Discussion*).

Escherichia coli *UshA has poor FAD hydrolysis activity*

Escherichia coli UshA has been found to hydrolyze FAD with only approximately 1% of the activity of AMP hydrolysis (Alves-Pereira et al., 2008). We verified that *E. coli* UshA hydrolyses FAD slowly using our fluorescence-based hydrolysis assay by incubating cell extracts from wild-type and Δ*ushA E. coli* strains with FAD. No fluorescence increase was observed with either cell extract (Figure S5). We also expressed *E. coli* UshA in *S. oneidensis*Δ*ushA* cells. *E. coli ushA* was cloned into the vector pBBR1MCS-2 under control of a *lac* promoter, ensuring constitutive expression in *S. oneidensis*. In order to confirm UshA expression, mutant cells complemented with the *E. coli ushA* plasmid were grown with AMP as the sole carbon source (Figure 3.6 A). *E. coli ushA* was able to restore growth on AMP, indicating that it folds and is properly targeted to the periplasm when expressed in *S. oneidensis*. We next tested whether *E. coli ushA* is able to complement FAD hydrolysis by *S. oneidensis*Δ*ushA* mutants (Figure 3.6 B). As expected, Δ*ushA* cell extracts expressing an empty pBBR1MCS-2 vector did not hydrolyze FAD, as monitored by fluorescence increase. Complementation of Δ*ushA* mutants with a vector containing *S. oneidensis ushA* restored FAD hydrolysis at a rate significantly faster than that of wild-type *S. oneidensis* expressing endogenous levels of *ushA*. However, cell extracts of Δ*ushA* complemented with *E. coli ushA* hydrolysed FAD

at a non-negligible, but much slower, rate (Figure 3.6 B). Additionally, expression of *S. oneidensis* UshA in an *E. coli ushA* deletion strain resulted in robust FAD hydrolysis activity in cell extracts while wild-type K12 extracts showed no appreciable activity (Figure S6). Together, these results suggest that *S. oneidensis* UshA hydrolyses FAD more readily than does the *E. coli* homologue of UshA.

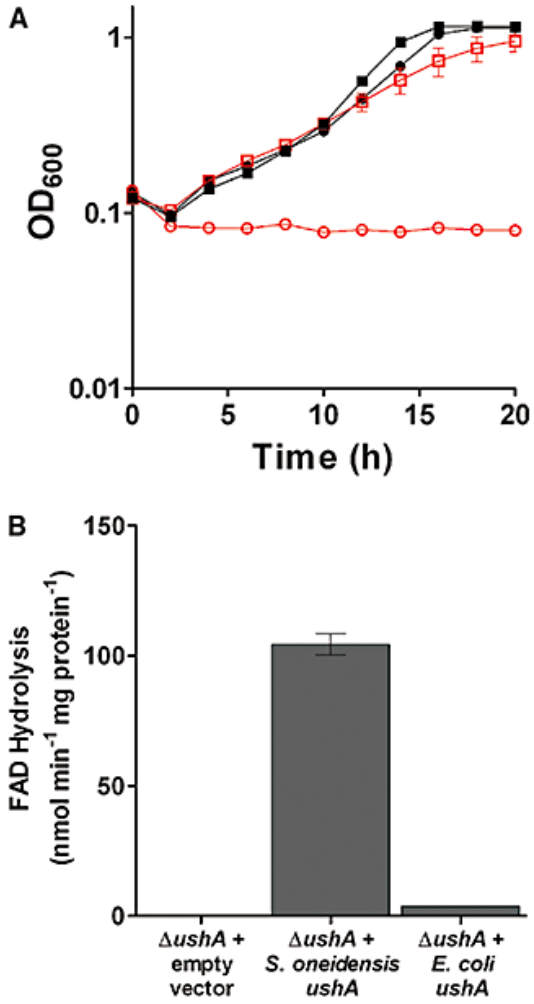

Figure 3.6

E. coli UshA enables growth of *S. oneidensis ushA* mutants on AMP but cannot restore FAD hydrolysis. A. Optical density of wild-type MR-1 with empty vector pBBR1MCS-2 (●), wild-type MR-1 complemented with *E. coli ushA* (■), *S. oneidensis*Δ*ushA* with

empty pBBR1MCS-2 (○) and *S. oneidensis*Δ*ushA* complemented with *E. coli ushA* (□), grown aerobically in SBM with 10 mM AMP as sole carbon source. Mean ± SEM of triplicate cultures. B. Mean rate (±SEM) of FAD hydrolysis by sonicated cell extracts of *S. oneidensis*Δ*ushA* expressing empty vector (left), *S. oneidensis ushA* (middle) or *E. coli ushA* (right). Triplicate samples; representative of two experiments.

Shewanella oneidensis *MR-1 can use FAD as an electron shuttle*

Previous studies have shown that *Shewanella* use riboflavin and FMN to mediate reduction of insoluble iron hydroxides and that supplementation of media with riboflavin or FMN enhances iron reduction rates (Canstein et al., 2008; Coursolle et al., 2010; Marsili et al., 2008). In one study, the ability of FAD to enhance iron reduction was also tested (Canstein et al., 2008); however, our results indicate that in wild-type cultures, extracellular FAD is quickly converted to FMN (Figure 3.3 A). Therefore, in order to test whether FAD itself, in the absence of conversion to FMN, can serve as an electron shuttle for extracellular iron reduction, we added exogenous flavins to both wild-type MR-1 and Δ*ushA* mutants cultured in minimal medium with Fe(III)-oxide. Fe(III) reduction was measured by monitoring the formation of Fe(II). In cultures of Δ*ushA* mutants, 10 μM exogenous riboflavin, FMN or FAD equally enhanced the rate of iron reduction compared with the rate when no flavins were added (Figure 3.7). When only endogenous levels of flavins were present, wild-type and Δ*ushA* cells reduced iron at identical rates (Figure 3.7), also indicating that the FAD secreted by Δ*ushA* cultures is capable of mediating iron reduction. Additionally, we measured rates of flavin reduction by Δ*ushA* cells and found that FAD is reduced by Δ*ushA* cells at a rate similar to riboflavin (data

not shown). From these results, we conclude that FAD can serve as an electron shuttle for the reduction of insoluble metals by *S. oneidensis*.

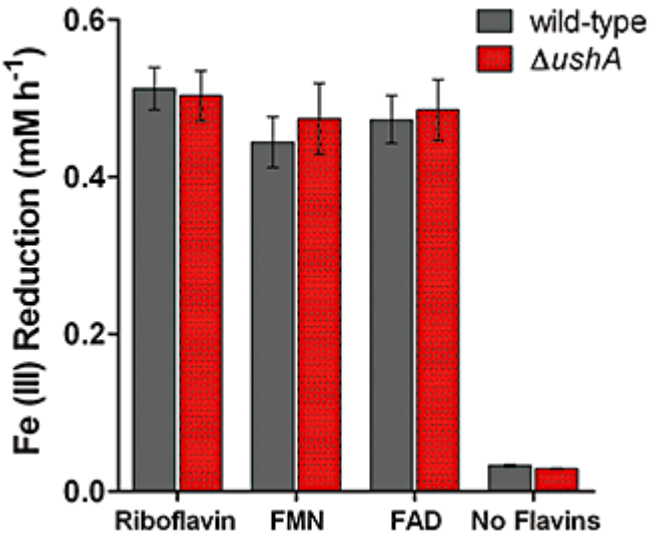

Figure 3.7

FAD enhances iron reduction by *S. oneidensis*. Mean rate (±SEM) of Fe(III) reduction by wild-type MR-1 (solid), and Δ*ushA* (patterned). Cells were incubated with 5 mM ferrihydrite, and reduction was monitored by measurement of Fe(II) production. The initial rate of iron reduction was measured by fitting the initial linear portion of the curve. Controls containing no cells did not reduce iron (data not shown).

3.4 Discussion

Although *Shewanella* rely on extracellular electron shuttling by flavin molecules to respire insoluble metal substrates, little is known about the mechanism of flavin secretion, release or processing. We have discovered that rather than directly secreting riboflavin and FMN as previously thought, *S. oneidensis* first transports the cofactor FAD into the periplasmic space, where the 5′-nucleotidase UshA hydrolyses it to FMN, adenosine and phosphate. Our current working hypothesis of *Shewanella* flavin

processing is outlined in Figure 3.8. FAD is synthesized from its riboflavin precursor in the cytoplasm and then exported across the inner membrane by an as yet unidentified mechanism. Secreted FAD may be incorporated into periplasmic proteins requiring an FAD cofactor, such as the flavocytochrome FccA. *Shewanella frigidimarina* strain NCIMB400 possesses a homologue of FccA called Fcc_3 and a second isoform called Ifc_3, both of which have been biochemically characterized and shown to contain a non-covalently bound FAD cofactor (Dobbin, Butt, Powell, Reid, & Richardson, 1999; Pealing et al., 1992), suggesting there are additional flavocytochromes made by *Shewanella* that are processed in a similar way to FccA. Excess FAD is hydrolysed by UshA into FMN and AMP. UshA further hydrolyses AMP into inorganic phosphate and the nucleoside adenosine, which may then be metabolized by the cell. FMN could then diffuse through OM pores into the extracellular space, where it begins a cycle of reduction by OM cytochromes and oxidation by the terminal electron acceptor. Conversion of FMN to riboflavin, also an effective electron shuttle (Canstein et al., 2008; Marsili et al., 2008), appears to be due to slow hydrolysis and is not dependent on UshA (data not shown).

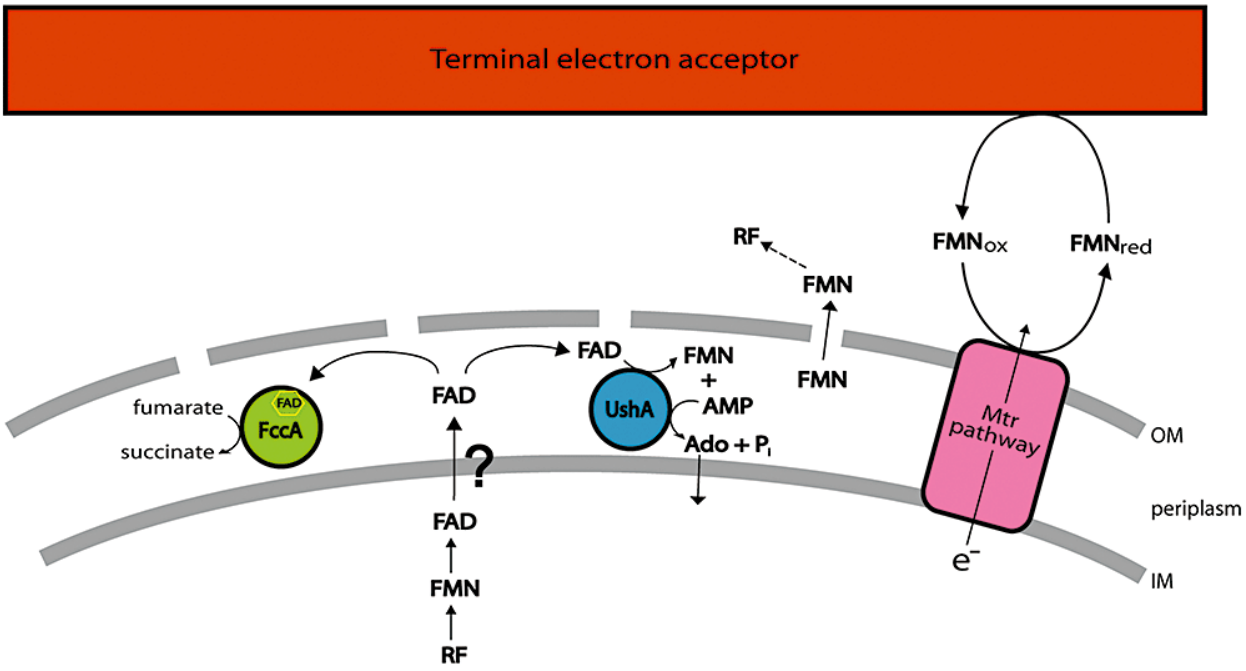

Figure 3.8

Working hypothesis/model for the role of UshA in periplasmic processing of flavin electron shuttles by *S. oneidensis*. Following synthesis in the cytoplasm, FAD is secreted across the inner membrane (IM) via an unknown mechanism into the periplasmic space, where it is incorporated into the periplasmic fumarate reductase, FccA. Excess FAD is hydrolysed by UshA into FMN and AMP. AMP is further hydrolysed by UshA into inorganic phosphate and adenosine (Ado), which may be recycled by the cell. FMN is free to diffuse through OM porins (shown as gaps in the OM) into the extracellular medium, where a fraction is spontaneously converted into riboflavin (RF). Flavins (both FMN and RF) can serve as electron shuttles for cycling of electrons between OM members of the Mtr pathway and an insoluble terminal electron acceptor.

In general, riboflavin and FMN have been the primary flavins detected in bacterial cultures (Canstein et al., 2008; Demain, 1972); when FAD has been detected it

has often been assumed to be evidence of lysis. Using an *ushA* deletion mutant, we have found that healthy, exponentially growing cultures of *S. oneidensis* primarily secrete FAD, previously undetected because UshA rapidly hydrolyses secreted FAD. Importantly, we were unable to detect any differences in membrane integrity between wild-type and *ushA* mutant cells, suggesting there is a specific secretion mechanism for FAD. It is consistent from a consideration of *Shewanella* physiology that secretion of FAD into the periplasm occurs under certain circumstances. Reduction of the widely available organic compound fumarate by *S. oneidensis* requires an unusual periplasmic tetraheme *c*-type cytochrome FccA that contains a non-covalently bound FAD cofactor (T. M. Maier et al., 2003). The maturation of *c*-type cytochromes in Gram-negative bacteria only occurs in the periplasmic space, where a linear polypeptide containing CxxCH motif(s) is processed by a suite of proteins (Thöny-Meyer, 2002). We hypothesize that apo-FccA is translocated from the cytoplasm into the periplasm and acquires its haem cofactors via *c*-type cytochrome maturation before acquiring its FAD cofactor. FccA binds FAD non-covalently (Dobbin et al., 1999; Pealing et al., 1992) and therefore must be folded to retain the cofactor. If our hypothesis regarding the order of cofactor acquisition is correct, FAD must be accessible to FccA in the periplasmic space.

In a contrasting example, the periplasmic methylmenoquinol : fumarate reductase complex of *Wolinella succinogenes* contains a protein, SdhA (locus tag WS1920), that contains a non-covalently bound FAD cofactor with a binding site similar to FccA from *Shewanella* (Juhnke, Hiltscher, Nasiri, Schwalbe, & Lancaster, 2009). SdhA was shown to be transported across the cytoplasmic membrane by the twin-arginine translocation pathway (TAT) (Juhnke et al., 2009). The TAT pathway translocates folded proteins

across the cytoplasmic membrane (Natale, Brüser, & Driessen, 2008) and is consistent with SdhA from *W. succinogenes* acquiring FAD as the protein is folded in the cytoplasm. Though SdhA appears to bind FAD in a manner similar to *Shewanella*, the *W. succinogenes* protein is not a *c*-type cytochrome, meaning it can fully mature in the cytoplasm before export.

Very little is known about excretion of flavins from bacteria. Although certain strains of riboflavin 'overproducers' have been isolated (Demain, 1972), research in bacteria has been focused on biosynthetic pathways and regulation rather than on transport mechanisms. Only two bacterial riboflavin transporters, RibU (Burgess et al., 2006; Kreneva et al., 2000) and RibM (Grill et al., 2007; Vitreschak et al., 2002; Vogl et al., 2007) have been confirmed. Thus far, these known flavin transporters have only been shown to be involved in uptake of flavins, not in their export. In fact, RibU binding assays have shown that it is not capable of transporting FAD (Duurkens, Tol, Geertsma, Permentier, & Slotboom, 2007). *S. oneidensis* possesses a putative transporter, *SO2713*, with approximately 25% sequence identity to RibM, but it is not known whether it is capable of transporting flavins. The *S. oneidensis* genome also includes a number of putative ABC transporters and multi-drug efflux proteins that could be involved in FAD secretion. Given that there are numerous candidate genes with potential for FAD transport, and given that no transporters were identified in this mutagenesis screen, it seems that *Shewanella* may have either multiple mechanisms for exporting FAD across the inner membrane, that the export mechanism shares functions essential to cell survival or that our screen was not saturated.

We have shown, both by monitoring fluorescence increases and by HPLC analyses, that *S. oneidensis* UshA hydrolyses FAD into the flavin derivative FMN. FAD has not previously been reported to be a major substrate for UshA (Alves-Pereira et al., 2008), and we have verified that FAD is not as rapidly hydrolysed by the *E. coli* homologue. The difference in substrate specificities suggests that *Shewanella* has adapted to high periplasmic concentrations of FAD. Consistent with the adaptation of *Shewanella* UshA to recognize FAD, *S. oneidensis* grows well on AMP, the nucleotide by-product of FAD hydrolysis. Wild-type *S. oneidensis* grows faster on AMP than on CMP or GMP, suggesting that it may be better adapted to growth on AMP. The constitutive export and hydrolysis of FAD by *Shewanella* ensure that AMP is constantly available for recycling.

The inorganic phosphate released by AMP hydrolysis is most likely reclaimed by the cell, just as is the adenosine nucleoside. AMP and DNA are capable of supporting growth of phosphate-starved cells, indicating that UshA-dependent hydrolysis of nucleotides could supply the phosphorus needs of the cell; however, UshA is not required. Δ*ushA* mutants grew just as rapidly with equimolar AMP or NaH_2PO_4 as sole phosphate source. We detected only approximately 10–30 μM free inorganic phosphate in AMP and DNA stock solutions (data not shown), well below the concentration at which phosphate is limiting (Pinchuk et al., 2008), leaving nucleotide hydrolysis as the only source for phosphate in our cultures. Under the conditions of our experiment, cells that have been starved for phosphate may upregulate an alternative nucleotidase or phosphatase capable of liberating phosphate from nucleotides.

The overall benefits of secreting a small amount of FAD to satisfy the enzymatic requirement of FccA (and possibly other enzymes) in the periplasm, coupled with the

release of FMN and RF, which act as accelerants for extracellular electron transfer to insoluble substrates, together must outweigh the energetic investment costs incurred, at least under laboratory conditions. It is critical to note that the concentration of flavins quantified in culture supernatants of *Shewanella* strains is very low, on the order of approximately 250 nM in minimal medium and approximately 1 μM in rich medium (Canstein et al., 2008; Coursolle et al., 2010; Marsili et al., 2008), and that this small amount is sufficient to dramatically accelerate electron transfer to insoluble electrode surfaces (Baron et al., 2009) and iron oxide minerals (Ross, Brantley, & Tien, 2009). It has been calculated that *Shewanella* growing on electrodes can produce ATP at a rate approximately 1000-fold faster than the rate of ATP consumption for riboflavin biosynthesis to concentrations of 250 nM (Marsili et al., 2008). Conversion of riboflavin to FAD requires two additional ATP molecules (Kearney, Goldenberg, Lipsick, & Perl, 1979) in addition to any energetic cost that may be associated with FAD export, but the total energetic investment remains quite low compared with the benefit gained. Additionally, we have found that *S. oneidensis* can grow on the adenosine resulting from UshA hydrolysis of FAD. By recycling a portion of the FAD molecule, *S. oneidensis* may therefore reduce the metabolic burden of its release.

Shewanella hold great promise for biotechnological applications. In order to optimize metal reduction in technologies employing *Shewanella* species, it will be critical to understand which electron shuttles are being produced, the mechanisms by which they are produced, and the ecological advantages of each. In this article we have described a previously unknown role for the nucleotidase UshA in processing of flavin electron shuttles by *Shewanella,* and we provide the first insight into the molecular mechanism of

shuttle production. Our results show that FMN and riboflavin are not the primary flavins secreted from *Shewanella* cells; rather, FAD is first secreted. Major questions remain to be answered, including the identity of the FAD secretion/release pathway(s) and whether FAD secretion is a common feature among bacteria known to accumulate FMN and/or RF in culture supernatants. Answering these questions will constitute a major advance in our understanding of the mechanism of electron shuttle production by *Shewanella*.

3.5 Experimental procedures

Strains and growth conditions

Wild-type *S. oneidensis* strain MR-1 has been described (C. R. Myers & Nealson, 1988). The mutant *ushA E. coli* strain JW0469 from the Keio Collection has been described (Baba et al., 2006). A complete list of strains and plasmids used in this study can be found in Table S1. Single colonies freshly streaked from a frozen stock were inoculated into 2 ml of LB medium containing the appropriate antibiotic and grown aerobically for 16 h before washing followed by inoculation into minimal medium. Anaerobic cultures were flushed with nitrogen gas for 10 min following inoculation. *Shewanella* strains were cultured at 30°C with shaking in SBM minimal medium consisting of (per liter) 0.225 g K_2HPO_4, 0.225 g KH_2PO_4, 0.46 g NaCl, 0.225 g $(NH_4)_2SO_4$, 0.117 g $MgSO_4$–$7H_2O$, and 10 mM (aerobic) or 100 mM (anaerobic) HEPES, adjusted to pH 7.2. In addition, 5 ml l^{-1} of vitamins excluding riboflavin (Balch et al., 1979), 5 ml l^{-1} trace minerals (Marsili et al., 2008) and 0.01% casamino acids (Difco) were added. Carbon source and electron acceptors, when applicable, were added as

indicated. *E. coli* strains were cultured at 37°C with shaking in M9 minimal medium (Sambrook & Russell, 2001) with the indicated carbon source.

For phosphate starvation experiments, cultures were first starved for 24 h in phosphate-free M1 medium (Pinchuk et al., 2008) supplemented with 10 ml l^{-1} vitamins and 10 ml l^{-1} trace minerals as above. A 30 mM lactate was added as a carbon source. Following depletion of phosphate reserves, cells were inoculated into M1 medium containing either 300 μg ml^{-1} filter-sterilized DNA, 1 mM filter-sterilized AMP or 1 mM NaH_2PO_4 as sole phosphate source. Cultures were grown at 30°C with shaking. Abiotic, no-carbon and no-phosphate controls were cultured alongside the samples.

Transposon mutagenesis

Transposon mutants were made by mating *S. oneidensis* MR-1 with *E. coli* WM3064 containing Tn*pho*A′-1 (Wilmes-Riesenberg & Wanner, 1992). Exconjugants were selected under aerobic conditions on LB plates containing 50 μg ml^{-1} kanamycin and were inoculated into 96-well plates containing LB with 50 μg ml^{-1} kanamycin. After overnight growth at room temperature, fluorescence was recorded at 440 nm excitation, 525 nm emission in a Molecular Devices SpectraMax M2 reader. Cultures exhibiting fluorescence intensity below that of wild-type cultures were re-inoculated into fresh medium and screened twice more. Mutants continuing to fluoresce at levels less than wild-type were analyzed with arbitrary polymerase chain reaction (PCR) and sequenced to determine the site of transposon insertion.

Deletion and complementation

*Shewanella oneidensis*Δ*ushA* (strain JG1079) was prepared following a described protocol (Saltikov & Newman, 2003). Briefly, regions flanking *S. oneidensis ushA* were amplified using the following primers:

UF SpeI: GGACTAGTCATGGGTTAGGCGATTCT,

UR EcoRI: GGGAATTCAGTCAGCACTGCAGTT,

DF EcoRI: ccGAATTCCCAGTGGGTGACATTGTG,

DR SacI: aaGAGCTCTGACAGACTTGCGGCTAA,

and were ligated into the suicide vector pSMV3 (Saltikov & Newman, 2003). After mating of the pSMV3 vector into *S. oneidensis* MR-1, recombination was confirmed by PCR and by sequencing. Cells were plated on LB supplemented with 5% sucrose to select for loss of *sacB* on the mutagenesis vector, and deletion of *ushA* was confirmed by PCR. Complementation was performed by PCR amplification of *S. oneidensis ushA* (ORF *SO2001*) using the following primers:

SOushA1 NNNTCTAGACCGATAAAACCATCATG, and

SOushA2 NNGGGCCCCTGTACTAGTCAGTATCT.

Products were purified, digested and ligated into the vectors pBBR1MCS-2 and pBBR1MCS-3 (Kovach et al., 1995). Similar results were obtained using either complementation vector. Inclusion of a 350 bp region upstream of *ushA* and a reversed orientation in the vector ensured that expression was under control of the endogenous promoter.

*Escherichia coli*Δ*ushA* (strain JG1145) was prepared by transformation of the JW0469 *ushA* mutant (Baba et al., 2006) with the temperature-sensitive plasmid pCP20

(Cherepanov & Wackernagel, 1995) encoding a FLP recombinase to excise the kanamycin-resistance cassette. Recombinants were verified by sensitivity to kanamycin and by PCR. pCP20 was removed by overnight passage at 43°C, leaving an unmarked *ushA* deletion. The *E. coli ushA* complement was prepared by PCR amplification of *E. coli ushA* (locus tag *b0480*) and the 20 bp region immediately upstream of the start codon using the following primers:

ECushA1 GGTACCATCAGGTCAGGGAGAGAAGT and

ECushA2 GAGCTCTTACTGCCAGCTCACCTCA.

E. coli ushA was ligated into the vector pBBR1MCS-2 in an orientation ensuring expression under the vector-encoded *lac* promoter, which is constitutively active in *S. oneidensis*.

HPLC

High-performance liquid chromatography was performed as follows using a 4.6 mm × 150 mm Eclipse XDB-C18 column with a 5 μm particle size (Agilent Technologies). Twenty-five microliters of sample was injected onto an LC-10AT liquid chromatograph (Shimadzu) equipped with an SIL-10AF autoinjector. The mobile phase consisted of 20% methanol, 1% glacial acetic acid in water at a flow rate of 1 ml min^{-1}. The column was maintained at 30°C. Flavins were detected with an RF-10AXL fluorescence detector (Shimadzu) at an excitation wavelength of 440 nm and an emission wavelength of 525 nm. Riboflavin (Fisher), FMN and FAD (Sigma) standards were prepared in SBM at concentrations ranging from 0.125 to 10 μM. At a 1 ml min^{-1} flow rate, FAD eluted at approximately 6 min, FMN 9 min and riboflavin 15 min. Flavin

concentrations were calculated by comparing the integrated area of each peak to the area of standard peaks.

Flavin fluorescence measurements

Five hundred microliter samples of anaerobic cultures were removed at the indicated times and centrifuged to remove cells. Supernatants were transferred to fresh Eppendorf tubes and frozen at −20°C until analyzed. For fluorescence measurements, 300 µl of each supernatant sample was transferred to a clear 96-well plate and read in a Molecular Devices SpectraMax M2 plate reader at 440 nm excitation, 525 nm emission.

FAD hydrolysis and phosphate assays

Phosphate concentration was assayed using a Colorimetric Phosphate Assay kit (Abcam) in a 96-well plate format, according to the manufacturer's instructions. For FAD hydrolysis assays, 10 ml overnight cultures of *S. oneidensis* or *E. coli* in LB were washed once with SBM and resuspended in 500 µl SBM. Before sonication, 30 µl were removed, diluted in SBM and set on ice to be used as intact cells. The remainder of each culture was sonicated in an ice-water bath with 20 pulses of approximately 2 s each using a Sonic Dismembrator Model 60 (Fisher) set at power level 10. Sonicated samples were centrifuged at 10 000 r.p.m., 3 min, and supernatants were stored on ice until use. Protein concentrations in each cell extract were measured with a bicinchoninic acid (BCA) protein assay kit (Pierce) according to the manufacturer's instructions. Cell extracts were diluted in SBM to the same final protein concentration before being aliquotted into 96-well plates alongside intact cell samples. Plates were then pre-warmed to 30°C before

addition of warmed FAD to a final concentration of 80 μM. Fluorescence acquisition was begun immediately and at 5 min intervals at 30°C. Plates were shaken for 5 s before each fluorescence acquisition. The rate of FAD hydrolysis was determined by fitting the initial linear portion of each curve and assuming that 1 mol FMN was produced per mol FAD consumed. Based on measurements of FMN standards, 1 nmol FMN was assumed to be equivalent to 770 RFU. Following the final time point, samples were heated at 95–100°C for 10 min to stop enzyme activity before storage at −20°C. Samples were thawed and diluted 1:10 before analysis by HPLC. Standards were heated to verify that heating of samples did not alter flavin concentrations.

Subcellular fractionation

Periplasmic, cytoplasmic and total membrane fractions were separated by the method of Kaufmann and Lovley (Kaufmann & Lovley, 2001). Following separation, total protein concentrations in each fraction were measured with a BCA assay. Fractions were stored overnight at 4°C before use in FAD and AMP hydrolysis assays.

Live/dead staining

Mid-log phase cultures of anaerobically grown *Shewanella* were stained with a BacLight Live/Dead Stain (Invitrogen), according to the manufacturer's instructions. Fluorescence of triplicate samples of stained cells was measured in a 96-well plate fluorescence reader (Molecular Devices). Fluorescence emission was obtained in 10 nm increments from 500 to 700 nm at 470 nm excitation. The green to red fluorescence ratio

was calculated from the integrated fluorescence intensities between 510–540 nm (green) and 620–650 nm (red).

Iron and flavin reduction assays

Overnight cultures of *S. oneidensis* in LB were washed once with SBM, and then resuspended in SBM to an OD of 1.3. Thirty microliters of each cell suspension was added to 270 µl SBM containing 20 mM lactate, vitamins, minerals, 5 mM ferrihydrite (iron oxide), and 10 µM riboflavin, FMN or FAD in 96-well plates. Plates were incubated at room temperature in a GasPak System anaerobic Petri dish holder that was flushed with nitrogen gas for 15 min between each time point. At each time point, 50 µl of 5M hydrochloric acid (HCl) was added to stabilize Fe(II). Thirty microliters was taken from this well and diluted 1:10 into 0.5 M HCl to yield concentrations within the range of standard curves. Fifty microliters of the diluted sample was mixed with 300 µl ferrozine reagent (Stookey, 1970), consisting of (per liter) 2 g ferrozine, 23.8 g HEPES, pH 7.0, and the absorbance was read at 562 nm. Standard curves were made as described (Coursolle et al., 2010). Flavin reduction assays were performed in 96-well plates, and reduction was monitored through the decrease of flavin fluorescence over time, as described (Coursolle et al., 2010).

3.6 Acknowledgements

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assistance with the mutagenesis screen, Dan Coursolle for measuring the reduction rate of FAD, Adam Spaulding for assistance in constructing the initial *ushA* complementation vector and Kristopher Hunt for measurements of inorganic phosphate.

3.7 Supporting Information

Table 3.2 Strains and plasmids used in this study.

Strain	Description	Source
S. oneidensis		
MR-1 (JG274)	wild-type MR-1	(C. R. Myers & Nealson, 1988)
JG1079	Δ*ushA*	this work
JG521	MR-1 + pBBR1MCS-3	this work
JG1097	Δ*ushA* + pBBR1MCS-3	this work
JG1093	MR-1 + pSO2001MCS-3	this work
JG1096	Δ*ushA* + pSO2001	this work
JG168	MR-1 + pBBR1MCS-2	(Hau *et al*., 2008)
JG1175	Δ*ushA* + pBBR1MCS-2	this work
JG1173	MR-1 + pSO2001MCS-2	this work
JG1174	Δ*ushA* + pSO2001MCS-2	this work
JG1237	MR-1 + pb0480MCS-2	this work
JG1236	Δ*ushA* + pb0480MCS-2	this work
E. coli		
UQ950	DH5α λ*pir*	(Saltikov & Newman, 2003)
WM3064	Donor strain for conjugation	(Saltikov & Newman, 2003)
MG1655	K12 wildtype strain	lab stock
JW0469 (JG1137)	*ushA* (b0480)::Km	(Baba et al., 2006)
JG1145	Δ*ushA* (b0480)	this work
JG1188	MG1655 + pBBR1MCS-2	this work
JG1189	JG1145 + pBBR1MCS-2	this work
JG1179	MG1655 + pSO2001MCS-2	this work
JG1180	JG1145 + pSO2001MCS-2	this work
JG1245	JG1145 + pb0480MCS-2	this work

Plasmid	Description	Source
pSMV3:ushAdel	Deletion vector for SO2001	this work
pSO2001MCS-3	SO2001 (*S. oneidensis ushA*) vector, Tc	this work
pSO2001MCS-2	SO2001 (*S. oneidensis ushA*) vector, Km	this work
pb0480MCS-2	b0480 (*E. coli ushA*) vector, Km	this work
pBBR1MCS-3	Broad host range vector, Tc	(Kovach et al., 1995)
pBBR1MCS-2	Broad host range vector, Km	(Kovach et al., 1995)
pCP20	Flp recombinase vector, Ap	(Datsenko & Wanner, 2000)

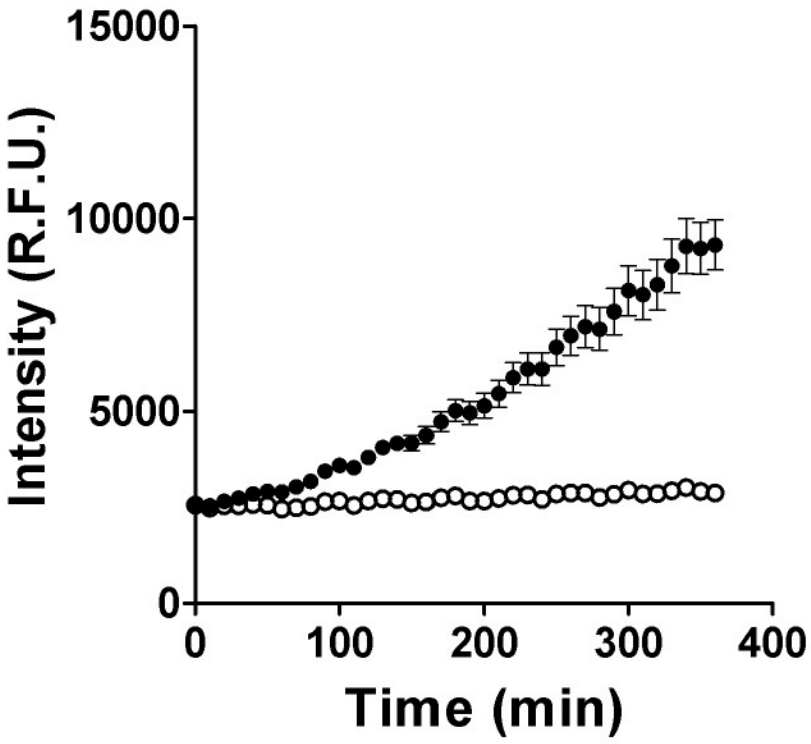

Figure 3.9

Filtered *S. oneidensis* culture supernatants hydrolyze FAD poorly. Supernatants from wild-type *S. oneidensis* cultures grown to mid-log phase in anaerobic SBM were added to solutions of FAD. Mean fluorescence intensity (± SEM) of triplicate samples of unfiltered supernatants (●) or supernatants passed through a 0.2-μm filter (○) is plotted versus time.

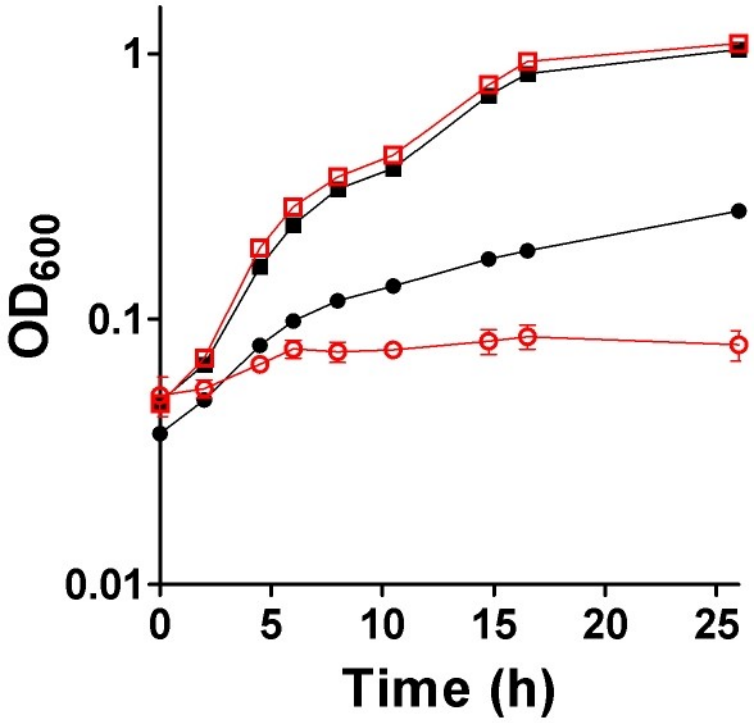

Figure 3.10

S. oneidensis ushA complements growth of *E. coli* Δ*ushA* mutants on AMP. Optical density of wild-type *E. coli* with empty vector pBBR1MCS-2 (●), wild-type *E. coli* complemented with *S. oneidensis ushA* (■), *E. coli* Δ*ushA* with empty pBBR1MCS-2 (○) and *E. coli* Δ*ushA* complemented with *S. oneidensis ushA* (□), grown aerobically in M9 medium with 10 mM AMP as sole carbon source. Mean ± SEM of triplicate cultures.

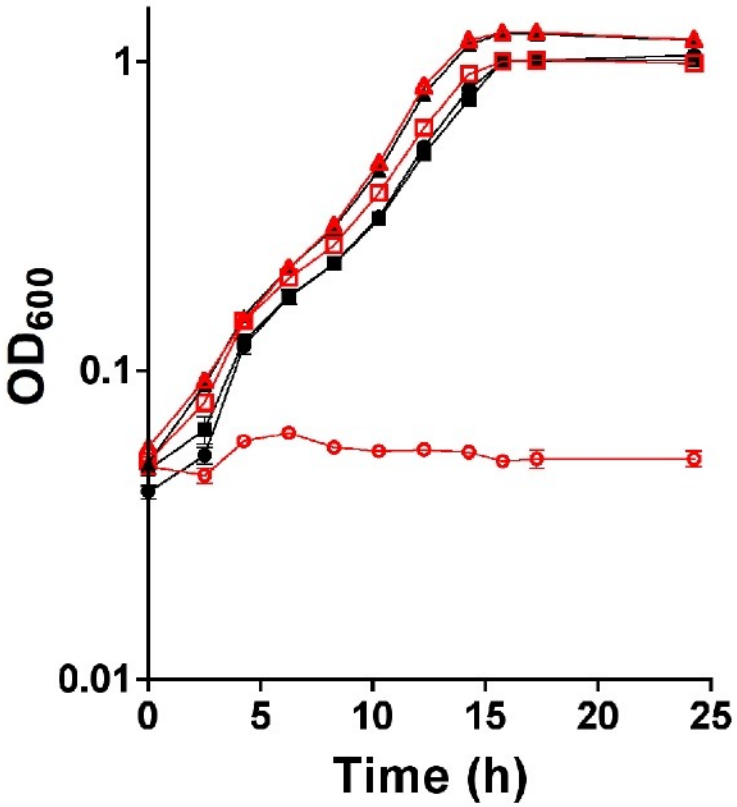

Figure 3.11

Complementation of *ushA* growth defect using AMP as sole source of carbon and energy. Optical density of wild-type MR-1 with empty vector (●) and with the *ushA* complementation vector (■) grown with AMP. Corresponding *ushA* mutant strains have the same labels but are in red and are empty circles or squares, respectively. As positive controls, MR-1 (□) and Δ*ushA* (□, red) were grown with adenosine (Ado) as the sole carbon and energy source. Mean ± SEM of triplicate cultures.

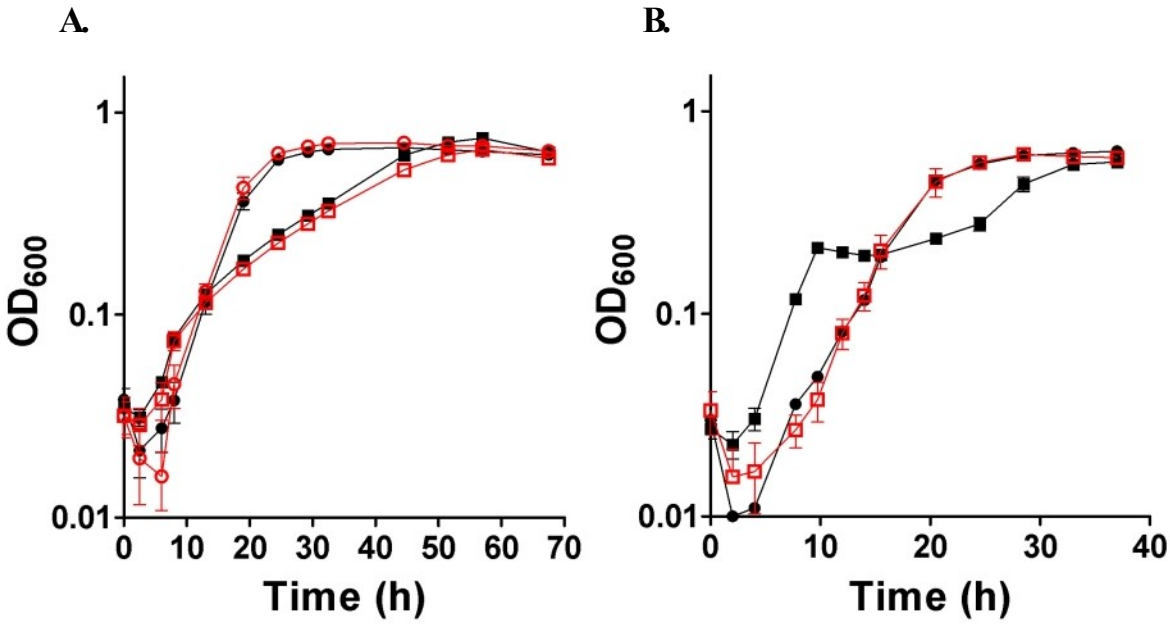

Figure 3.12

UshA is not required for growth of *S. oneidensis* on AMP or DNA as sole phosphate source. (A) Optical density of wild-type MR-1 (black, solid symbols) and Δ*ushA* (red, open symbols) grown aerobically in M1 minimal medium with 1 mM DNA (■) or 1 mM NaH_2PO_4 (●) as sole phosphate source. (B) Optical density of wild-type MR-1 (black, solid symbols) and Δ*ushA* (red, open symbols) grown aerobically in M1 minimal medium with 1 mM AMP (■) or 1 mM NaH_2PO_4 (●) as sole phosphate source. Mean ± SEM of triplicate cultures.

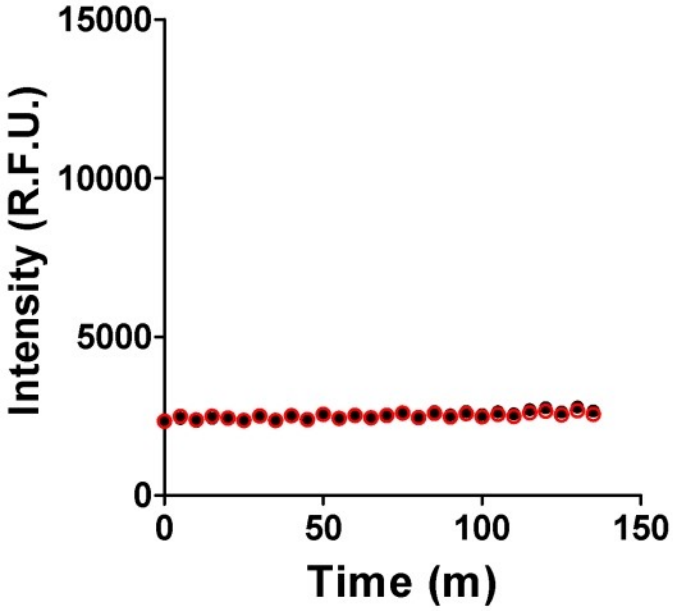

Figure 3.13

E. coli ushA does not efficiently hydrolyze FAD. (A) Sonicated cell extracts (0.18 mg/ml total protein) of wild-type *E. coli* (●) and Δ*ushA* (○) were added to solutions of FAD. Mean fluorescence intensity (± SEM) is plotted versus time as FAD is hydrolyzed to FMN. Data were obtained from triplicate cultures.

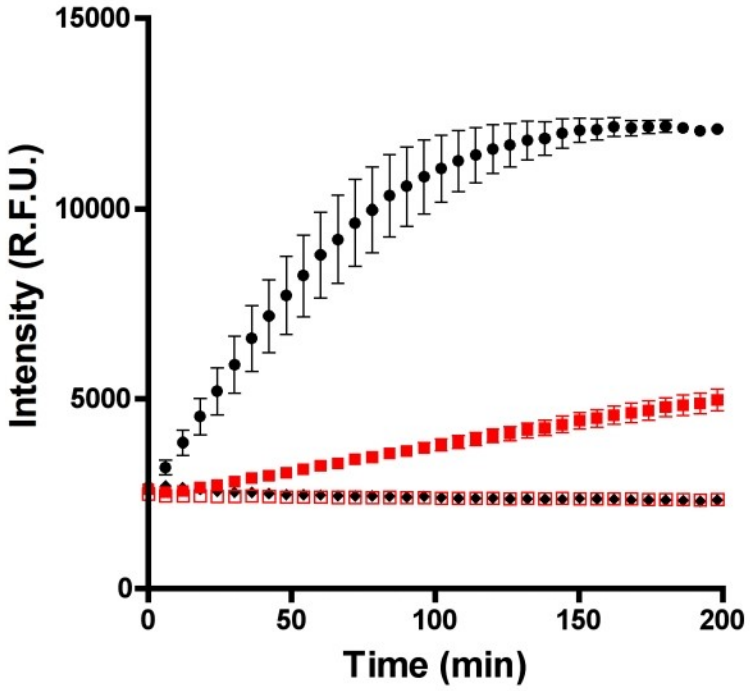

Figure 3.14

Expression of *S. oneidensis ushA* in *E. coli* confers FAD hydrolysis activity. Whole cell activity assays were performed with wild-type *E. coli* K12 (♦), Δ*ushA E. coli* (□) and the Δ*ushA E. coli* carrying complementation vectors for either *S. oneidensis ushA* (□) or *E. coli ushA* (□). Mean fluorescence intensity (± SEM) is plotted versus time as FAD is hydrolyzed to FMN. Data were obtained from triplicate cultures.

Chapter 4: The Flavin Co-Factors of FccA and UrdA Facilitate Turnover during Growth with Poor Electron Donors in *Shewanella oneidensis*

4.1 Introduction

Shewanella oneidensis strain MR-1 (MR-1) is a nonfermentative, facultative anaerobic bacterium. Under anaerobic conditions, MR-1 synthesizes numerous *c*-type cytochromes and flavoproteins to couple metabolic electrons to the reduction of electron acceptors in the periplasm or outside of the cell. FccA and UrdA are both periplasmic, flavin-containing proteins that allow for respiration of fumarate and urocanate, respectively.

FccA is the most abundant *c*-type cytochrome in the periplasm under anaerobic conditions, and is the sole periplasmic fumarate reductase capable of reducing extracellular fumarate (Maier et al., 2003; Morris et al., 1994; Pealing et al., 1992). FccA is capable of receiving electrons from its physiological donor, CymA, or electrons from an artificial electron donor, methyl viologen. In many organisms, fumarate is used as an intracellular terminal electron acceptor (Cole, Condon, Lemire, & Weiner, 1985). For example, in *Escherichia coli*, the fumarate reductase is bound to the inner-membrane, interacts with quinones, consists of four subunits, contains both covalently-bound FAD and iron sulfur clusters, and is reversible (Dickie & Weiner, 1979; Morningstar, Johnson, Cecchini, Ackresll, & Kearney, 1985; Walker & Singer, 1970; Westenberg, Gunsalus, Ackrell, Sices, & Cecchini, 1993). Unlike the fumarate reductase in *E. coli*, FccA from *Shewanella* is periplasmic, monomeric, four heme *c*-type cytochrome containing noncovalently-bound FAD, and is irreversible (Leys et al., 1999; Maier et al., 2003; C. R. Myers & Myers, 1992; Pealing et al., 1992). Since FccA is a periplasmic cytochrome, the apoenzyme is exported into the periplasm where *c*-type cytochrome maturation factors fold and incorporate heme groups into the protein (Verissimo & Daldal, 2014). It is

unclear when the FAD co-factor is incorporated in FccA, but Bfe exports FAD from the cytoplasm to the periplasm (Kotloski & Gralnick, 2013). Excess FAD is cleaved by the periplasmic UshA protein and the resulting adenosine monophosphate is recycled by the cell (Covington et al., 2010).

In addition to functioning as a fumarate reductase, FccA may have a role in providing a buffer for excess electrons. During extracellular respiration in MR-1, electrons flow from an oxidoreductase to the quinone pool and to CymA. Once at CymA, electrons flow to FccA in the case of fumarate respiration or to the Mtr pathway in the case of direct metal or flavin-mediated respiration. However, electrons are also able to flow between MtrA and FccA in both extracellular electron transfer and during reverse electron transfer (Ross, Flynn, Baron, Gralnick, & Bond, 2011; Schuetz, Schicklberger, Kuermann, Spormann, & Gescher, 2009).

Initially misidentified as a fumarate reductase based on homology, UrdA is also a periplasmic protein (Bogachev, Bertsova, Bloch, & Verkhovsky, 2012). Like FccA, UrdA contains a noncovalently-bound FAD co-factor, but also contains a covalently-bound FMN co-factor (Bogachev et al., 2012; Mattevi et al., 1999; Punta et al., 2012; Yeats, Bentley, & Bateman, 2003). UrdA is the sole protein responsible for urocanate respiration by coupling metabolic electrons to the reduction of urocanate to make deamino-histidine (Bogachev et al., 2012). Based on structural similarities between UrdA and FccA, Bogachev et al. proposes that the FMN co-factor of UrdA accepts electrons from CymA and the FAD co-factor is involved in the active site. Urocanate was first isolated from dog urine, but histidine found in peptides could be a natural and abundant source of urocanate. When MR-1 is grown anaerobically in liquid culture using lactate as

an electron donor and urocanate as an electron acceptor, there is an extended lag phase, because MR-1 requires both anaerobic conditions and the presence of urocanate to induce *urdA* expression (Bogachev et al., 2012).

The presence of UshA in the periplasm of MR-1 poses the question of why FAD is exported from the cell only to be cleaved into FMN and AMP. If FMN was exported, there would be no need for UshA. Moreover, FAD is more metabolically expensive to produce, and both FAD and FMN can function as electron shuttles (Abbas & Sibirny, 2011; Canstein et al., 2008; Velasquez-Orta et al., 2010). The existence of FAD co-factors in FccA and UrdA suggest either that the FAD co-factor is associated with the protein in the cytoplasm or the FAD is exported from the cell and associates with the proteins in the periplasm. If flavin co-factors associate with proteins in the periplasm, a *Shewanella* mutant unable to export FAD should be deficient in these functions. Using a *Shewanella* Δ*bfe* strain, the importance of flavin co-factors in FccA and UrdA was evaluated.

4.2 Results and Discussion

Growth on lactate and fumarate

It was predicted that a strain unable to export FAD to the periplasm would be deficient in fumarate reduction via FccA. To test this prediction, *Shewanella* strains were grown anaerobically in minimal medium using 20 mM lactate as an electron donor and 40 mM fumarate as an electron acceptor (Figure 4.1). Strains unable to export flavins (Δ*bfe*) or cleave FAD (Δ*ushA*) grow at a similar rate to MR-1. Strains complemented with *bfe* on the vector grow slightly slower than uncomplemented strains conceivably due

to increased FAD export or deficiencies from overproducing Bfe (data not shown). The observation that Δ*bfe* strains grow at a comparable rate to MR-1 could mean that FccA can reduce fumarate to succinate without an FAD co-factor or there is sufficient extracellular FAD from cell lysis to fulfill the requirement. It is unlikely that the amount of extracellular FAD from cell lysis is sufficient to fulfill the FccA requirement, because UshA quickly cleaves excess FAD (Covington et al., 2010). It is also unknown what percent of FccA proteins need to have an FAD co-factor to allow for wild-type growth on fumarate.

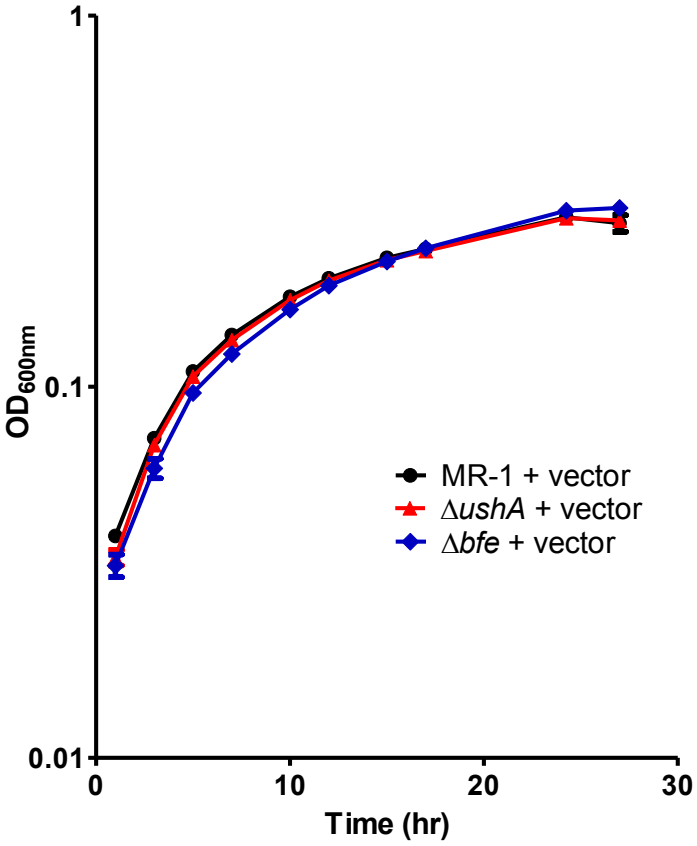

Figure 4.1

Flavin export from Bfe is not required for growth lactate and fumarate. Δ*bfe* and Δ*ushA* strains grow at a similar rate as MR-1 anaerobically in SBM with 20 mM lactate and 40 mM fumarate.

Methyl viologen assay with fumarate

To more directly assess the functionality of FccA, a whole cell methyl viologen assay was performed. In the assay, dithionite is used to chemically reduce methyl viologen. When reduced, methyl viologen changes from a colorless to a blue solution. Reduced methyl viologen is able to transfer electrons to FccA thru hemes or FAD co-factor. If fumarate is present, FccA can transfer electrons received from methyl viologen

to produce succinate. The now oxidized methyl viologen becomes colorless and the rate of enzyme turnover can be monitored by the disappearance of color using a spectrophotometer.

In the absence of exogenous FAD, only MR-1 and Δ*ushA* strains are able to oxidize methyl viologen and reduce fumarate causing a disappearance of the blue color (data not shown). Δ*bfe* strains, including the double mutant (Δ*ushA*, Δ*bfe*), are unable to oxidize methyl viologen without exogenous flavins and rates are equivalent to the Δ*fccA* negative control (data not shown). When 1 μM FAD is added to the assay, MR-1, Δ*ushA*, and Δ*ushA*-Δ*bfe* strains are now able to oxidize methyl viologen (Figure 4.2). The Δ*bfe* strain is still unable to oxidize methyl viologen, presumably due to the activity of UshA cleaving FAD into FMN and AMP leaving FccA without an FAD co-factor (Figure 4.2). To further demonstrate that FAD is required for oxidation of methyl viologen activity, 1 μM of FMN or B_2 was added to a Δ*ushA*-Δ*bfe* strain. No activity was detected under these conditions.

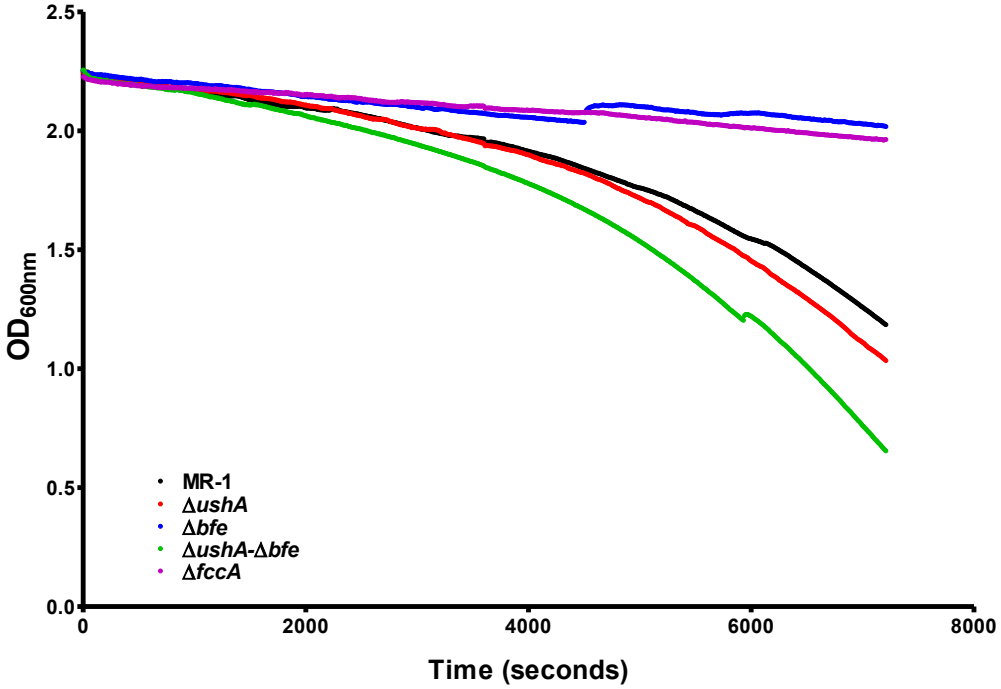

Figure 4.2

Methyl viologen reduction assay with *Shewanella* strains in SBM with 3.125 mM fumarate and 1 μM FAD. MR-1 (black), Δ*ushA* (red), and Δ*ushA*-Δ*bfe* (green) strains are able to reduce fumarate and when provided extracellular FAD. Δ*bfe* (blue) and Δ*fccA* (purple) are unable to be supplemented with exogenous FAD.

With regards to FccA activity, there is a discrepancy between the data in figure 4.1 and figure 4.2. Based on the data in figure 4.1, it appears that FccA functions without its FAD co-factor in Δ*bfe* strains. However, the Δ*bfe* strain in figure 4.2 is unable to reduce fumarate suggesting that FccA is unable to function without an FAD co-factor. It is likely the methyl viologen oxidation assay with fumarate works in a non-physiological way, and it is unknown which heme or whether the FAD co-factor is reduced by methyl viologen. The FAD co-factor in FccA may be required to accept electrons from methyl

viologen. In this scenario, the heme groups of FccA are completely bypassed and oxidation of methyl viologen and subsequent reduction of fumarate to succinate would only occur when FccA has a FAD co-factor. Conversely in liquid cultures, the electrons would come from CymA and be transferred along heme groups in FccA. When at the terminal heme group in FccA, the electrons could transfer to fumarate whether or not an FAD co-factor is present.

In summary, FccA seems to function physiologically without its FAD co-factor. It is difficult to determine an oxidation rate in the methyl viologen assay, because of the dithionite used to reduce methyl viologen. The dithionite could be removed from the assay and the methyl viologen could be electrochemically reduced (Thorneley, 1974). Another possibility would be to purify FccA, confirm the absence of an FAD co-factor, and repeat the methyl viologen assay with and without exogenous FAD. If purified FccA cannot be supplemented with FAD, then there might be another protein or mechanism involved in adding the co-factor to FccA.

Growth on lactate and urocanate

UrdA is a periplasmic protein that contains both a covalently bound FMN and a non-covalently bound FAD. A *Shewanella* strain unable to export FAD into the periplasm, or a strain unable to convert FAD into FMN, might be deficient when grown anaerobically on lactate and urocanate. To test this, *Shewanella* strains were grown anaerobically in minimal medium using 10 mM lactate as an electron donor and 20 mM urocanate as an electron acceptor (Figure 4.3). Δ*bfe* (blue) and Δ*ushA*-Δ*bfe* (green) strains were unable to grow in these conditions. Whereas, MR-1 (black) and Δ*ushA* (red)

strains grew at similar rates. If UrdA requires both FAD and FMN co-factors, then it would be expected that a Δ*ushA* strain would be unable to grow due to lack of FMN. When exogenous flavins (1 µM FMN, 1 µM FAD, or 1 µM FMN and 1 µM FAD) were added to the liquid cultures, Δ*bfe* and Δ*ushA*-Δ*bfe* strains were still unable to be grown (data not shown).

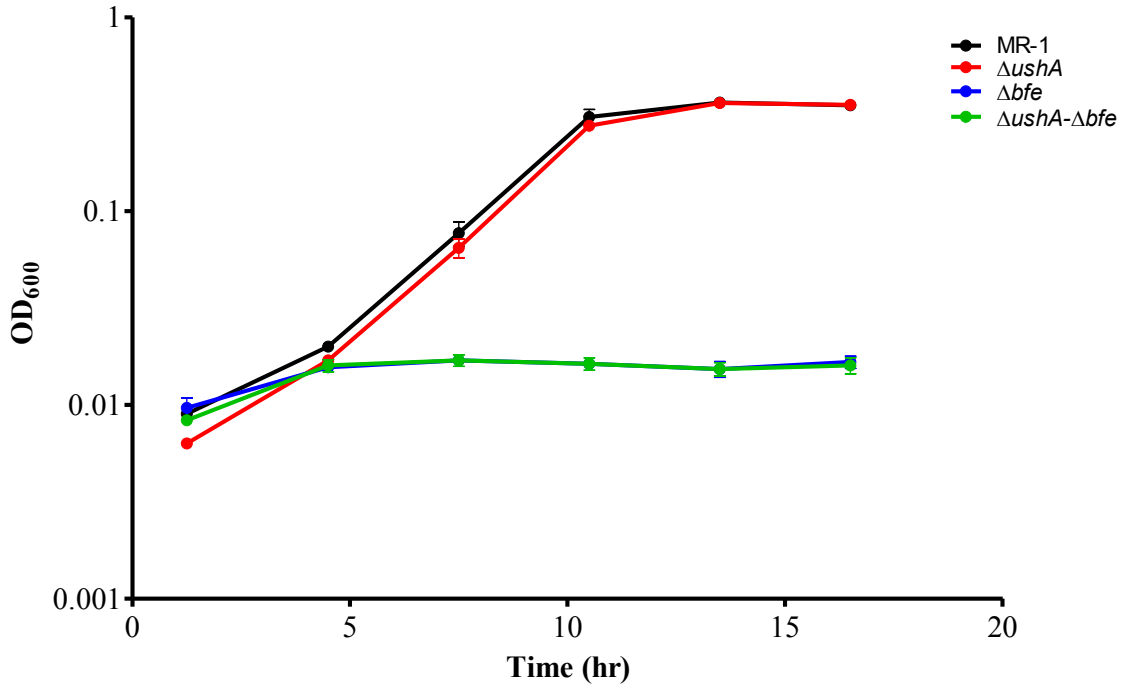

Figure 4.3

Flavin export from Bfe is required for growth with lactate and urocanate. Δ*bfe* and Δ*ushA*-Δ*bfe* strains are unable to grow anaerobically in SBM with 10 mM lactate and 20 mM urocanate.

A number of possible explanations exist for the inability to supplement Δ*bfe* and Δ*ushA*-Δ*bfe* strains. UrdA may require another protein to incorporate the FMN co-factor into the protein like the flavinator, SdhE, in the succinate dehydrogenase complex

(McNeil, Hampton, Hards, Watson, & Cook, 2014). Additionally, *urdA* requires both anaerobic conditions and urocanate to be expressed (Bogachev et al., 2012). It is possible *urdA* is being repressed in Δ*bfe* strains due to the lack of the Bfe protein itself or a sub-stimulatory concentration of exogenous flavins. Using a 10 μM concentration of FAD and FMN or expressing *urdA* on a plasmid without native regulation could cause activation.

Growth on N-*acetylglucosamine and fumarate*

To attempt to find a condition where FccA in a Δ*bfe* background is deficient, *N*-acetylglucosamine (NAG), a different electron donor, was used in anaerobic SBM cultures with fumarate as the terminal electron acceptor. Much like the result with lactate and fumarate, mutants unable to export flavins grew at a similar rate to MR-1 (Figure 4.4).

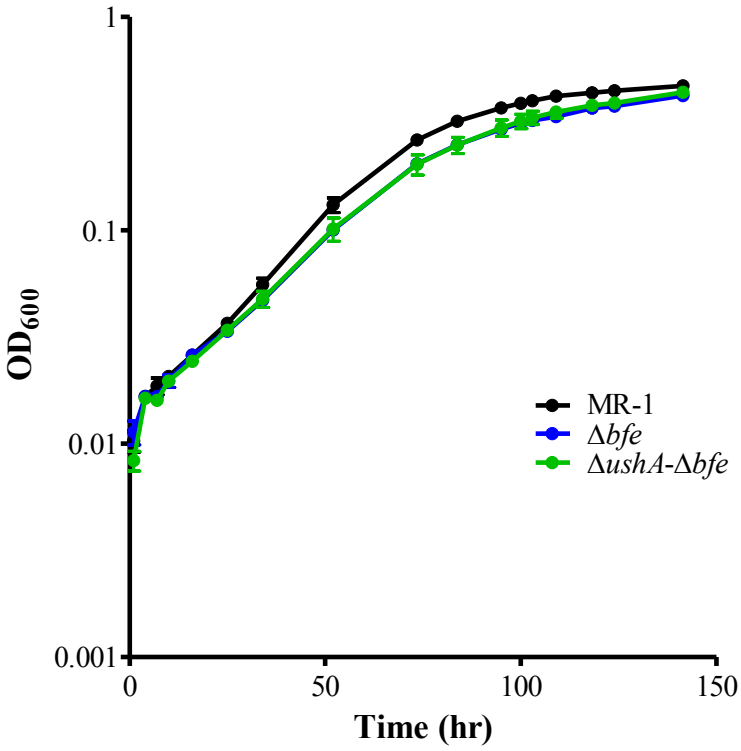

Figure 4.4

Flavin export from Bfe is not required for growth on an alternative electron donor, NAG. Δ*bfe* strains grow at a similar rate as MR-1 in SBM with NAG 10 mM and fumarate 60 mM.

The aim of this research was to identify deficiencies that exist in Δ*bfe* backgrounds, besides the absence of flavin electron shuttles. Two periplasmic proteins, FccA and UrdA, require flavin co-factors that should be lacking in Δ*bfe* strains. UrdA function appears to be deficient in Δ*bfe* backgrounds, but FccA seems to function normally in growth curves and abnormally in methyl viologen experiments. The existence of these two proteins could explain why FAD specifically is exported into the periplasm and then cleaved into FMN.

4.3 Materials and Methods

Bacterial strains and growth conditions

MR-1 was described by Myers and Nealson (C. R. Myers & Nealson, 1988). A frozen freezer stock was struck for isolation on a Luria-Bertani (LB) agar plate and after incubation, a single colony was inoculated into 2ml of LB medium and incubated aerobically while shaking at 230 r.p.m. for 16 hours at 30°C. For experiments with minimal medium, *Shewanella* basal medium (SBM) consisting of 0.225g K_2HPO_4, 0.225g KH_2PO_4, 0.46g NaCl, 0.225g $(NH_4)_2SO_4$, 0.117g $MgSO_4$-$7H_2O$, and 10mM HEPES adjusted to pH 7.2 per liter of double distilled water was used. One liter of SBM was supplemented with 5ml of vitamins excluding riboflavin (Balch et al., 1979), 5ml of trace minerals (Marsili et al., 2008), and 0.05% casamino acids (Difco). Other substances were added as indicated. When required, anaerobic cultures were made by flushing nitrogen through Balch tubes sealed with butyl rubber stoppers for 15 minutes.

Methyl viologen assay

Shewanella strains were struck from a -80°C freezer stock onto LB agar plates. Plates were incubated for approximately 16 hours at 30°C. After colonies appear on plates, a single colony is transferred to 2 ml of liquid LB and shaken at 230 r.p.m. for 16 hours. Next, 20 µl of the LB cultures was transferred to aerobic 2 ml of SBM with 20 mM lactate and incubated for 16 hours and 30°C. Then, 50µl of the SBM culture was transferred to 5 ml of SBM culture with 20 mM lactate. Cells were centrifuged down at 8000 x g for 3 minutes and washed once with SBM. After washing, cells were

resuspended in SBM to an optical density of 0.3. To each well of a 96 well plate 80 µl of 1 mM methyl viologen, 30 µl of 10 mM sodium dithionite, 50 µl of 100 mM HEPES, 60 µl of 0.3 OD cells, and the reaction was started by adding 100 µl of 10 mM sodium fumarate.

Chapter 5: Electrosynthesis in *Shewanella*: Production of Reducing Equivalents and ATP

5.1 Introduction

In the last few decades the field of microbial electrochemistry has rapidly grown. This field started off as a scientific curiosity and now encompasses the use of microbial electrochemical systems to generate energy, balance redox fermentations, remediate the environment, and fix carbon dioxide (Clauwaert et al., 2007; Flynn, Ross, Hunt, Bond, & Gralnick, 2010; Gregory & Lovley, 2005; Nevin, Woodard, Franks, Summers, & Lovley, 2010; Rabaey & Verstraete, 2005). There are almost as many acronyms for microbial electrochemical systems as there are applications. For a list of 47 acronyms and applications see the review by Wang and Ren (Wang & Ren, 2013). One of the reasons this field has grown quickly and includes a diverse range of applications is due to the work of both engineers and microbiologists. One of the organisms that has been influential in the development of microbial electrochemical systems is *Shewanella oneidensis* strain MR-1.

MR-1 has the ability to move electrons to external electron acceptors via direct contact and flavin electron shuttles. The ability to move electrons from inside the cell to an external electron acceptor creates the possibility for production of electricity and other biotechnological applications. Moreover, electrons can be moved from an external electron source into a cell and alter cellular metabolism to create preferred compounds or pathways in a process termed electrosynthesis. The electron conduit in *Shewanella* is functionally reversible; meaning cells are able to attach to the electrode and donate electrons or the electrode can donate electrons to the cell. However, the capability of *Shewanella* to produce NADH or ATP from proton motive force from electrons donated from an electrode has never been demonstrated. If cellular NADH or ATP can be

produced using electricity, then it will be a critical step in producing high value compounds by artificially driving the metabolism of *Shewanella*.

A practical way to study electrosynthesis in living cells is to use three-electrode bioreactors (Figure 5.1). The bioreactors have working, counter, and reference electrodes. The bioreactors are made anaerobic via a gas-in valve and exhaust gas leaves through the gas-out. Additionally the gas-out serves as a port to either add or remove substances with a syringe. Bioreactors are stirred using a magnetic stir bar and a glass frit allows for ion exchange between the medium and the reference electrode while minimizing liquid transfer. The electrode in these bioreactors is able to be poised at a range of potentials and measures electrons entering or leaving the electrode in real-time.

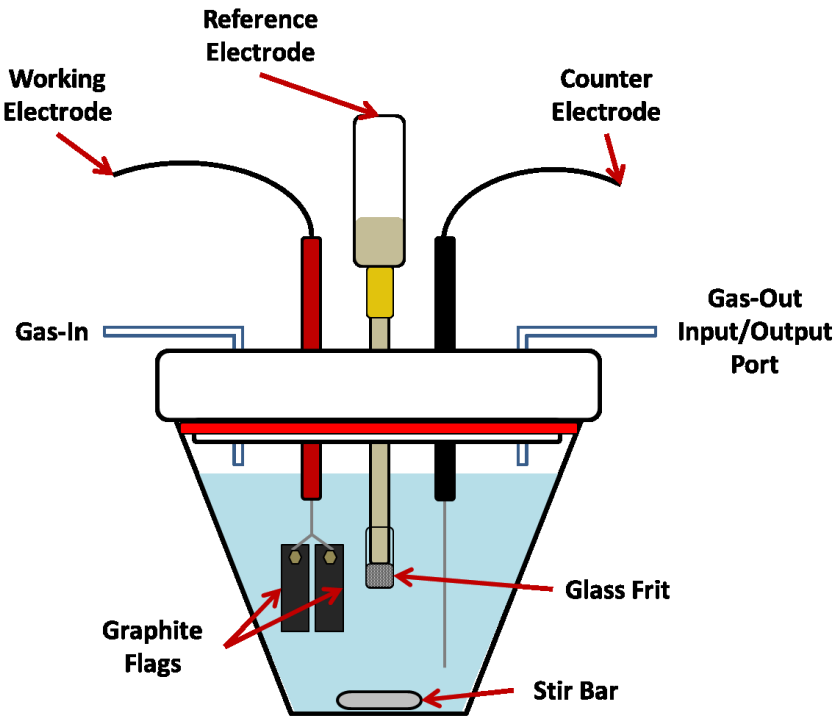

Figure 5.1

The standard set up for three-electrode bioreactors with two graphite flags. The two graphite flags provide a surface for cells to respire and can be removed independently to provide duplicate test conditions within the same bioreactor.

5.2 Results and Discussion

General bioreactor setup

Numerous iterations of bioreactor setups were used in an attempt to produce ATP in *Shewanella* cultures. Bioreactors were inoculated at time zero with a *Shewanella* culture. During the first phase the bioreactor headspace was degassed with argon gas. The electron donor was lactate and the electron acceptors were graphite flags and a limiting amount of fumarate. The limiting amount of fumarate provides a way for cells to respire without attaching to the graphite flags and may help by providing energy for transcription

and translation of genes needed to utilize the graphite flags. During the second phase the bioreactors are washed by swapping the glass bioreactor cone with a sterile cone containing minimal medium. Electrodes in the bioreactor are held in place by the bioreactor top. In this phase, the point is to remove unused lactate and deplete energy sources. There is no added electron donor in this phase, and the graphite flags are still poised to accept electrons. During the third and final phase, the bioreactors are washed again by the same method used previously, the headspace gas is changed to atmospheric gas, and the potential of the graphite flags are changed from an electron acceptor to an electron donor. In dual graphite flag setups, one flag was harvested at the start of the final phase and the second flag was harvested at the end of the final phase. There is a negative current in phase three, because electrons are flowing from the graphite flag and ultimately to oxygen. The phases are outlined in figure 5.2. Unfortunately, there was no positive correlation between current and ATP or NADH production. These experiments resulted in new and better methods to build and inoculate bioreactors.

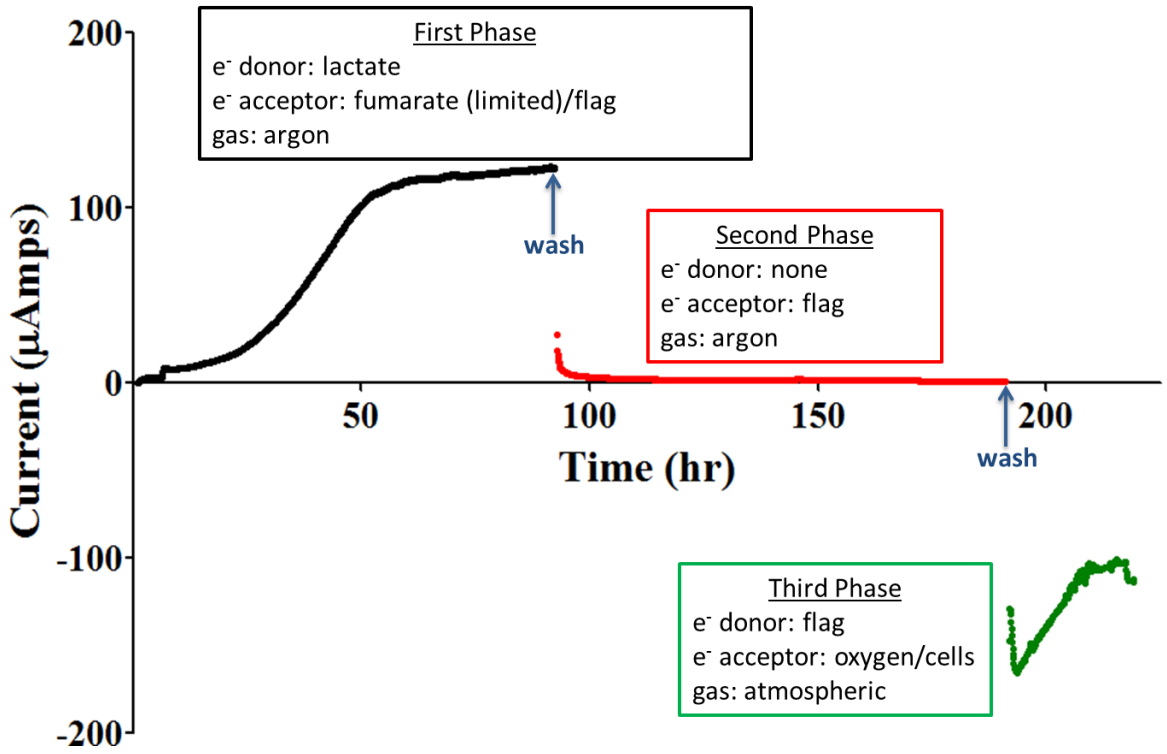

Figure 5.2

Description of the three phases of a typical electrosynthesis experiment.

Cultures grown in bioreactors versus slurry method

When optimizing the bioreactor setup, two methods of inoculation were examined trying to maximize the number of cells on the graphite flag and turnaround time between experiments. In method one, the bioreactors were inoculated with 1 mL of a 0.5 OD culture in SBM. The medium inside the reactor contained 15 mL of SBM with 60 mM lactate and 40 mM fumarate. After inoculation, cultures grew in bioreactors and current increased as demonstrated in the first phase of figure 5.2. After approximately 50 hours, current plateaued indicating maximum biomass on the graphite flag and equilibrium between flavin export and breakdown.

The second method of inoculation is referred to the slurry method. In this method, *Shewanella* is grown aerobically in 400 mL of LB for 16 hours in a 30°C shaking incubator. Even though the culture is shaking, oxygen is limiting due to the gas-liquid mass transfer rates (Maier & Büchs, 2001). After 16 hours, shaking is stopped for 3 hours to create partial anaerobic conditions to induce proteins and pathways for extracellular respiration such as the Mtr pathway. Next, the culture is centrifuged, washed with SBM, and resuspended in 15 mL of SBM. All 15 mL of washed culture (OD > 10) is added to an empty bioreactor.

There are multiple advantages/disadvantages associated with the two reactor setup methods. The advantage of the first method is that the bioreactors produce on average 100-150 µamps per bioreactor compared to 10-15 µamps per bioreactor in the slurry method. The first method is also much easier to wash planktonic cells from the reactors. The slurry inoculated reactors still contain large numbers of cells after washing. The disadvantage of the first method is that it takes approximately 2-3 days from inoculation to reach a stable current plateau. The slurry method reaches a stable current plateau after 1-2 hours. In the slurry method, the culture used to inoculate the bioreactors is grown aerobically. The aerobic growth allows the testing of strains that are unable to grow under anaerobic conditions. For example, a Δ*cymA* or a Δ *fccA* mutant cannot grow under anaerobic SBM with lactate and fumarate.

Length of phase two

Length of phase two or the amount of time cells in a bioreactor were on a poised electrode without an exogenous electron donor was examined. The reasoning for phase

two was to deplete the electron donor remaining in the medium or stored within the cell. Once residual electron donor was depleted, intracellular ATP concentrations should decrease. When ATP concentrations are at a minimal level, phase three will begin. In phase three, ATP will possibly be produced using a cathode and oxygen as a terminal electron acceptor. The reason for depleting ATP prior to phase three is to be able to demonstrate the greatest difference between strains making ATP from using a cathode and strains not using a cathode. A period length of 1-3 days was assessed along with studying under anaerobic, aerobic conditions, or in the presence of 100 μM carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), an inhibitor of oxidative phosphorylation. CCCP uncouples the proton gradient across the inner-membrane by acting as a protonophore. While the use of CCCP or ΔATPase *Shewanella* strains did lower ATP concentrations, none of the variations with the length of phase two had an appreciable effect on biomass or ATP concentrations when measured at the end of phase three.

Amount of time on cathode before harvesting

The length of phase three was also examined. The time ranged from 15 minutes to 24 hours between the harvesting of the first graphite flag and the second. The purpose for phase three was to change the potential of the graphite flag from an electron acceptor to an electron donor to make a proton gradient in the cells. Electrons should flow from the graphite flag, to the Mtr pathway in *Shewanella* cells, CymA, quinone pool, through the electron transport chain, and finally to oxygen. During this process, protons might be translocated from the inner-membrane to the periplasm. Additionally, two protons will be consumed at the terminal oxidase when oxygen is reduced to water. The proton gradient

generated from this process would be used to generate ATP through ATP synthase. The difference of ATP concentrations would be measured at the beginning when the first electrode is harvested and at the end of phase three when the second electrode is harvested.

The results of all experiments in these bioreactors determined that there were higher concentrations of ATP per unit of protein on the first electrode than the second electrode. In other words, a cathodic graphite flag was a hostile environment causing a cellular decrease of intracellular ATP, and a longer time between the harvesting of the first and second graphite flag meant a lower ATP concentration. To demonstrate the negative effects on MR-1 of a cathodic graphite flag, two bioreactors were assembled. Both reactors were exposed to atmospheric oxygen and filled with 15 mL of sterile SBM containing 20 mM lactate and 40 mM fumarate. One reactor was poised at -360 mV (vs. SHE) and the other reactor was poised at 0 mV for 16 hours. After 16 hours, both reactors were inoculated with MR-1 from an overnight culture and incubated at 30°C with stirring. In these conditions, *Shewanella* cells do not need to interact or respire the electrode in order to grow. However, in the bioreactor poised at -360 mV, no turbidity was observed and in the 0 mV bioreactor visible growth occurred (Figure 5.3). This experiment establishes that a graphite flag poised at -360 mV in the presence of oxygen produces toxic compounds, likely reactive oxygen species.

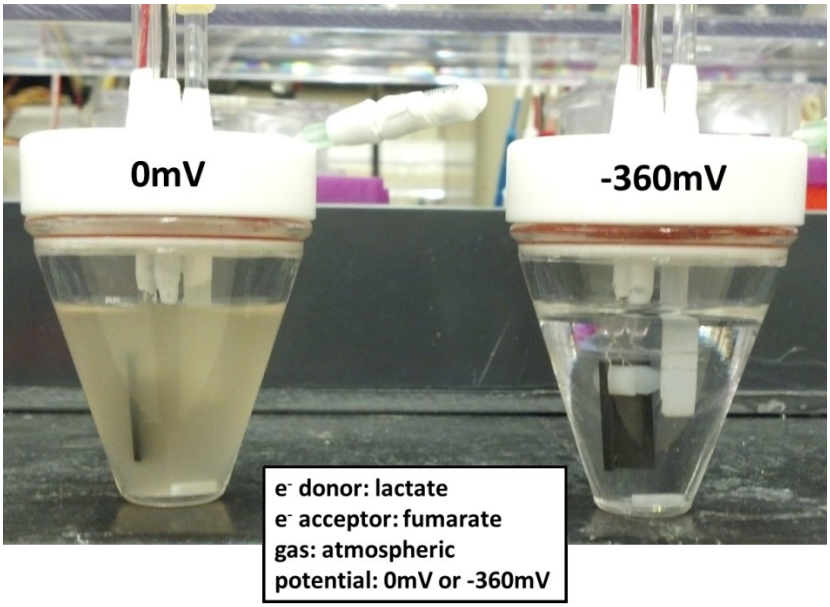

Figure 5.3

An electrode poised at -360 mV in a bioreactor is not permissible to *Shewanella* growth. Whereas an electrode poised at 0 mV allows for growth.

Even though a cathodic graphite flag is a harsh environment for *Shewanella*, there are ways to mitigate the effects. An increase in the amount of cells bound to the graphite flag would reduce the surface available for the abiotic reaction of oxygen with the graphite flag. This could be accomplished by making mutations in *Shewanella* or overexpressing the catalase gene. If oxygen is the problem, a different terminal electron acceptor could be substituted. However, the use of oxygen would be ideal for biotechnology applications. Increasing the electrode potential will alleviate the negative effects of oxygen and a surface other than graphite might also help. Otherwise, a porous ceramic cup can quench the reactive compounds before harming cells (Li et al., 2012). Besides varying bioreactor conditions or the strain, changes could be made to how the amount of ATP is assessed. It is possible to build a bioreactor inside a luminometer and

express luciferase inside the cells. This method would provide real-time measurements of ATP without having to destroy the cells using trichloroacetic acid.

5.3 Materials and Methods

Three-electrode bioreactors

Bioreactors were assembled as described by Marsili *et al*. One milliliter from an aerobic SBM culture with vitamins, minerals, 0.01% casamino acids, and 20 mM DL-lactate was added to 9 ml of anaerobic SBM with vitamins, minerals, 0.05% casamino acids, 50 mM DL-lactate, and 40 mM fumarate (Marsili et al., 2008). Cultures were grown at 30°C and shook at 200 r.p.m. until an OD of 0.4 was reached. The entire culture was added to the bioreactor. Bioreactors were continuously flushed with nitrogen gas and electrodes were poised at a potential of +0.240 V versus standard hydrogen electrode using a 16-channel VMP potentiostat (Bio-Logic SA).

Chapter 6: Conclusions and Future Directions

6.1 Conclusions

The research presented in this thesis demonstrates the importance of flavins in the physiology of *Shewanella oneidensis* strain MR-1. The observation that *Shewanella* species export and utilize FMN and B_2 for extracellular respiration of insoluble electron acceptors has been known since 2008. However, it was not clear that only FAD was exported from the cytoplasm until a transposon screen was performed in *Shewanella* to identify the flavin exporting protein. The initial transposon screen did not identify a flavin transporter, but instead, identified a protein responsible for processing exported FAD, UshA. After UshA was characterized, it was realized that FAD is processed after being exported, and a second transposon screen was performed in a Δ*ushA* background. The second transposon screen identified the FAD transporter, Bfe. The identification and subsequent deletion of the gene allowed for the first construction of a *Shewanella* strain unable to export flavin electron shuttles. The creation of a Δ*bfe* strain allowed the measurement of extracellular respiration of both soluble and insoluble electron acceptors without flavin electron shuttles. The Δ*bfe* strain was critical for determining the contribution of flavin electron shuttles to rates of extracellular respiration.

The identification of an FAD specific transporter and a periplasmic FAD processing protein led to an interesting question about why FAD is exported only to be cleaved into FMN by the nucleotidase. There are a number of proteins in the periplasm of MR-1 that contain an FAD co-factor, FMN co-factor, or both. Initially, it was suspected that these proteins would be non-functional in a Δ*bfe* background. However, growth conditions requiring the fumarate reductase, FccA, appear to be unaffected by the presence or absence of an FAD co-factor. On the other hand, growth conditions requiring

urocanate reductase, UrdA, appear to require the Bfe transporter and cannot be supplemented with exogenous FAD and FMN. The seemingly contradicting results of growth experiments with FccA and UrdA can offer significant insight into *Shewanella* physiology.

In the process of elucidating *Shewanella* physiology, three-electrode bioreactors will be essential. These bioreactors are able to measure respiration rates in real-time by counting electrons. The number of electrons measured by the potentiostat can be compared to HPLC measurements of metabolite concentrations in the bioreactor. These compared measurements can yield information regarding where metabolic electrons are coming from and in the case of electrosynthesis, where electrons are going.

The monitoring of electrons and charge will be especially important for future work on electrosynthesis. On the journey to making ATP and reducing equivalents, it might be more advantageous to take smaller steps and confirm each step is occurring as expected by using various *Shewanella* mutants deficient in one or more proteins required for extracellular respiration.

6.2 Future Directions

A common criticism against the flavin shuttling model is that flavins are metabolically expensive to produce and export. In the environment, other organisms could consume the flavins exported by *Shewanella* which would deplete the amount available for electron shuttling. Moreover, to respire an insoluble electron acceptor, the amount of flavins exported by a cell would need to exponentially increase with distance between the outer-membrane and the electron acceptor.

To address this criticism of the flavin shuttling model, it is important to experimentally determine the cost of producing and exporting flavins. The metabolic cost of flavin export could be determined using MR-1 and Δ*bfe* strains grown under various conditions. In aerobic conditions, extracellular flavins should not be advantageous for growth and it would be predicted that the Δ*bfe* strain would outgrow MR-1. From previous experiments using a spectrophotometer measuring optical density, there was no difference in growth between MR-1 and Δ*bfe*. Additionally, there might only be a slight growth advantage in aerobic conditions which would be undetectable by measuring optical density. In order to increase sensitivity, a fluorescent protein could be cloned into one of the strains for detection in flow cytometry. The converse experiment would also need to be performed to determine any fitness cost of expressing the fluorescent protein. The two strains would be mixed at a 1:1 ratio at the start of the experiment and grown to late exponential or stationary phase. At that point, the culture would be transferred to fresh medium and grown to late exponential or stationary phase again. After a series of transfers, the ratio of strains would be measured using flow cytometry. If there is a slight growth advantage, it would be clear after a series of transfers.

Alternatively, the Δ*bfe* strain may have a growth defect when grown anaerobically using fumarate as an electron acceptor. In a Δ*bfe* strain, the periplasmic fumarate reductase lacks its FAD co-factor. Without its co-factor, the fumarate reductase may turnover slower. Again, a competition between MR-1 and Δ*bfe* could be used to determine a growth defect. The flavins exported by MR-1 could potentially complement the Δ*bfe* strain, but the periplasmic UshA in the Δ*bfe* strain should quickly convert FAD into FMN which is unusable by FccA.

The growth competition experiments in an aerobic environment would help determine the metabolic cost of exporting flavins under conditions in which flavins are not used for respiration. Competition experiments using fumarate as an electron acceptor would provide a reason why flavins are exported when a soluble electron acceptor is present. Under anaerobic conditions with a soluble electron acceptor, flavins would be used as co-factors instead of electron shuttles.

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