Shewanella secretes flavins that mediate extracellular electron transfer

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Bacteria able to transfer electrons to metals are key agents in biogeochemical metal cycling, subsurface bioremediation, and corrosion processes. More recently, these bacteria have gained attention as the transfer of electrons from the cell surface to conductive materials can be used in multiple applications. In this work, we adapted electrochemical techniques to probe intact biofilms of *Shewanella oneidensis* MR-1 and *Shewanella sp.* MR-4 grown by using a poised electrode as an electron acceptor. This approach detected redox-active molecules within biofilms, which were involved in electron transfer to the electrode. A combination of methods identified a mixture of riboflavin and riboflavin-5′-phosphate in supernatants from biofilm reactors, with riboflavin representing the dominant component during sustained incubations (>72 h). Removal of riboflavin from biofilms reduced the rate of electron transfer to electrodes by >70%, consistent with a role as a soluble redox shuttle carrying electrons from the cell surface to external acceptors. Differential pulse voltammetry and cyclic voltammetry revealed a layer of flavins adsorbed to electrodes, even after soluble components were removed, especially in older biofilms. Riboflavin adsorbed quickly to other surfaces of geochemical interest, such as Fe(III) and Mn(IV) oxy(hydr)oxides. This *in situ* demonstration of flavin production, and sequestration at surfaces, requires the paradigm of soluble redox shuttles in geochemistry to be adjusted to include binding and modification of surfaces. Moreover, the known ability of isoalloxazine rings to act as metal chelators, along with their electron shuttling capacity, suggests that extracellular respiration of minerals by *Shewanella* is more complex than originally conceived.

bioelectrochemistry | biogeochemistry | redox mediator | riboflavin

Electrons require a discrete pathway to traverse distances >0.01 μm (1–3), yet bacteria such as *Shewanella* demonstrate an ability to transfer electrons to metals located >50 μm from cell surfaces (4, 5). For example, in experiments by Nevin and Lovley (5), *Shewanella. alga* BrY reduced iron oxides trapped within porous alginate beads. A more recent study by Lies *et al.* (4) also demonstrated reduction of Fe(III) oxides precipitated within nanoporous glass beads by *Shewanella oneidensis* MR-1 (4). Importantly, these studies could not detect a compound to explain these observations or differentiate between a model where a redox active compound produced by *Shewanella* diffused into the bead and a model where *Shewanella* produced a molecule to chelate ferric iron to facilitate its return to the cell.

S. oneidensis MR-1 was also reported to secrete compounds that could rescue menaquinone biosynthesis mutants (6). Later experiments supported the hypothesis that these compounds were intermediates of quinone biosynthesis released by lysed cells, rather than intentionally secreted shuttles (7). Recent analysis of *Shewanella putrefaciens* 200 provided new evidence for an unidentified organic Fe(III) chelator, which was required for maximal rates of Fe(III) reduction (8). Protein-based structures ("nanowires") have also been proposed as mechanisms for mediating electron transfer beyond the immediate surfaces of these bacteria (9).

In this article, we exploit the ability of *Shewanella* to grow as biofilms on electrodes, using electrodes as electron acceptors for respiration, to show that electron transfer by two strains of *Shewanella* to these surfaces is mediated by flavins, which are actively secreted by the cells. Flavins adsorbed to electrode surfaces, especially when colonized by biofilms. Along with this mixed shuttling/binding behavior, flavins are known to be capable of metal chelation (10–12). Thus, experiments conducted under conditions thought to remove soluble molecules from this organism's environment likely contained compounds that altered surface reactivity, mediated electron transfer, and increased the concentration of soluble metals. These combined properties explain the abilities of many *Shewanella* isolates.

Results and Discussion

Evidence for a Redox Mediator Involved in Electron Transfer. When midexponential phase *S. oneidensis* MR-1 or *Shewanella sp.* MR-4 cells were inoculated into a reactor containing a polished 2-cm^2 carbon electrode poised at +0.24 V [vs. standard hydrogen electrode (SHE)], an oxidation current of 3–6 μA, reflecting lactate oxidation by cells, and electron transfer from cells to electrodes, was immediately observed. Anodic (oxidation) current increased steadily for ≈72 h and stabilized at a plateau characteristic for each strain [32 μA (±4, $n = 4$) for MR-1, 45 μA (±5, $n = 4$) for MR-4]. Addition of lactate at this stage did not increase the rate of electron transfer, indicating that this rate was not caused by substrate limitation, but was likely caused by saturation of electrode surfaces by bacteria.

Once a stable oxidation current was observed, the medium surrounding biofilm-coated electrodes was removed and replaced with fresh anaerobic medium containing lactate as the electron donor. In similar experiments with bacteria such as *Geobacter* (13–15), medium replacement rarely affects the electron transfer rate >5%. Surprisingly, replacement with fresh medium immediately reduced oxidation currents by both strains of *Shewanella* an average of 73% (±4.5%, $n = 6$). An example of a typical medium replacement experiment for MR-4 is shown in Fig. 1*A*. For consistency, all subsequent figures show MR-4, although identical behavior was observed for MR-1.

These results suggested that either an unknown soluble compound mediated electron transfer from attached *Shewanella* cells to electrodes or planktonic *Shewanella* were responsible for the majority of electron transfer. When medium was removed, centrifuged to remove planktonic cells, and returned to chambers containing electrode-attached biofilms, current was immediately restored to 94% of its original level (±6.1%, $n = 6$) (Fig. 1*A*). These experiments indicated that the biofilm remained intact and that a soluble compound mediated electron transfer from *Shewanella* cells to the electrode. This finding was unexpected, especially in light of reports that *Shewanella* produces structures postulated to directly "wire"

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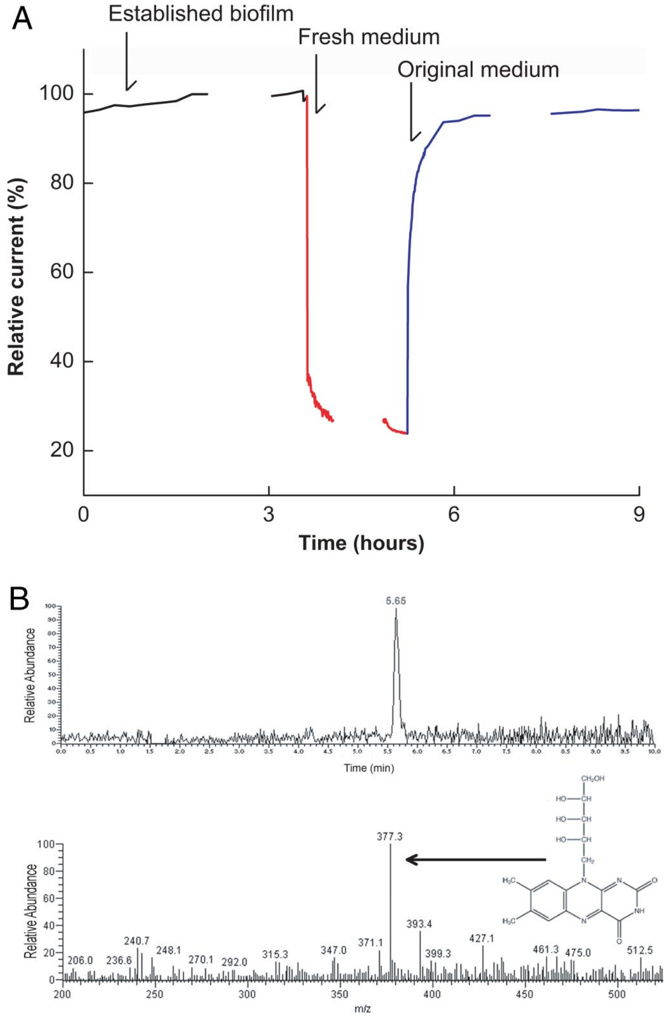

Fig. 1. Evidence for riboflavin secretion by electrode-attached bacteria. (*A*) Oxidation current by an established *Shewanella* MR-4 biofilm (black trace), decline after addition of fresh medium (red trace), and recovery after replacement of original medium found to contain secreted riboflavin (blue trace). Gaps indicate CV and DPV analysis. (*B*) LC-MS identification of microbially produced riboflavin from biofilm chambers after 96 h of incubation. The LC peak at 5.65 min (*Upper*) corresponds to compound with a *m/z* of 377.2, whereas analysis of the 377.2 peak yielded an ion with a *m/z* ratio of 243 (*Lower*).

cells to surfaces and metals, theoretically eliminating the need for a soluble redox mediator (9).

In separate experiments, after addition of fresh medium caused a decline in oxidation current by >70%, current slowly recovered back to original levels over a 72-h period. Repeated rounds of medium replacement, decrease of current by >70%, and slow (72–96 h) recovery could be observed, consistent with production by attached cells of a soluble redox mediator able to enhance the rate of electron transfer to electrodes.

To further investigate this hypothesis, cell-free medium obtained from electrode reactors was placed in sterile electrochemical cells containing identical glassy carbon electrodes and analyzed by linear sweep cyclic voltammetry (CV). These experiments produced reversible voltammetric peaks, centered at −0.21 V (vs. SHE), that increased in height with the age of the culture. After exposure of the medium to indoor light for 8 h, the height of these peaks decreased, and higher potential peaks were observed. No peaks could be detected in fresh medium.

Cell-free media from electrode-attached cultures demonstrated an absorbance maximum at 450 nm, and a fluorescence emission maximum at 525 nm, consistent with a flavin. With supernatants from 96-h biofilms, reverse-phase liquid chromatography (LC)-MS coupled with secondary MS analysis detected a new constituent with a mass-to-charge ratio (*m/z*) of 377.2 (Fig. 1*B*), identical to protonated riboflavin. Secondary MS analysis of the 377.2 peak yielded an ion with a *m/z* ratio of 243, consistent with riboflavin standards (16). No riboflavin-5′-phosphate (FMN) was detected in these samples. LC-MS/MS analysis showed riboflavin accumulated to a range of 250 to 500 nM. Samples taken at inoculation revealed a mixture of FMN and riboflavin (≈50:50), but biofilm growth was always associated with a shift to >90% riboflavin. MR-4 shifted to >95% riboflavin within 48 h, thus data shown (Figs. 1 and 2) are from cultures containing riboflavin as the dominant compound.

When riboflavin was added to sterile electrodes and growth medium, peak heights and potentials were identical to those observed with culture supernatants. FMN produced peaks centered 3 mV more negative than riboflavin, suggesting that these flavins could not be distinguished, especially where small pH alterations could cause potential changes. Although redox shuttles are often added in the micromolar range to stimulate reduction of metals by bacteria, the addition of only 250 nM riboflavin to biofilms produced the same stimulatory effect as the addition of cell-free medium supernatants (Fig. 2*A*).

After each experiment, biomass was measured to standardize rates to levels of attached protein. After 96 h of growth, 52 μg (±11, $n = 4$) of electrode-attached protein was detected, (equivalent to <0.1 OD_{600} units, if all cells were released from the electrode). An electron transfer rate of 35 μA (3.6×10^{-10} mol electrons transferred to electrodes s^{-1}) was thus equivalent to a specific rate of electron transfer of 25.1 μmol electrons·mg protein^{-1}·h^{-1}. As this rate could be achieved in the absence of planktonic cells, it represented a measure of the rate of respiration by *Shewanella* biofilms. Because lactate is oxidized to acetate by *Shewanella* (confirmed by HPLC), requiring transfer of four electrons to an exogenous electron acceptor, a lactate consumption rate of 6.3 μmol lactate·mg protein^{-1}·h^{-1} could be inferred. These rates were similar to reports of MR-1 reducing Fe(III)-minerals (≈3 μmol lactate·mg protein^{-1}·h^{-1}), and lower than rates observed for planktonic MR-1 respiring nitrate or Fe(III) citrate (≈15–30 μmol lactate·mg protein^{-1}·h^{-1}) (17, 18).

It is often assumed that bacteria could not use redox mediators in natural ecosystems, as synthesis of a molecule to attain a useful concentration represents a biosynthetic cost, especially if the molecule must be built from a simple precursor. Assuming no recovery of energy from pyrophosphate produced during biosynthesis, 1 mol of riboflavin could require as much as 25 mol ATP to produce (19, 20). To accumulate to 250 nM within a 72-h period would be equivalent to an ATP cost as high as 6.7×10^{-3} μmol ATP·mg protein^{-1}·h^{-1}.

To place this rate in context, an ATP production rate for *Shewanella* biofilms was estimated. Lactate metabolism proceeds via oxidation to pyruvate, acetyl-CoA, acetyl-phosphate formation, and excretion of acetate for generation of 1 ATP/lactate. During this metabolism, four electrons are respired to external acceptors, possibly generating ATP via proton pumping and an F-type ATP synthase. The total yield of substrate-level ATP generation plus chemiosmotic coupling was estimated from the energy available in lactate oxidation. Oxidation of lactate to acetate ($E^{\circ\prime} = -0.42$ V) linked to riboflavin reduction ($E^{\circ\prime} = -0.21$ V) provides −81 kJ/mol free energy, which, at a cost of ATP synthesis of ≈60 kJ/mol ATP, allows 1.5 ATP/lactate. This estimate is similar to those based on midpoint potentials of outer membrane cytochromes implicated in metal reduction by *Shewanella* such as OmcA (−0.24 to −0.32 V) (21), and MtrC (approximately −0.1 V) (22), making 1.5 ATP/lactate a conservative assumption for ATP yield.

Thus, at an oxidation current of 35 μA, *Shewanella* produced ATP at a rate of ≈9.6 μmol ATP·mg protein^{-1}·h^{-1}, which represented a rate of ATP production ≈1,000-fold faster than the rate

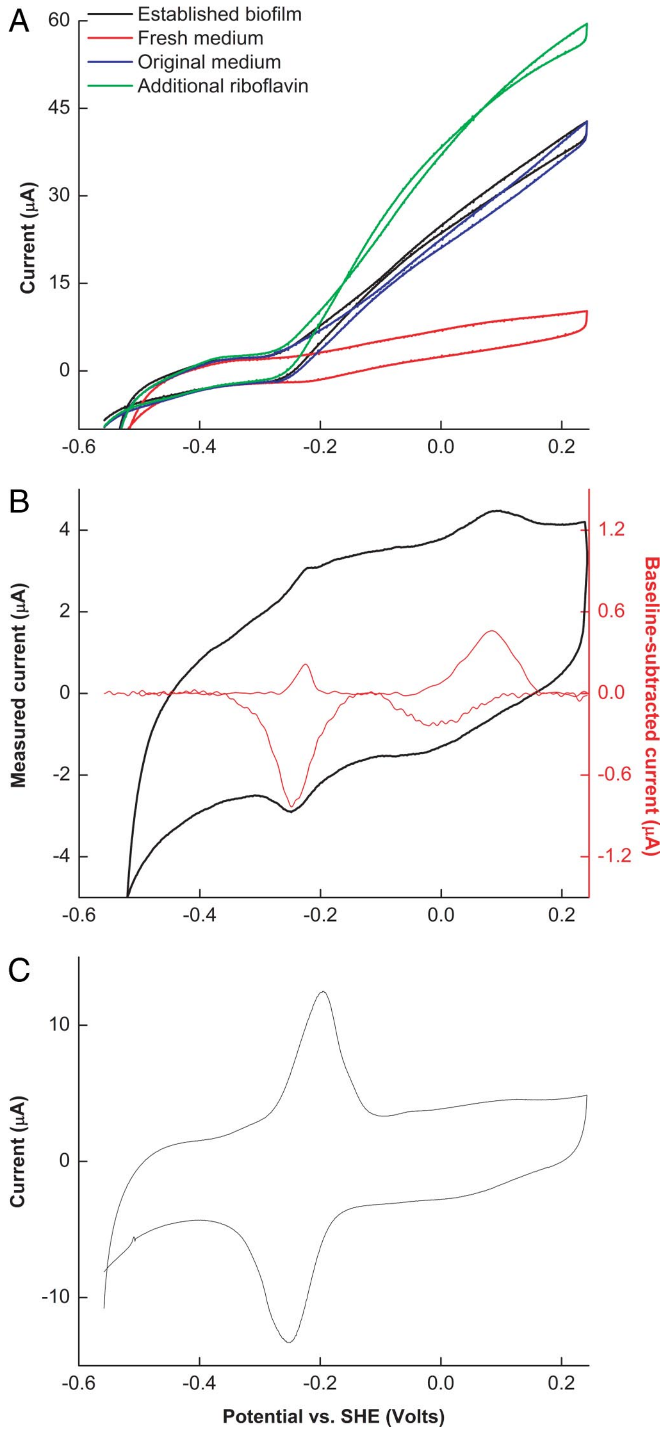

Fig. 2. Evidence for flavins controlling the rate of electron transfer to electrodes. (*A*) CV of *Shewanella* MR-4 in the presence of electron donor (lactate), showing current-voltage relationships for established biofilms (black trace), the same biofilms washed in medium free of redox mediators (red trace), and reimmersed in the original medium (blue trace). The green trace shows the effect of adding 250 nM riboflavin, to approximately double the concentration. (*B*) Nonturnover CV, showing a peak centered at −0.21 V. Raw data are shown in black, baseline-subtracted data are in red. (*C*) CV of riboflavin at identical sterile carbon electrodes.

of ATP consumption for flavin synthesis. Even with flaws in these assumptions (doubling ATP produced/lactate, doubling the cost of flavin synthesis, etc.), <0.1% of *Shewanella's* ATP budget would be required to account for the observed levels. This estimate is significantly lower than previous estimates in organisms such as *Geobacter*, which were based on higher levels of secreted shuttles (μM range), oxidation of electron donors such as acetate, which yield much less ATP, and use of two-carbon precursors that require more ATP in biosynthesis (23).

Therefore, growth of electrode-attached *Shewanella* cells was associated with accumulation of flavins that became dominated by riboflavin, at levels that increased the rate of electron transfer by at least 370%, yet biosynthesis of these molecules was estimated to cost <0.1% of the cell's ATP budget. Based on these results, the electrochemical activity of flavins at the biofilm-electrode interface was investigated further.

Evidence for Riboflavin at the Biofilm-Electrode Interface. We have recently shown that slow scan-rate voltammetry (SSRV) can detect electron transfer between bacteria and electrodes (15). When cells are attached to electrodes, a slow (1 mV/s) linear sweep from low to high potential results in an increasing anodic (oxidation) current that initiates at a characteristic potential. Reversing this linear sweep produces a nearly identical oxidation current. In a similar technique, known as protein film voltammetry (24), redox enzymes adsorbed on an electrode also demonstrate electron transfer from added electron donors to the electrode above a threshold potential (25–30).

When SSRV was performed on *Shewanella* MR-1 and MR-4 electrode-attached cultures oxidizing lactate, oxidation currents with an identical onset potential for electron transfer were observed (Fig. 2*A*). Removal of riboflavin-containing medium reduced the maximum current of this oxidation process, which was restored when centrifuged medium was replaced. When additional riboflavin was added to *Shewanella* cultures, the rate of the electron transfer process increased, yet the onset potential remained the same.

When the medium was replaced with fresh medium lacking lactate, and incubated for >12 h to deplete residual electron donor, the oxidation current dropped to background levels (<2 μA). These conditions were designed to allow detection of redox species at slow scan rates, without interference from the current resulting from lactate oxidation. In these experiments, an anodic (oxidation) peak was detected, centered at −0.2 V (Fig. 2*B*). These observations were consistent with peaks observed when sterile electrodes were incubated with riboflavin.

Evidence for Bound Riboflavin at the Electrode Surface. An observation with implications in this and many other experiments was that riboflavin demonstrated an affinity for carbon electrode surfaces and biofilms. To explore binding further, sterile carbon electrodes were incubated with riboflavin, and electrodes were scanned repeatedly with time via differential pulse voltammetry (DPV) (Fig. 3). When scanning from negative to positive potential, a peak centered at −0.208 V was detected within 15 min, and the height of this peak reached a maximum within 60 min. When these electrodes were placed in riboflavin-free supporting electrolyte, the height of the peak at −0.208 V decreased by <50% over a 24-h period. FMN also demonstrated rapid adsorption and binding to electrodes in DPV analyses.

When sterile electrodes were exposed to riboflavin and analyzed at increasing scan rates by CV, the potential difference between oxidation and reduction peaks was small (<20 mV) at slow scan rates. Peak potentials shifted with increasing scan rate and peak separation increased (Fig. 4*A*), characteristic of adsorbed redox species. In addition, peak heights increased as a linear function of scan rate (R^2 = 0.999) (Fig. 4*B*), another indication of adsorbed redox species (31). Based on peak heights in CV data, the amount of riboflavin adsorbed to electrodes was estimated at 0.2 nmol/cm^2 (31), which indicated that as much as 8% of the riboflavin in the reactor could be adsorbed.

Similar results were obtained with *Shewanella*-colonized elec-

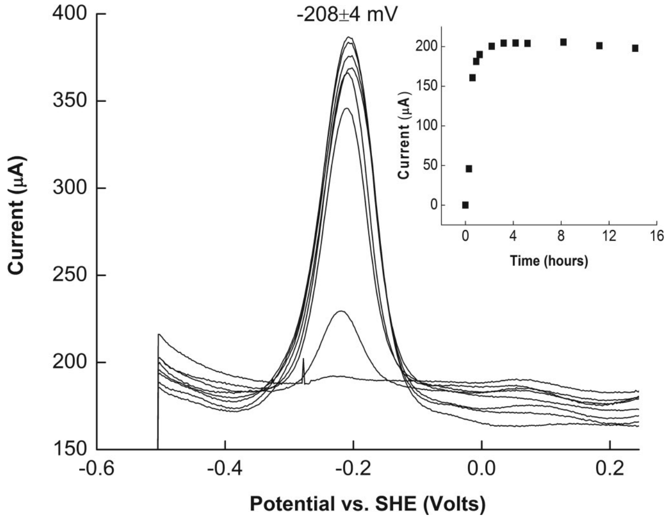

Fig. 3. DPV (from negative to positive potentials) of sterile carbon electrodes in growth medium after different times of exposure to riboflavin. (*Inset*) Increase in baseline-subtracted peak height over time.

trodes. For example, during initial biofilm growth, DPV scans from negative to positive potentials showed a peak centered at −0.2 V that increased in height as the culture aged (Fig. 5*A*). DPV voltammograms from older biofilms (and substrate-limiting CV experiments) also revealed higher-potential peaks representing possible secondary compounds present at lower concentrations. However, the potential of these peaks was too high (approximately +0.1 V) to be involved in the primary electron transfer process, which was observed in the range of −0.25 to 0 V.

When medium surrounding young biofilms (<3 days old) was exchanged with fresh medium, the peak height in DPV analyses decreased by at least 70%, indicating that much of the flavin detected by DPV could be removed (Fig. 5*B*). This finding was consistent with the large drop in oxidation current (>75% in young biofilms) described earlier (e.g., Fig. 1*A*). However, in more mature biofilms (>6 days old), exchange with fresh medium only decreased the peak height in DPV by 30%. Consistent with this observation, addition of fresh medium to older biofilms decreased oxidation current by a lesser degree (50–60%; data not shown).

These results demonstrated that whenever soluble flavins were present, a percentage was adsorbed to the electrode, and possibly also to cell material. The broad peaks and electrochemical behavior seen in our experiments were consistent with observations (32) that electron transfer between carbon electrodes and flavins can be relatively slow, suggesting that surfaces designed to better interact with these compounds would support higher electron transfer rates. However, expressed per unit electrode surface area, rates obtained with *Shewanella* (0.15 A/m^2) remain many orders of magnitude lower than what is obtained in chemical fuel cells (≈1,000 A/m^2), similar to what has been observed for other microbial catalysts (13, 33).

Implications of Flavin Secretion, Shuttling, and Binding. The term "mediator" is often used to evoke a soluble compound that can facilitate electron transfer. The acceleration of the electron transfer rate from *Shewanella* to electrodes by flavins fits the definition of an endogenously produced mediator. However, functionalization of surfaces by redox-active compounds is a common electrochemical theme. For example, flavin-based linkers can wire glucose oxidase to gold nanoparticles (34), electrodes modified to contain phenazines increase electron transfer rates from bacteria to electrodes (35), and sorption of polyaniline-based compounds to stainless steel protects surfaces from corrosion (36). Flavins, and phenazines

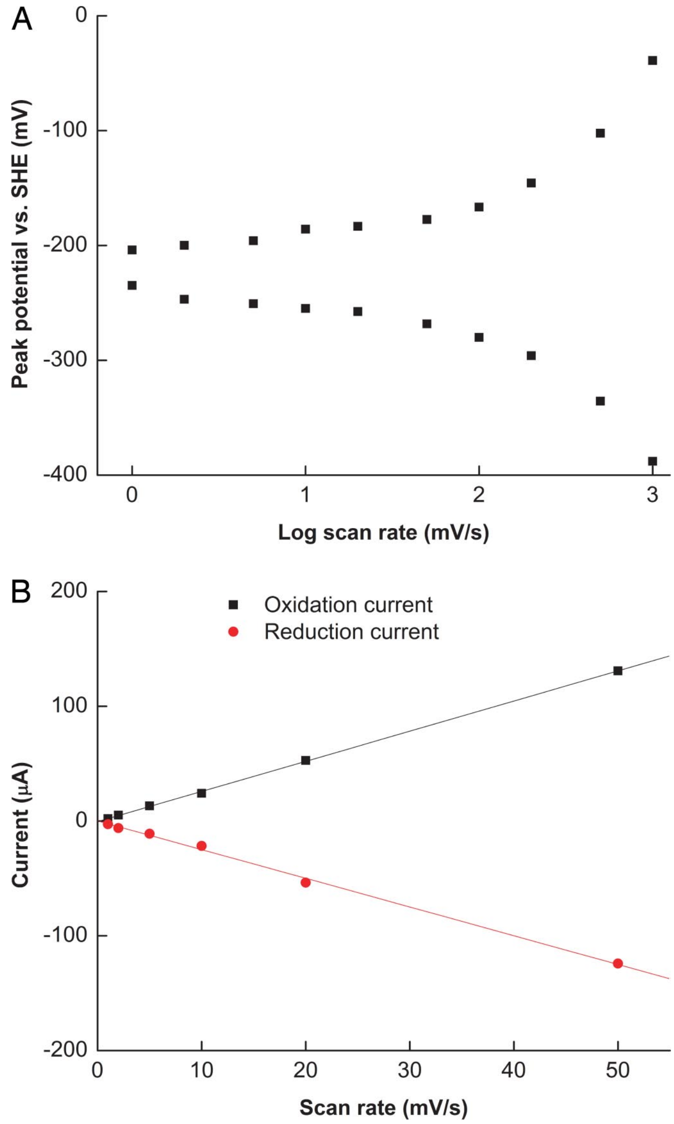

Fig. 4. Relationship between peak potential (*A*) or peak height (*B*) and scan rate for sterile carbon electrodes in *Shewanella* growth medium containing riboflavin.

produced by *Pseudomonas* representatives (37) should be viewed as compounds that have been present not only in the soluble phase, but on surfaces in experiments where analytical methods indicated they were removed.

How has a compound that exerts such a strong influence on surface reduction by *Shewanella* species evaded detection? One factor may be binding to filters used to filter-sterilize medium samples [supporting information (SI) Table 1], which caused underestimation in our early experiments. Coupled with their light sensitivity (38), effectiveness at only nanomolar concentrations, and yellow color only when oxidized and present at >1 μM, flavins were likely contributing to reduction of Fe(III) oxides (e.g., ref. 4) in previous studies. We observed flavin production by *Shewanella* species in addition to MR-1 and MR-4 in exponential, biofilm, and planktonic growth phases, indicating it is always present at some level (data not shown). As most *Shewanella* work uses dense cell suspensions, even washed cultures can quickly reestablish levels able to mediate electron transfer. For example, peaks can be seen in mediator-free voltammograms from *Shewanella* (39). As we took care to conduct experiments with low biomass and electrode

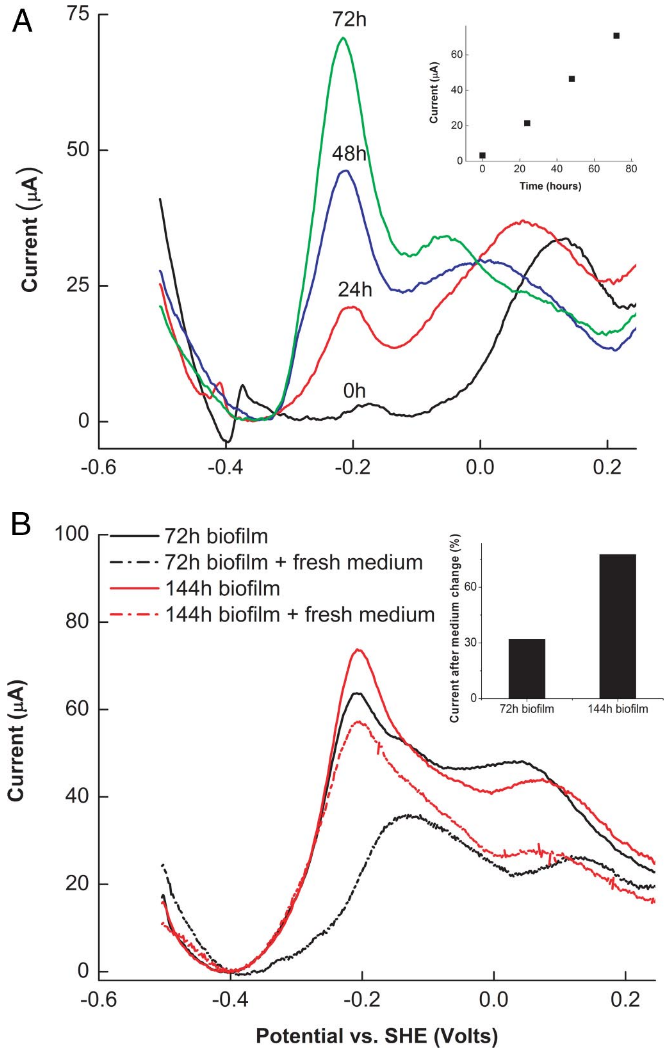

Fig. 5. DPV of carbon electrodes colonized by *Shewanella* MR-4 (from negative to positive potentials). (*A*) Increase in peak height (at −0.21 V) with growth of culture. (*B*) Changes in peak height after addition of medium, showing retention in older biofilms. Black trace shows DPV of a young biofilm in presence (solid line) and absence (dotted trace) of soluble riboflavin. Red trace shows DPV of an older biofilm in presence (solid line) and absence (dotted trace) of soluble riboflavin. (*Inset*) Change in peak height (at −0.21 V) in each treatment.

surface areas, relative to reactor volume, medium changes were able to significantly deplete mediator concentrations to allow detection of these compounds.

We also observed binding of riboflavin to Fe(III) and Mn(IV) oxide surfaces commonly used as substrates for this organism (SI Table 2). Riboflavin and FMN will also bind to forms of smectite clay [e.g., Fe(III) and Ca(II)], forming specific 1:1 interactions with Fe(III) in clays (40, 41). Such interactions may increase recycling, altering the reactivity of the surface, or creating a gradient so *Shewanella* can sense its proximity to electron acceptors. Such binding would further hamper detection in the soluble phase.

Another important property of isoalloxazine rings is that of a chelator. Early studies by Albert (10, 11) showed that the combination of the ionizable hydroxyl group and tertiary heterocyclic nitrogen atom created an effective chelator. Also interesting is that this binding site is one of the few that does not follow the Irving-Williams (Cu>Ni>Zn>Fe) series for binding stability, and demonstrates an unusually higher avidity for Fe [Log K for Fe(II) = 7.1] than Cu, Ni, and Zn, (10, 11).

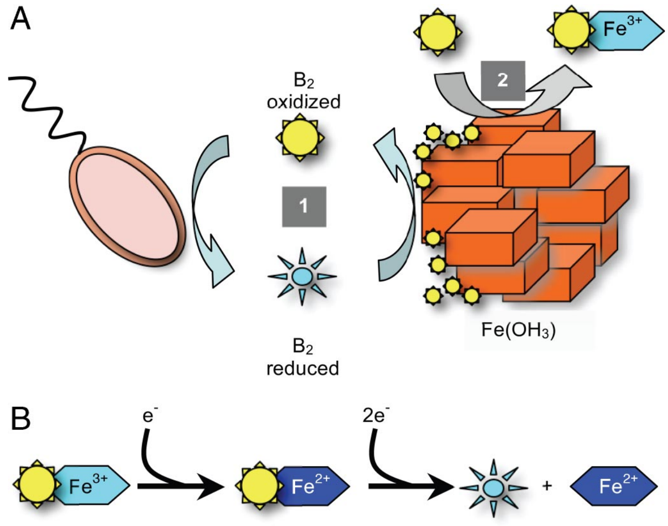

Fig. 6. Combined electron shuttling and chelator (shelator) activity by FMN or riboflavin (abbreviated as vitamin B2). (*A*) Model of interactions with metal oxides. (*B*) Electron-accepting abilities of solubilized metal.

Bacteria, yeast, and plants use the combined abilities of flavins in metal acquisition. Specifically, *Helicobacter pylori* does not produce siderophores under Fe-limited conditions, but produces riboflavin that can reduce Fe(III) in ferritins (12). Sugar beet and sunflower roots excrete micromolar amounts of riboflavin-5′ and riboflavin-3′ sulfate when grown in Fe-poor soils, leading to reduction and uptake of Fe (42). In *Pichia gulliermondii*, growth in low-Fe(III) medium leads to riboflavin excretion, whereas mutations preventing riboflavin overproduction significantly slow Fe-limited growth (43).

Together, these observations suggest that extracellular respiration by *Shewanella* is more complicated than previously determined. Flavins with a midpoint potential of −0.2 to −0.25 V can reduce most iron oxides and soluble forms of Fe(III) (Fig. 6) and have a natural avidity for many metals. As the midpoint potentials of the semiquinone and hydroquinone states differ by only ≈0.06 V, flavins are suited to single-electron and two-electron reactions (e.g., to interact with cytochromes and metals requiring one or two electrons for reduction). Chelation of Fe(III) (step 2 in Fig. 6) could increase local Fe(III) concentrations. Upon returning to the cell, complexes can accept as many as three electrons or be used for nutritional purposes. In addition, a gradient of redox-active molecules and metals, becoming more oxidized near the metal surface, could guide cells to favorable sites.

These electrochemical and analytical observations demonstrate that biofilms of *Shewanella* use secreted flavins in electron transfer to external acceptors, and that many environmentally relevant surfaces exposed to *Shewanella* are coated by electroactive flavins that may affect interactions with bacterial surface proteins. In metal-containing environments, flavin electron shuttling, metal chelation, and surface binding could act in concert to promote respiration and metal oxide dissolution phenotypes associated with this organism. Many activities catalyzed by *Shewanella*, and other organisms that secrete electroactive compounds, should be reexamined in light of this complex and ecologically important behavior.

Materials and Methods

Microbiological Methods. *S. oneidensis* strain MR-1 and *Shewanella sp.* MR-4 were cultivated in minimal salts medium with 10 mM Hepes. All vitamins were eliminated. Medium contained (per liter): 0.46 g NH_4Cl, 0.225 g K_2HPO_4, 0.225 g KH_2PO_4, 0.117 g $MgSO_4$ 7 H_2O, 0.225 $(NH_4)_2$ SO_4, plus 10 ml of a mineral mix (containing per liter: 1.5 g NTA, 0.1 g $MnCl_2{\cdot}4H_2O$, 0.3 g $FeSO_4{\cdot}7H_2O$, 0.17 g $CoCl_2{\cdot}6H_2O$, 0.1 g $ZnCl_2$, 0.04 g $CuSO_4{\cdot}5H_2O$, 0.005 g $AlK(SO4)_2{\cdot}12H_2O$, 0.005 g H_3BO_3, 0.09 g Na_2MoO_4, 0.12 g $NiCl_2$, 0.02 g $NaWO_4{\cdot}2H_2O$, and 0.10 g Na_2SeO_4).

Medium was adjusted to pH 7, sparged with oxygen-free N_2, sealed with butyl stoppers and aluminum seals, and autoclaved. Filter-sterilized casamino acids were added (0.05% vol/vol) after autoclaving. Cultures for each reactor were grown from frozen stocks, then transferred into anaerobic medium with 20 mM fumarate as the electron acceptor until an OD of 0.4 and transferred into the electrochemical cell, and lactate was added (20 mM) to ensure excess electron donor. Cultures were discarded at the end of each experiment.

Assembly of Biororeactor for Electrode Studies. Glassy carbon working electrodes (E-Tek), machined to $2 \times 0.5 \times 0.1$ cm, were polished (400 particles/inch), rinsed in DI, cleaned in 1 M HCl, and stored in deionized water. Electrodes were connected to a 0.1-mm Pt wire (Sigma–Aldrich) and washed with two changes of acetone and water. Counter Pt electrode wires were inserted into glass capillaries (Kimble) and soldered to copper wires. Reference electrodes were connected via a 3-mm glass capillary and vycor frit (Bioanalytical Systems). The resistance of each electrode assembly was <0.5 ohm (SI Fig. 7). Cells were autoclaved, and the salt bridge filled with 0.1 M Na_2SO_4 in 1% agar. A calomel reference electrode (Fisher Scientific) was placed in this layer and covered in Na_2SO_4. Reactors were operated under a flow of sterile humidified oxygen-free N_2 at 30°C and mixed with a magnetic stirrer. Sterile reactors were analyzed before each experiment to verify the absence of redox compounds. Cells showing residual peaks in DPV, anodic current in CV, or baseline noise were discarded.

Growth in Electrochemical Cells. Electrochemical measures were typically performed with a VMP potentiostat (Princeton Applied Research). A constant potential of 0.24 V vs. SHE was applied to electrodes, and current was averaged over 15-min periods. The working electrode was also monitored by DPV and CV. Analyses were performed without stirring enabled. The parameters were: for DPV, $E_i = -0.558$ V vs. SHE, $E_f = 0.242$ V vs. SHE, pulse height 50 mV, pulse width 300 ms, step height 2 mV, step time 500 ms, scan rate 4 mV/s, current average over the last 80% of the step (1 s, 12 points), accumulation time 5 s; and for CV, equilibrium time 5 s, scan rate 1 mV/s, $E_i = -0.558$ V vs. SHE, $E_f = 0.242$ V vs. SHE, current averaged over the whole step (1 s, 10 points). Scan rate analysis was performed with a Gamry PCI4 Femtostat (Gamry Instruments).

To exchange medium, medium was removed with a sterile nitrogen-flushed syringe. This original medium was transferred to a foil-wrapped, sterile, anaerobic tube, passed into an anaerobic chamber, and centrifuged (10 min at 5,000 × *g*) to remove biomass. This cell-free medium was returned to an anaerobic sealed tube and used as described. After medium was removed, 3 ml of fresh medium was added to the chamber, then discarded to rinse chambers. Ten milliliters of fresh medium with 20 mM lactate was then added.

Analytical Methods. Planktonic cells were removed by dipping electrodes in sterile medium. The electrode was incubated in 1 ml of 0.2 M NaOH, at 96°C for 20 min to solubilize biomass. Samples were analyzed by bicinchoninic acid assay.

For LC/MS/MS analysis, medium samples and standards were not filtered to prevent loss of compounds caused by binding. Centrifuged samples were analyzed according to Midtun *et al.* (16), by using a ZORBAX Eclipse C8 reverse-phase column (Agilent) with a 5-μm particle size. Eluted compounds were analyzed by MS and MS-MS using a Thermo Electron LCQ Ion Trap Spectrometer (Thermo Scientific) operated in the positive ion mode. Flavins were monitored by using HPLC as described (44). The column was a 4.6-mm × 525-mm Eclipse XDB-C18 (Agilent) (5-μm particle size). A fluorescence detector (Waters) was used with an excitation wavelength of 440 nm and an emission wavelength of 525 nm.

Note added in Proof: As this article went to press, von Canstein *et al*. (45) reported secretion of flavins by multiple planktonic *Shewanella* cultures and noted a role for these compounds in azo dye decoloration and metal reduction. These results are consistent with our findings using biofilm cultures.

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