Flavin Electron Shuttles Dominate Extracellular Electron Transfer by *Shewanella oneidensis*

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ABSTRACT ***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.**

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*.**

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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 (1). 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 (2). 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) (3, 4). While both organisms utilize a variety of multiheme *c*-type cytochromes, only *Shewanella* is able to respire insoluble substrates without direct contact (5, 6). Both organisms are proposed to produce conductive "nanowires" that may facilitate respiration of insoluble substrates (7, 8); 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 (9, 10), including multiple forms of Fe(III) oxide (11), and facilitate sensing of redox gradients (12). Secreted flavins are reduced by the Mtr respiratory pathway in MR-1 (13), and the crystal structure of

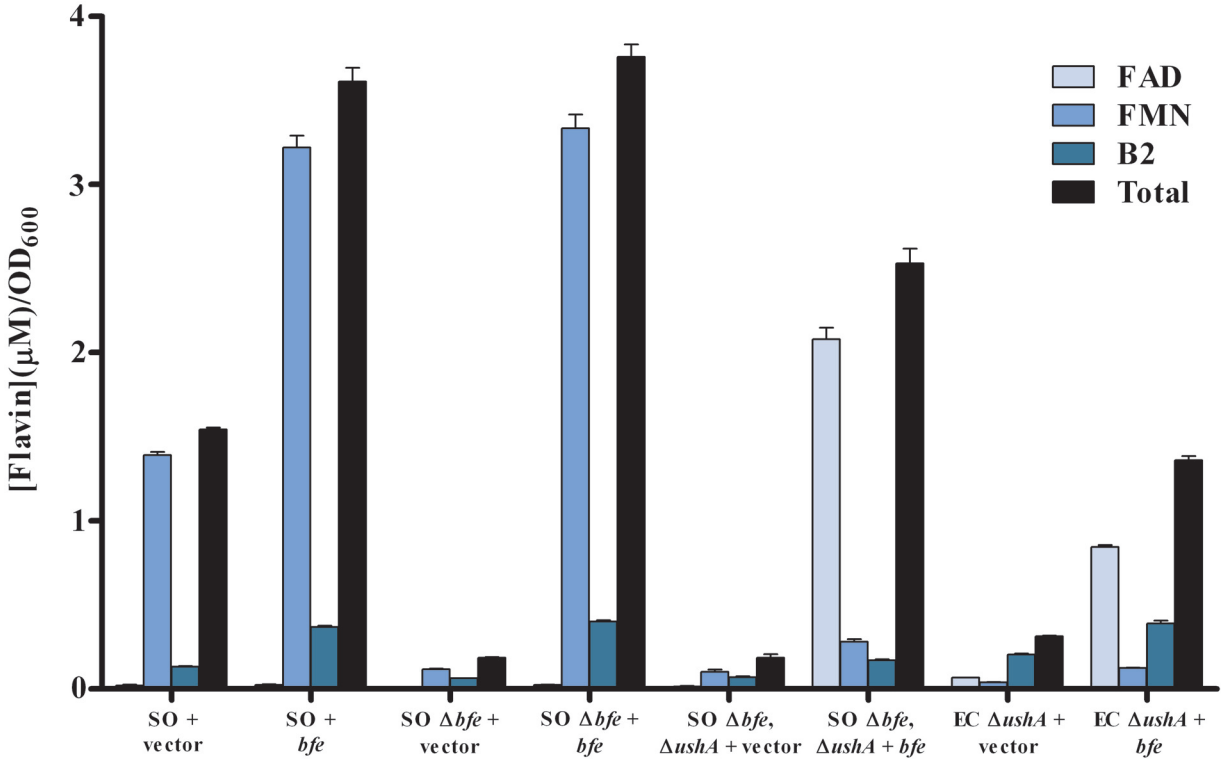

FIG 1 Flavin profile of *S. oneidensis* (SO) or *E. coli* (EC) strains quantified by HPLC. *S. oneidensis* cultures were anaerobically grown in SBM with 20 mM lactate and 40 mM fumarate at 30°C. Balch tubes were made anaerobic by flushing nitrogen gas through butyl rubber stoppers for 15 min. After 15 h of incubation, a sample was taken and cells were removed by centrifugation. HPLC was performed as previously described (19). The Δ*ushA E. coli* strain was grown in SBM with 20 mM lactate overnight at 37°C. Error bars indicate SEM ($n = 3$).

a paralog of the outer-membrane-associated decaheme cytochrome MtrC reveals FMN binding domains near two solvent-exposed heme groups (14), 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 (13, 15). 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 (13, 16). *S. oneidensis* Δ*ushA* was mated with *Escherichia coli* WM3064 (17) containing Tn*phoA*′-1 (18) to create transposon mutants. Transposon selection occurred under aerobic conditions on *Shewanella* basal medium (SBM) (19) 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) (20).

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 (19). 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) (Fig. 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.

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 (Fig. 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 (Fig. 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 (21) as previously described (13). Cells were provided 5 mM Fe(III) oxide (ferrihydrite) as the sole anaerobic electron acceptor (Fig. 2A). 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 (10, 13). Overexpression of *bfe* increased the amount of supernatant flavins (Fig. 1), resulting in strains that reduce Fe(III) oxide faster than MR-1 (Fig. 2A). Complementation (Fig. 2A) or addition of 10 μM FMN was able to alleviate the Fe(III) oxide reduction defect (see Fig. S1A in the supplemental material). As predicted, flavin electron shuttles were not necessary for reduction of soluble Fe(III) citrate (see Fig. S1B). 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.

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 (9). 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 (Fig. 2B) due to increased availability

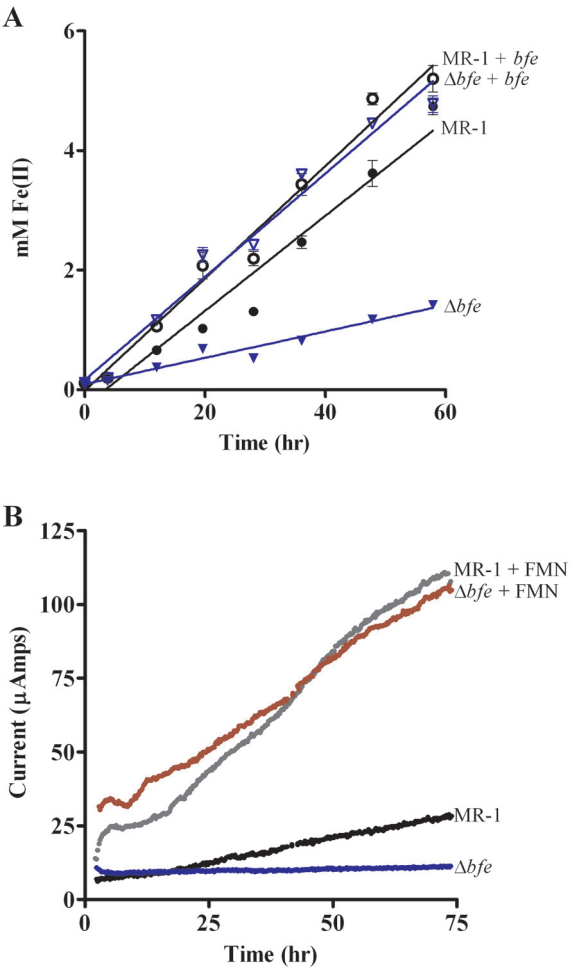

FIG 2 Electron shuttles accelerate reduction of insoluble extracellular electron acceptors. (A) Fe(III) oxide (ferrihydrite) reduction was quantified as previously described (13) for the following strains: MR-1 + vector (●), MR-1 + *bfe* (○), Δ*bfe* strain + vector (▼), and Δ*bfe* strain + *bfe* (▽). Error bars indicate SEM ($n = 3$). (B) Bioreactors were assembled as previously described (9). 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.

of flavin electron shuttles (9). 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 (19).

Implications. Electron shuttling has been a controversial hypothesis for extracellular electron transfer since it was first suggested (22). 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 (9, 10, 13). While *G. sulfurreducens* strain PCA has a MATE-like domain efflux pump homolog 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 (14, 23), 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.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00553-12/-/DCSupplemental.

Figure S1, PDF file, 0.6 MB.

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