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Electron Transfer Beyond the Outer Membrane: Putting Electrons to Rest

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Keywords

Shewanella, *Geobacter*, extracellular electron transfer, cytochrome, electron shuttles

Abstract

Extracellular electron transfer (EET) is the physiological process that enables the reduction or oxidation of molecules and minerals beyond the surface of a microbial cell. The first bacteria characterized with this capability were *Shewanella* and *Geobacter*, both reported to couple their growth to the reduction of iron or manganese oxide minerals located extracellularly. A key difference between EET and nearly every other respiratory activity on Earth is the need to transfer electrons beyond the cell membrane. The past decade has resolved how well-conserved strategies conduct electrons from the inner membrane to the outer surface. However, recent data suggest a much wider and less well understood collection of mechanisms enabling electron transfer to distant acceptors. This review reflects the current state of knowledge from *Shewanella* and *Geobacter*, specifically focusing on transfer across the outer membrane and beyond—an activity that enables reduction of highly variable minerals, electrodes, and even other organisms.

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Contents

1. BACKGROUND

The enzymes responsible for biogeochemical cycling of carbon, nitrogen, and sulfur are typically located inside the cytoplasm or within the cytoplasmic membrane. The chemistry of each compound is unique, the active sites for catalysis are highly conserved, and the sequences encoding these enzymes are easily identified in genomes. The insolubility and diversity of abundant electron acceptors such as Fe(III) and Mn(IV) and their ability to accept electrons from multiple redox-active cofactors with a sufficiently low redox potential place very different constraints on electron transfer strategies used by dissimilatory metal-reducing bacteria such as *Shewanella* and *Geobacter*.

In the last decade, new molecular details have emerged to show how electrons from the periplasm cross cell membranes and reach distant electron acceptors. Multiple porin-cytochrome complexes, where multiheme *c*-type cytochromes are threaded through a β-barrel pore, reveal a strategy for transmembrane electron transfer that can now be identified in multiple metal-reducing and metal-oxidizing organisms. In contrast, mechanisms for delivering electrons beyond the cell are more diverse, and less well understood. In *Shewanella*, an exporter in combination with a periplasmic nucleotidase releases a pool of redox-active flavins that can shuttle electrons to acceptors not in contact with the cell, but *Shewanella* also can produce outer membrane nanowire extensions. In *Geobacter*, three different multiheme *c*-type cytochrome–based filament cryo-electron microscopy (EM) structures are a new component in the complement of nanowires known for decades to be essential for direct electron transfer to surfaces and other cells (111, 127, 128). In all these examples, from multiple nonhomologous porin-cytochrome conduits to cytochrome nanowires with unique heme packing arrangements, the mechanism of creating

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conductivity appears conserved whereas the complexes themselves show evidence of arising independently. This lack of a single signature enzyme or protein among metal-reducing bacteria suggests this ability could be highly widespread yet invisible to most genome annotation methods.

2. THE OUTER SURFACE: WHAT PRESENTS THE ELECTRONS TO THE OUTSIDE WORLD?

2.1. *Shewanella*: The Porin-Cytochrome Solution

Genetic and biochemical evidence helped address the initial skepticism that a respiratory process could be connected across two membranes in gram-negative bacteria. The first indication that *Shewanella* requires proteins localized to the outer membrane to reduce insoluble substrates came from genetic studies that identified a transposon insertion in *mtrB* (5), which encodes an outer membrane β-barrel protein. In the same genetic locus is *mtrA*, encoding a decaheme *c*-type cytochrome, and *mtrC*, encoding an outer membrane decaheme *c*-type cytochrome lipoprotein (**Figure 1**).

Two multiheme cytochromes, OmcA and MtrC (also known as OmcB in some early literature), were shown to be surface exposed using protease degradation assays (104) and later shown to be lipoproteins, facilitating their association with the surface of the cell (105). A role for these proteins in reduction of iron and manganese minerals was also demonstrated genetically (6, 104). MtrF is a third outer membrane, multiheme *c*-type cytochrome that is encoded directly upstream of the *omcA* locus (40) (**Figure 1**) but does not appear to have a role in metal reduction in the presence of OmcA or MtrC (103). Interestingly, MtrF does have the functional capacity to facilitate iron reduction (19) and is largely responsible for the residual iron reduction activity of an *omcA/mtrC* double mutant (18).

Localization of OmcA and MtrC (and presumably MtrF) requires the type II secretion pathway, first demonstrated in *Shewanella putrefaciens* (21) and later verified in *Shewanella oneidensis* (131). The integral outer membrane protein MtrB also appears to be required for proper localization of

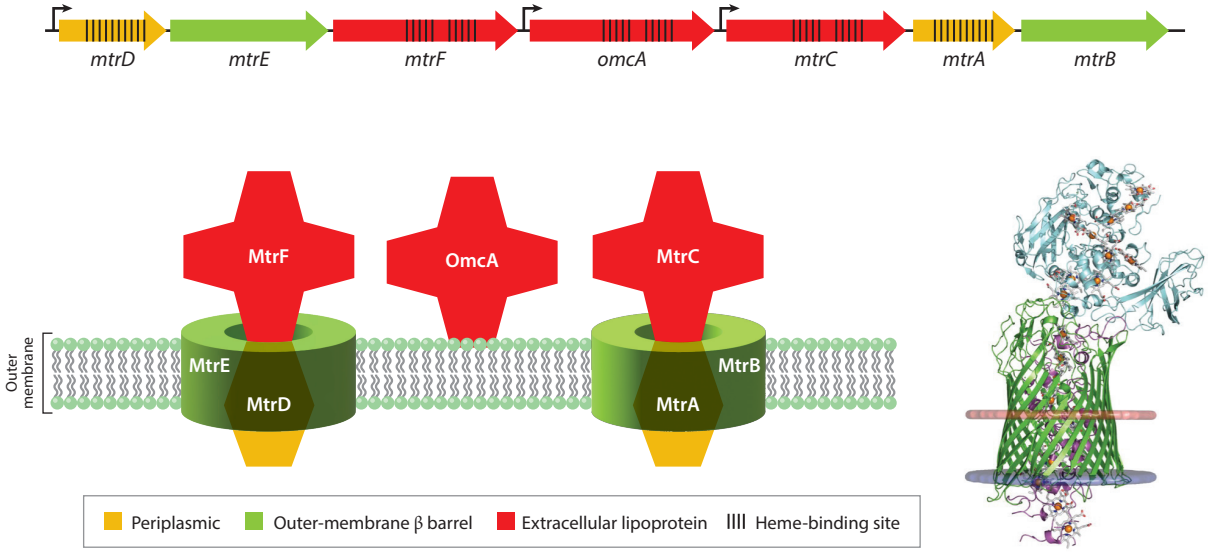

Figure 1

The *mtr* gene cluster from *Shewanella oneidensis* (*top*) encoding porin-cytochrome complexes (*bottom*).

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OmcA and MtrC (103), though how MtrB influences the localization of these decaheme *c*-type cytochromes remains unclear.

Structures of MtrF (15), OmcA (23), and MtrC (25) have been experimentally determined, with these proteins exhibiting a "staggered cross" arrangement of heme groups. A functional understanding of how electrons flow across the outer membrane was gained from determining the structure of the entire MtrCAB complex from *Shewanella baltica* (26), providing structural validation for the porin-cytochrome model initially proposed by Richardson et al. (122) (**Figure 1**). Periplasmic electron carrier proteins [FccA and CctA (138)] transfer electrons to the N-terminal, periplasmic-exposed heme group of MtrA. The MtrA protein itself is almost fully embedded in the integral outer membrane protein MtrB. MtrC forms the third portion of the complex, with one of its heme groups in close proximity to the C-terminal heme of MtrA.

2.2. OmcA

The majority of sequenced genomes from the genus *Shewanella* contain homologs of *mtrCAB*, suggesting that extracellular electron transfer is a general metabolic capability of these bacteria. Upstream of *mtrCAB* in these genomes is a gene encoding an OmcA-like protein, sometimes with 11 heme groups rather than 10 (23, 25, 132) and sometimes with up to 3 related copies (3). OmcA mutations in *S. oneidensis* do not manifest significant extracellular electron transfer (EET)-related phenotypes on their own, but they become important when MtrC is removed (17). OmcA may have an accessory role in the process of EET, perhaps in binding insoluble substrates (54, 55, 88, 102, 151). Direct interactions between OmcA and MtrC were seen in experiments with histidine-tagged proteins in vitro (130), but subsequent experiments with native proteins have not supported this observation (25). OmcA and MtrC also appear to have distinct mobility dynamics (14), consistent with a more transient association. The conservation and diversification of *omcA* and its homologs seen in the *Shewanella* genome are good evidence for their importance, though the factors driving variability of this component are not known.

2.3. MtrDEF

Encoded upstream of *omcA* is a second gene cluster that also encodes a membrane-spanning complex, MtrDEF. While the components encoded by MtrDEF share a high degree of similarity to MtrCAB, the gene order differs, likely due to the resolution of a duplication event prior to the speciation of the genus *Shewanella*. The paralog pairs in *S. oneidensis* are MtrC/MtrF (34% identity), MtrA/MtrD (68% identity), and MtrB/MtrE (35% identity). While inactivating *mtrDEF* on their own does not lead to a significant metal reduction defect in *S. oneidensis*, *mtrDEF* can partially complement reduction of soluble Fe(III) citrate in a mutant strain lacking nearly all Mtr-related genes (19). Like OmcA, its conservation within the genus *Shewanella* suggests an important role. Increased expression (~2–3-fold) of the *mtrDEF* genes was observed in $CaCl_2$-induced aggregated particles that might correlate to O_2 limitation under these conditions (98). The multiheme cytochrome MtrF may be preferentially matured and/or localized to the cell surface under conditions of high iron, as suggested by the polarizability phenotype of a mutant strain expressing *mtrDEF* when grown with fumarate and Fe(III) citrate compared to the phenotype when grown only with fumarate (147). While the *mtr* gene cluster is largely conserved across the genus *Shewanella*, the inventory of porin-cytochrome capability in *Geobacter* is much more broad and diverse.

2.4. The Highly Diversified Porin-Cytochrome Strategy of *Geobacter*

Compared to *Shewanella*, less is known about electron transfer across the *Geobacter* outer membrane. While a similar strategy of porin-cytochrome conduits is employed, the proteins identified

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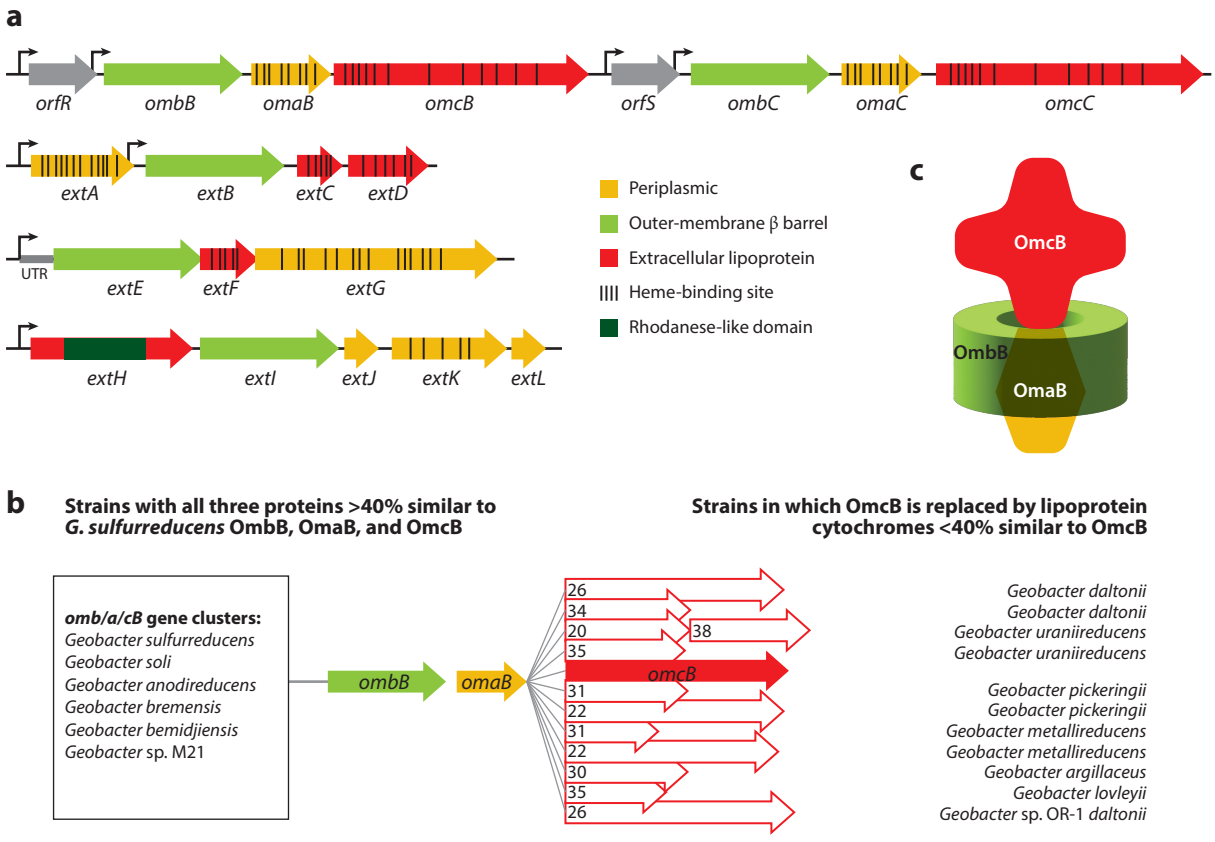

Figure 2

Diversity of gene clusters encoding porin-cytochrome complexes in *Geobacter sulfurreducens*. (*a*) Based on the presence of lipoprotein signal sequences, putative extracellular-facing cytochromes and subunits are shown in red. Likely membrane-spanning β-sheet proteins are in green, and periplasmic cytochromes in yellow. (*b*) Examples of non-homologous replacement of the outer cytochrome in the system, where multiple genomes have the same gene organization of *omb/oma*, but lipocytochromes of different sizes replace the *omc* subunit. Locus tags on the left refer to genomes containing the entire three-gene cluster as it is found in *G. sulfurreducens*, while locus tags on the right are clusters in which *omb/oma* are conserved but the OmcB gene has been replaced. (*c*) Model of a porin-cytochrome complex, using OmcB as an example. Figure adapted from Reference 53 (CC BY 4.0).

to date share no homology with Mtr proteins in *Shewanella*. In addition, *Geobacter* encodes and expresses multiple unrelated conduits (**Figure 2**).

2.5. OmcB-Based Porin-Cytochrome Conduits

A large Fe(III)-reducing complex enriched from *Geobacter sulfurreducens* membranes (90) led to early identification of a duplication within the genome encoding two 89-kDa dodecaheme lipoprotein cytochromes (91) sharing 73% identity, now known as OmcB and OmcC. Deletion of *omcB* caused a defect in soluble Fe(III) reduction, while deletion of *omcC* had little effect (65, 66). The importance of OmcB in electron transfer became increasingly evident in genetic experiments where deletion of cytochromes unrelated to OmcB led to Fe(III) reduction deficiencies that were later traced back to a loss of OmcB. For example, deletion of *macA*, encoding a periplasmic diheme peroxidase (12, 62, 129), lowered *omcB* expression and caused poor Fe(III) reduction, which

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could be restored by expression of *omcB* from a constitutive promoter. Removal of the multiheme cytochromes OmcG and OmcH also lowered OmcB levels in the outer membrane even though *omcB* mRNA was still present, leading to a decrease in Fe(III) reduction (60).

With the discovery of the porin-cytochrome paradigm in *Shewanella*, re-examination of genes adjacent to *omcB* revealed a small octaheme cytochrome (*ombB*) and a hypothetical β-barrel-forming protein (*omaB*) that suggested an unpronounceable OmcB-OmbB-OmaB transmembrane complex analogous to the MtrCAB complex, even though there is no homology between proteins in these two systems (**Figure 2**). Within the duplicated region also was the similar *omcC* next to *ombC* and *omaC*, which shared 100% identity with their *omb/oma* homologs. Intact ~155-kDa complexes consistent with OmcB-OmbB/C-OmaB/C and OmcC-OmbB/C-OmaB/C units could be purified from the *G. sulfurreducens* outer membrane, and isolated complexes inserted into liposomes catalyzed transmembrane electron transfer (71). Genetic experiments confirmed that deletion of other constituents from these operons (*omaB/C* or *ombB/C*) caused similar Fe(III)-reduction defects as Δ*omcB* (73). The OmcB/C system remains the only porin-cytochrome complex in *Geobacter* with biochemically confirmed transmembrane electron transfer abilities. Operons containing this *omcB-ombB-omaB* cluster are found in nearly every close relative of *G. sulfurreducens*, but as the regions of 100% identity within the duplicated region often cause sequencing and/or assembly errors (especially in metagenomes), the origin, stability, and role of the OmcB/C duplication are less clear.

2.6. Are Specific *Geobacter* Conduits Used for Specific Acceptors?

While OmcB-family conduits are clearly involved in electron transfer to soluble Fe(III), bioinformatic analyses find at least three other putative outer membrane electron conduits encoded on the genome of *G. sulfurreducens*, raising the possibility that different conduits could be used for different acceptors (133). Additional evidence supporting this hypothesis came from genome-wide transposon studies where insertions in *omcB* or *omcC* had no effect on *G. sulfurreducens* growth with electrodes, but insertions within an unrelated four-gene cluster (GSU2645-2642) containing a predicted dodecaheme periplasmic cytochrome (*extA*), β-barrel-like protein (*extB*), and two penta- and dodecaheme lipoprotein cytochromes (*extCD*) caused significant growth defects (13).

Along with the *omcB/C* and *extABCD* systems, *G. sulfurreducens* contains *extEFG* (GSU2726-2724), encoding a predicted outer membrane ExtE β barrel, outer membrane lipoprotein pentaheme *c*-cytochrome ExtF, and periplasmic dodecaheme *c*-cytochrome ExtG (**Figure 2**). Also known is *extHIJKL* (GSU2940-2936), which instead of an outer membrane lipoprotein *c*-cytochrome, encodes a rhodanese-family lipoprotein ExtH; β-barrel-like ExtI; a small periplasmic protein, ExtJ; and ExtK, a periplasmic pentaheme *c*-cytochrome that could also include downstream protein ExtL due to a stop codon encoding a possible selenocystine (32). Markerless deletion mutants lacking all single, double, and triple combinations of *omcBC*, *extABCD*, *extEFG*, and *extHIJKL* are available (52).

While conclusions regarding specific roles of different complexes are influenced by expression levels, and remain to be confirmed in related strains, some general trends have emerged. Repeatedly, significant soluble Fe(III) reduction defects are found in Δ*omcBC* strains, but deletion of *extABCD* in Δ*omcBC* is necessary to most severely affect soluble Fe(III) reduction. Expression of either the *omcB-ombB-omaB* operon or the *extABCD* cluster completely restores soluble Fe(III) reduction in mutants lacking all conduit genes. In contrast, deletion of *extEFG* in Δ*omcBC* is necessary to eliminate most insoluble Fe(III) reduction, strains containing only the *extEFG* cluster perform better with Mn(IV) oxides than Fe(III) oxides, and deletion of all five clusters is required

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to remove residual reduction of all metals. When electrodes are the electron acceptor, only strains lacking *extABCD* show a growth defect (52). In fact, a strain containing *extABCD* but lacking all other conduit clusters grows faster, forms denser biofilms, and produces a higher final density on electrodes (53). Complemented strains expressing *extABCD* from a vector also grow faster than wild type on electrodes.

While OmcBC and ExtEFG are linked to Fe(III) and Mn(IV) reduction, and ExtABCD is essential for electrode growth, there is evidence for ExtHIJKL participating in selenite and tellurite respiration, along with biochemical evidence that this cluster encodes a complex. Deletion of *extI* in *G. sulfurreducens* decreases the cell's ability to reduce both selenite and tellurite (49), and *extI* deletion affects localization of the rhodanese-related ExtH lipoprotein to the outer membrane (50). Using antibodies specific to ExtI and ExtH, these can be shown to exist in complexes with a larger molecular weight, but the presence of other subunits is yet to be determined.

While the unique extracellular rhodanese-domain protein in ExtHIJKL provides a possible explanation for its reduction of selenite or tellurite, it is more difficult to explain why OmcB-based conduits do not support electrode reduction, or why ExtABCD does not participate in metal oxide reduction. Transcriptional differences cannot explain these preferences, as the entire *omcB-omcC* duplicated operon shows tenfold higher expression in electrode-grown cells compared to *extABCD* (52), while *extABCD* has a similar expression level as *extEFG* and *extHIJKL*. One hypothesis could be the need to interface with nanowires or extracellular proteins involved in electron transfer beyond the cell membrane. Nanowires such as OmcE and OmcS, described in later sections, as well as the cell-associated triheme lipoprotein cytochrome PgcA (155) are needed for metal oxide reduction, while the nanowire OmcZ is the only cytochrome linked to formation of conductive electrode biofilms. It is possible that only specific porin-cytochrome complexes can inject electrons into these different extracellular wires and proteins; e.g., only ExtABCD can interface with OmcZ. There are no ExtABCD structures or studies testing nanowire interactions, but this speculation would also explain why both ExtABCD and OmcB-based complexes still support growth with soluble metals.

2.7. Diversity of *Geobacter* Porin-Cytochromes

Along with a greater variety of porin-cytochrome complexes, another notable difference between *Geobacter* and *Shewanella* is swapping of cytochromes with nonhomologous proteins. For example, in operons containing the *ombB* β-barrel and *omaB* periplasmic cytochrome genes, *omcB* is often replaced in *Geobacter* genomes with a range of completely different lipoprotein multiheme cytochromes, differing in both size and predicted heme number (**Figure 2**). In the ExtEFG family, the ExtF lipoprotein cytochrome is common, while the ExtG cytochrome predicted to be in the periplasm is replaced by cytochromes half as large, and in many cases, these also contain lipoprotein signal sequences. ExtABCD shows yet another pattern, where the lipoprotein cytochrome ExtC is highly conserved, but ExtD is more often than not replaced by a different lipoprotein cytochrome, up to four to five times as large as ExtD. These examples suggest that, especially compared to MtrCAB, the exterior-facing components of *Geobacter* complexes are constantly evolving or being replaced, and there may not be a single porin-cytochrome cluster that marks a genome as being from an Fe(III)-reducing or electrode-reducing cell.

3. ELECTRON SHUTTLING: EXTRACELLULAR ELECTRON TRANSFER DISCONNECTED

Once at the surface of the bacterial cell envelope, distribution of electrons to electron acceptors can occur in a direct manner where the substrate interacts specifically with the terminal reductase(s), a

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redox active conduit, or indirectly through a mediator that facilitates electron transfer to substrates located some distance from the cell surface. In the case of direct electron transfer, cells must be in direct contact with insoluble substrates such as iron or manganese minerals. Mediated EET is now primarily referred to as electron shuttling, a term initially coined during work in *Shewanella*, which was proposed to have this capability (112), and in *Geobacter*, which did not (106). Newman & Kolter (112) proposed that *S. oneidensis* secreted quinones as redox-active small molecules that could be used to facilitate respiration of insoluble substrates away from the cell surface. Whereas the *Shewanella* observation was later shown to be due to cross feeding (101, 105), the idea of electron shuttling pushed the field forward and provided a key point of distinction between the mechanism of EET in *Shewanella* and *Geobacter*.

Initial experiments separating cells from their insoluble substrate were done with semipermeable membranes (e.g., dialysis tubing), resulting in the conclusion that direct contact was required (108). Nevin & Lovley (108) performed key control experiments using dialysis tubing with a 300-kDa MW cutoff and showed that this material still prevented a low-molecular-weight electron shuttle [anthraquinone-2,6-disulfonate (AQDS)] and a chelator [nitrilotriacetic acid (NTA)] from facilitating electron transfer to the sequestered Fe(III) oxide. The interest in exploring the potential mechanism of electron shuttling and the realization that semipermeable membranes were not an effective method to study this process motivated the development of other approaches. The first approach was to incorporate Fe(III) oxide into microporous alginate beads, estimated to have a molecular mass cutoff of 12 kDa (108). *Geobacter metallireducens* was able to reduce only a small fraction of the total Fe(III) available, likely located at or near the surface of the beads; however, nearly all the iron could be reduced to Fe(II) with the addition of AQDS (108). A similar observation was also made using cultures of *G. sulfurreducens* and using 1% agar as a method of separating cells from substrate (136). Unlike *Geobacter*, a *Shewanella* strain (*S. alga* BrY) was capable of reducing a significant amount of iron that was sequestered in alginate beads without addition of an exogenous electron shuttle (109). The different capacities to facilitate reduction of entrapped iron by *Shewanella* and *Geobacter* are illustrated in **Figure 3**.

The second approach was to precipitate Fe(III) oxide in and around nanoporous glass beads, which were ~100 μm in diameter with interconnected pores with an average diameter of ~50 nm (70). Several *Shewanella* strains (*S. oneidensis*, *Shewanella* sp. ANA-3, and *S. putrefaciens* CN-32) were shown to mediate reduction of iron beyond what was localized to the outer cortex of the beads (70). These key experiments demonstrate a fundamental difference between EET strategies of *Geobacter* and *Shewanella* and motivated additional work to identify and characterize the electron shuttle produced by *Shewanella* strains that enable EET at a distance.

3.1. Electron Shuttling: The Flavin Awakens

The identity of the electron shuttle produced by *Shewanella* was determined independently by two groups using different approaches. One used bioelectrochemical reactors with a poised graphite electrode as the electron acceptor (97), while the other used insoluble Fe(III) oxides (144). Adding additional flavins to cells reducing electrodes (97) or Fe(III) oxide (144) enhanced the rate of reduction by *Shewanella* strains. The primary flavin found in culture supernatants is flavin mononucleotide (FMN), but the dephosphorylated form, riboflavin (B_2), also can be found (97, 144).

Flavins are essential for all living organisms, as FMN and flavin adenine dinucleotide (FAD) are cofactors for a wide range of critical biochemical reactions throughout metabolism in all domains of life (1). The genes encoding flavin biosynthesis in *S. oneidensis* combine components from both *Escherichia coli* and *Bacillus subtilis* with several proteins encoded more than once (10), including

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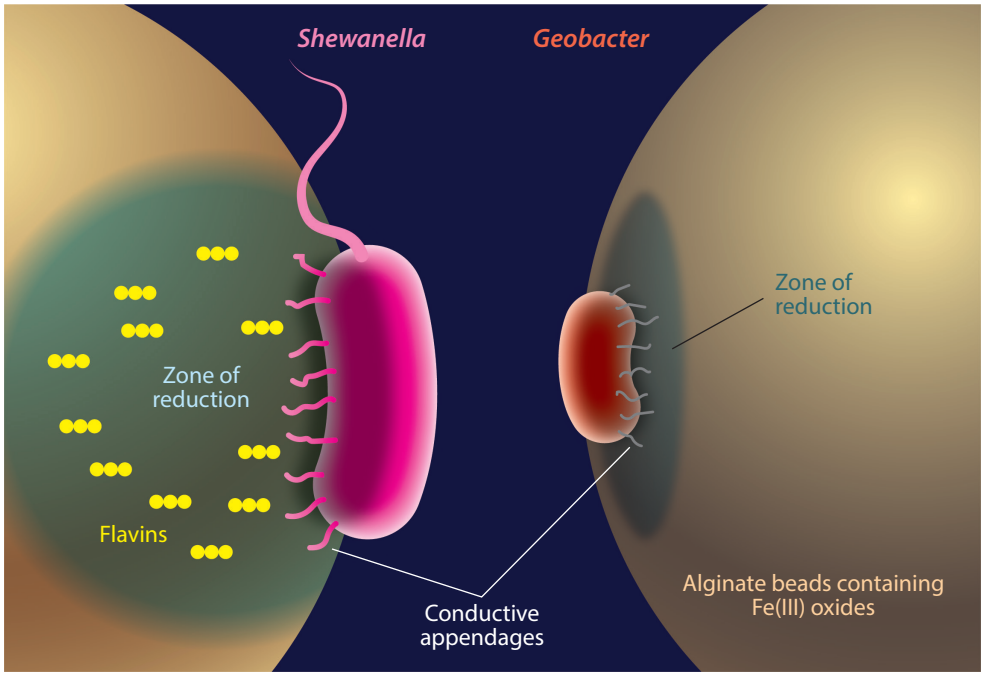

Figure 3

Fe(III)-loaded alginate beads have been used to help differentiate between direct and shuttle-mediated mechanisms for Fe(III) reduction by bacteria. *Shewanella* cells (*left*) are able to access Fe(III) embedded deep within porous matrices, resulting in a large zone of reduction, while *Geobacter* cells (*right*) require direct contact with Fe(III) located at or near the surface, resulting in a smaller zone of reduction. Yellow molecules represent flavins secreted by *Shewanella*. Small structures emanating from the cells represent outer-membrane extensions or cytochrome nanowires for *Shewanella* or *Geobacter*, respectively.

a fused protein, RibBX, that appears to be repurposed for posttranscriptional regulatory control of flavin biosynthesis (11). Surprisingly the form of flavin secreted into the periplasm of *S. oneidensis* was determined to be FAD (20) by the multidrug and toxin efflux family protein Bfe (64). Periplasmic FAD is rapidly cleaved by the enzyme UshA, yielding FMN and AMP (20). Perhaps one driving force behind the secretion of FAD into the periplasm is the requirement of the unusual fumarate reductase of *Shewanella*, the flavocytochrome FccA, that requires a FAD cofactor for activity (57, 69, 92).

One criticism of the electron shuttle model of extracellular electron transfer is that a shuttle-producing cell may experience a metabolic burden, leading to a competitive disadvantage. While flavin secretion provided a strong fitness advantage when cells were respiring fumarate, which is a flavin-dependent process due to FccA, other electron acceptors did not show this effect (57), suggesting that the energy invested into the production and secretion of extracellular flavins was not significant.

Beyond a role in extracellular electron transfer, flavins may also play a role in chemotaxis and/or energy taxis to help *Shewanella* find insoluble substrates (116, 135). A cloud of flavins surrounding a potential electron acceptor would form a redox gradient—more oxidized closer to the substrate and more reduced away from it—which could be sensed and/or used by *Shewanella*. Such a model could explain observations of enhanced swimming speed exhibited by strains of *Shewanella* when approaching insoluble electron acceptors (38, 39, 116).

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3.2. Flavins and the Mtr Pathway

In *Shewanella*, there exists some critical connections between flavins and the Mtr pathway. Strains lacking Mtr components are unable to catalyze the reduction of flavins (18) and putative flavin-binding sites were identified in the initial structural work with MtrF (15). MtrC and OmcA interact with various electron shuttles, including FMN and B2, and were suggested to have a 1:1 and a 2:1 stoichiometry of FMN to protein, respectively, using stopped-flow kinetics and NMR (nuclear magnetic resonance) (117). Edwards et al. (25) found that MtrC binds a single molecule of either FMN or B2, but only in the presence of the cellular reductant glutathione, which is thought to reduce a disulfide bond formed between two highly conserved cysteine residues. They observed similar flavin binding with OmcA and, to a lesser extent, with MtrF and UndA. They hypothesized that disulfide bond formation results in less favorable flavin binding under oxidizing conditions, whereas the highly reactive flavocytochrome would be made only under reducing conditions where the disulfide bond is not formed. The dynamics of disulfide bond formation and flavin binding appear to act as a throttle to help control electron transfer through these outer membrane decaheme proteins. Flavin binding is also consistent with electrochemical observations where a bound semiquinone form of flavin was observed to enhance the rate of EET (113, 114, 152).

3.3. Electron Shuttles in *Geobacter*

With the exception of *Geobacter uraniireducens*, which may secrete soluble FMN (44, 140), no *Geobacter* strain has been shown to reduce Fe(III) trapped in alginate beads, to be stimulated by addition of spent growth medium, or to be negatively affected when medium is exchanged from anode biofilms (7–9, 36, 109, 110). Based on these experiments, and measurements of culture supernatants (100), there is little evidence that *G. sulfurreducens* secretes abundant soluble extracellular compounds (>20 nM) to assist reduction of insoluble acceptors. However, addition of chelators or redox-active shuttles will strongly accelerate rates of Fe(III) oxide reduction (16, 76–79, 125). Naturally occurring humic substances have weak chelating ability, and abundant quinone groups at midpoint potentials that can act as electron acceptors for *Geobacter* (125). As humics are able to be oxidized by most Fe(III) forms, *Geobacter* likely utilizes them as electron shuttles in the environment (4).

Another possible extracellular mediator, although much more expensive for a cell than a small molecule, is a loosely attached or soluble extracellular cytochrome. One of the best candidates for this role is the *c*-type triheme lipoprotein PgcA (GSU1761). PgcA expression and abundance increase dramatically when insoluble Fe(III) oxides are the acceptor (2, 22, 58, 107), and selection for rapid growth with Fe(III) oxides enriches for upstream cAMP-GMP binding riboswitch mutations that greatly enhance *pgcA* expression in both *G. sulfurreducens* PCA and KN400 (134, 143). Mutants lacking *pgcA* are impaired in Fe(III) oxide reduction but are unaffected in growth with soluble metals or electrodes (155). The fact that accidental laboratory evolution can select for large changes in *pgcA* expression leads to phenotypic differences that can hide other phenotypes, such as the requirement for extracellular nanowires.

PgcA is produced as a 57-kDa lipoprotein, and processed to a smaller, non-lipoprotein form that can be detected in culture supernatants (134, 143, 154, 155). PgcA expressed and purified from *Shewanella* shows affinity for some laboratory Fe(III) oxides, but not bacterially produced magnetite. When added back to resting cell suspensions of both wild-type and Δ*pgcA G. sulfurreducens*, purified PgcA increases Fe(III) reduction rates similar to when FMN is added. The predicted structure of PgcA suggests each heme is separated by a long, flexible string of up to 29 proline-threonine repetitions. Similar repeats were reported by Lower et al. (89) to induce hydrogen bonding with metal oxide surfaces, and short threonine-proline-serine motifs are also

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found near exposed heme groups in the *S. oneidensis* OmcA crystal structure (24). These features indicate PgcA could act as a mobile shuttle between cells and Fe(III) oxides, using its natural affinity to limit loss to the environment, but such a strategy would be most useful under artificial conditions where cell densities are high and secreted proteins remain contained in a test tube.

While free FMN in *G. sulfurreducens* cultures is typically near or below the limits of detection (20–50 nM) (44, 100), a few reports indicate flavins could still participate in some aspects of *Geobacter* electron transfer. A hypothesis based on electrochemical data suggests *G. sulfurreducens* cytochromes or biofilm components might bind flavins to accelerate interfacial electron transfer (115), and riboflavin appears to bind OmcZ when purified from *E. coli* (142). Soluble FMN has been detected in cocultures of *G. metallireducens* and *G. sulfurreducens* after evolution for interspecies electron transfer, and additional exogenous riboflavin can improve growth of such syntrophic cocultures (45). No flavin secretion systems or flavin-binding sites in cytochrome structures of *Geobacter* are known, but these molecules are easily released by cell lysis and are highly facile at interfacial electron transfer, so flavins should be considered present in most laboratory experiments.

4. APPENDAGES: REACH OUT AND TOUCH

4.1. Microbial Nanowires in *Geobacter*

The need for outer membrane cytochrome conduits shows that Fe(III) reduction, including reduction of soluble and chelated forms, primarily occurs at or beyond the outer membrane. The inability of *Geobacter* to reduce Fe(III) oxides entrapped within alginate (109, 110) or agar (136) implies additional components are required to facilitate contact or attachment to insoluble acceptors. In 2005, cells undergoing metal reduction were shown to release two abundant cytochromes when subjected to shearing treatments (99). Deletion of these cytochromes, named OmcE and OmcS, affected reduction of Fe(III) oxides, but not soluble Fe(III) (99). A few months later, a new paradigm in extracellular electron transfer was introduced, as similar shearing experiments identified filaments with surprising electron transport properties under conductive probe atomic force microscopy, compared to globular materials or proteins sheared from other cells. These filaments were named nanowires. Insertional deletion of the *pilA-N* pilin gene (GSU1496) within a type IV pili operon eliminated most of these filaments and impaired growth with both Fe(III) oxides and electrodes (121). The hypothesis that bacteria utilized nanowires revolutionized all models of *Geobacter* electron transfer, as nanowires would eliminate the need for soluble shuttles to access materials not in contact with the cell and explain how cells formed conductive biofilms. Simply as an energetic solution, filling the space between cells and metals with enough redox proteins to create conductivity, akin to redox hydrogels, would take more protein than exists inside cells. Creating wires from proteins on the order of 3–4 nm per subunit could reach 1 μm beyond a cell for the cost of only ~300 proteins.

A great body of work exists regarding the hypothesis that nanowires are conductive filaments composed of a single pilin subunit (PilA-N). This is documented and well summarized in multiple reviews (80–85, 95), as well as in critiques of other publications (75, 86, 87, 94, 137). There is little question that *Geobacter* produces nanowires. Mutations in pili genes usually affect *Geobacter*'s ability to reduce metal oxides, form biofilms on electrodes, and produce filaments of different sizes and conductivities. What complicates interpretation of these studies is the fact that *G. sulfurreducens* also produces multiheme cytochrome filaments with dimensions and properties nearly identical to what were previously assumed to be pili (30, 148). In some cases, electron micrographs can be reanalyzed to show that images labeled as pili are actually cytochrome nanowires (149). Cryo-EM has shown that sheared preparations also contain abundant B-DNA

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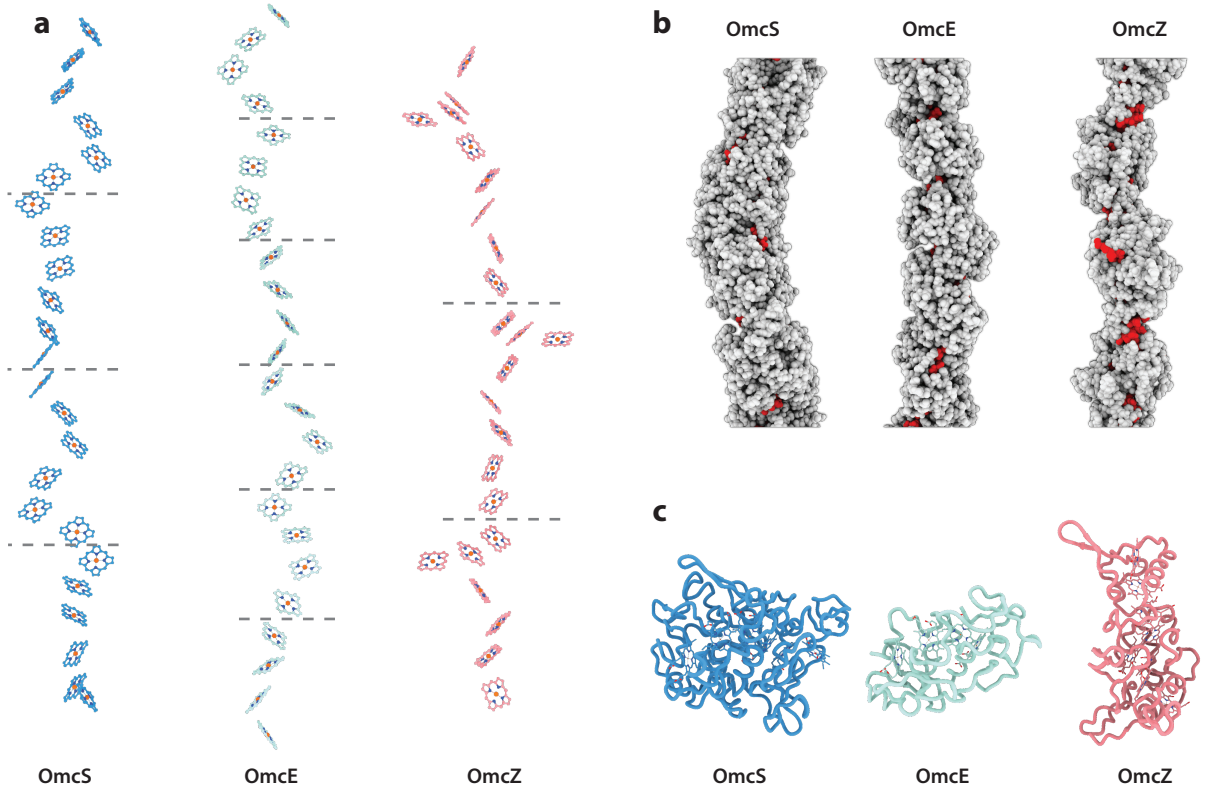

Figure 4

Cytochrome nanowires from *Geobacter sulfurreducens*. (*a*) Packing of *c*-type hemes in the three wire structures, with dashed lines showing interfaces of individual subunits. Wires are rotated to show overlapping OmcE and OmcS motifs within the fourth OmcE subunit. (*b*) Structures of the three nanowires showing differences in thickness, shape, and solvent exposure of hemes (*red*). (*c*) Structure of individual subunits for the hexaheme OmcS, tetraheme OmcE, and octoheme OmcZ. Figure adapted from Reference 150 (CC BY 4.0).

with similar dimensions as cytochrome nanowires and hypothetical pili (37, 149). The production of many extracellular cytochromes is affected by even subtle modifications of pilins, such as in the case where single–amino acid changes within the pili sequence alter the ratio of different extracellular cytochrome nanowires (153). There are no solved structures or publicly shared cryo-EM images for any *Geobacter* PilA-N-based nanowire at this time.

To date, three solved structures for cytochrome nanowires are available. The first two consist of OmcE (149) and OmcS (30, 148), the cytochromes originally found by shearing proteins from metal-reducing *Geobacter*. The third nanowire is OmcZ (150), a cytochrome essential for formation of conductive electrode biofilms (111) (**Figure 4**). The structure of the *G. sulfurreducens* type IV pilus is also known, but it is much larger than predicted and comprised of both PilA-N and PilA-C subunits. This differs from the in silico models that propose electron transport could occur through a thinner, conductive PilA-N-only filament (37, 150). As the identified type IV pilus is nonconductive, one hypothesis is that it plays a secretory or regulatory role, explaining defects in cytochrome production when pili are deleted, but this remains unconfirmed (37, 46, 63, 74, 124).

As evidenced by the electron transport properties of synthetic nanofibers comprised of aromatic-free peptides (47), CsgA curli fibers (56), tetratricopeptide repeat proteins (157), and even individual proteins lacking any redox cofactors (156), *c*-type hemes are not needed for

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laboratory demonstrations of electron transport through a protein. To distinguish cytochrome nanowires and known forms of type IV pili from previous work with theories of conductive pili, the term e-pili is used to refer to filaments hypothesized to form from only the PilA-N subunit to make a conductive protein that is proposed to have metallic qualities (43, 145). Models of e-pili filaments place aromatic residues in a central core to justify the hypothesized conductivity, but these models lack features seen in all homologous bacterial and archaeal pilin structures, including the melting and unraveling of the core α helix in order to polymerize into a mature filament (28, 29, 33, 96, 146). Advancements in cryo-EM or isolation of an intact PilA-N-only filament from cells will help resolve this mystery.

4.2. The OmcE and OmcS Cytochrome Nanowire Family

The tetraheme OmcE nanowire differs from the hexaheme OmcS nanowire in both size and morphology, with a rise per subunit of ~34 Å with 6.1 subunits per turn for OmcE, compared to a 46.7 Å rise per subunit and 4.3 subunits per turn in OmcS (**Figure 4**). OmcE filaments, which are ~3 nm at their thinnest point and ~6 nm at their thickest, are easily distinguished from ~5–7.5-nm OmcS in EM images. Due to size and characteristic sinusoidal morphology, images labeled as pili can now be identified retroactively as OmcS (68, 72, 93). In some cases (such as the case of the strain containing the Aro-5 mutant pilus lacking aromatic residues in Reference 96), images can be used to compute an averaged power spectrum to conclusively identify the filaments as OmcS.

OmcE and OmcS lack any similarity in overall appearance of their filaments, internal protein fold, thickness, helical pitch, and primary sequence. However, the four-heme motif within OmcE subunits can be superimposed on the first four hemes within OmcS. Atom pairs from coordinating cysteine and histidine residues also show three-dimensional alignment between the two proteins (149).

A unique feature shared by both OmcE and OmcS is coordination of a heme in one subunit by a histidine from the next, stabilizing the interface and helping bring all hemes within 3–5 Å (edge-to-edge) for rapid electron transfer through the continuous bis-His heme core (30, 148, 149). Both proteins lack significant amounts of secondary structure (such as only 13% helices and 6% β strands in OmcS), a trait that should tolerate mutations, insertions, and deletions in unstructured regions. This could help explain the great sequence divergence between OmcE and OmcS while maintaining the same heme packing arrangement. On their surfaces, additional electron density suggests at least five exposed residues of OmcE are modified by glycans, while two residues are modified on OmcS by sugars or other small molecules (30, 148, 149).

4.3. The Unique Structure of the OmcZ Nanowire

The structure, dimensions, and heme packing of the OmcZ filament show no relation to OmcE or OmcS. OmcZ forms a 3- to 5-nm fiber with a rise of 58 Å between adjacent subunits. Unlike OmcE and OmcS, there is no coordination of hemes between subunits. The interfacial buried area of OmcZ is similar to that of OmcE (~1100 Å^2) and smaller than the OmcS interface (~1900 Å^2). All eight hemes of the OmcZ subunit are bis-His coordinated, with a unique heme (heme 6) in each subunit that does not fit into the central linear heme chain. All hemes in OmcZ are thus close to two other hemes, with the exception of the off-axis heme, which is only accessible to heme 5 at an edge-to-edge distance of 4.6 Å. The extra heme off the central core gives OmcZ a distinctive z shape in EM images (150, 153).

Unlike OmcE/S, which share a conserved heme motif, no hemes or OmcZ can be aligned with other nanowires. However, all three nanowires do share a general pattern common to most multiheme proteins where a pair of parallel hemes are followed by another doublet of parallel

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hemes. As the second pair is roughly perpendicular to the first, this is sometimes referred to as a T-shape repeat (51). Technically, pairs of hemes that appear parallel are rotated nearly 180° with respect to each other, making them anti-parallel, and this heme-heme angle pattern is seen in nearly every multiheme cytochrome in the Protein Data Bank, as discussed in References 51 and 149. In all nanowires, there is an antiparallel pair at the interface. Also different from OmcE and OmcS, OmcZ is first produced as an ~50-kDa protein and then posttranslationally cleaved to reveal the 30-kDa heme–containing monomer, after which there are no further modifications.

A striking feature of OmcZ is the solvent exposure of heme 6, which is estimated at 326 $Å^2$, on the order of the periplasmic-exposed heme in MtrA (292 $Å^2$), and greater than any in OmcE (27–85 $Å^2$) or OmcS (52–142 $Å^2$). This exposure should facilitate electron transfer to other OmcZ wires, and/or to electrodes. Individual OmcZ filaments are reported to be much more conductive than OmcS (153). As there is little in the structure to explain this measurement, one hypothesis is that the solvent exposure of OmcZ hemes improves electron exchange with surfaces used to estimate electron transport properties.

4.4. Physiological Roles of OmcS, OmcE, and OmcZ

The new nanowire structures, pilus structures, and reports of DNA in samples resolve a long-standing mystery regarding the identity of most filaments on the *Geobacter* cell exterior, and the conductivity of cytochrome nanowires strongly suggests a role in electron transfer. However, many of these cytochromes are simultaneously expressed by cells, and their production can be affected by deletion of other cytochromes or pili, clouding results from genetic data. For example, deletion of a gene for a small monoheme cytochrome (*omcF*) increases transcript levels of both *omcS* and *omcE* (59), although neither OmcS nor OmcE is then detected on the outer surface of an *omcF* mutant. Deletion of *omcE* can increase levels of OmcS on the outer surface, while deletion of *omcS* increases OmcE (61). Deletion of *pilA-N* leads to loss of OmcS and OmcZ filaments from the cell surface (37). Mutation of aromatic amino acids within the PilA-N protein causes cells to produce more OmcZ filaments (153). Effects such as these make it difficult to interpret results from single deletions, especially those conducted before cytochrome nanowires were known to exist.

Like the highly expressed *omcB* conduit, both *omcE* and *omcS* are upregulated during growth with extracellular acceptors, even when genetic data show they are unnecessary. It is worth considering that growth with metal oxides, compared to soluble or easily reduced compounds, could be viewed by the cell as an energy-limited or acceptor-limited condition, rather than a response to a metal per se. Expression of *omcE* is substantial during growth with both fumarate and Fe(III) citrate and increases threefold during Fe(III) oxide growth, but it increases as much as ninefold with electrodes [compared to Fe(III) citrate] (2, 42, 99). However, OmcE has only been shown to be necessary for Fe(III) oxide reduction. OmcS transcripts are also present during growth with fumarate and electrodes but repressed during growth with Fe(III) citrate. This repression partially explains large increases (13- to 28-fold) reported during growth with insoluble metal oxides, as they are compared to the repressed Fe(III) citrate condition rather than fumarate (2, 42, 99). OmcS can also be one of the most abundant cytochromes in the proteome of Fe(III) oxide–grown cultures when compared to Fe(III) citrate–grown cultures (22) and can be visualized with gold-conjugated antibodies extending from cells undergoing Fe(III) oxide reduction (67). Deletion of either *omcE* or *omcS* has been linked to defects in reduction of Fe(III) oxides, Mn(IV) oxides, U(VI), and humic acids (2, 126–128), but in each case, cells still retained at least one of the two nanowires.

The *omcZ* gene is upregulated over 40-fold in conductive electrode biofilms (31, 111), and it is abundant in the extracellular matrix between electrode-grown cells (48, 120). In fact, samples for atomic force microscopy imaging and OmcZ structure determination have been obtainable only from anode biofilms (150, 153). OmcZ is the only cytochrome out of over 80 multiheme proteins

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in the *G. sulfurreducens* genome for which there is strong evidence that it is necessary for long-distance conductivity during electrode growth (111, 118, 123). Deletion of *omcZ* primarily affects anodic electron transfer to electrodes at low redox potentials [<0 V versus standard hydrogen electrode (SHE)], and it also slows cathodic corrosion of Fe^0 (141). In most studies, *omcZ* deletion does not affect reduction of other extracellular metals, with the caveat again that such single-deletion strains still have at least two other nanowires, plus the extracellular triheme cytochrome PgcA, and other extracellular proteins (2). The fact that OmcE, OmcS, OmcZ, OmcB, and PilA functions are deeply intertwined is further illustrated by the fact that deletion of the GSU1771 transcriptional regulator triggers 5- to 100-fold changes in expression of all five of these genes (41, 143).

4.5. Microbial Nanowires in *Shewanella*

While initial observations suggested that *S. oneidensis* made nanowires similar to the protein-based conductive structures of *Geobacter* (34), these structures were later determined to be extensions of the outer membrane that contain membrane-embedded porin-cytochrome components in addition to soluble periplasmic proteins (119). Conductivity across (34) and along (27) these structures in a manner that requires Mtr pathway components was demonstrated. The outer membrane extensions are related to the production of outer membrane vesicles (139), which have previously been shown to be capable of EET to iron minerals (35). Mobility of the outer membrane multiheme cytochromes MtrC and OmcA was observed in both the outer membrane and outer membrane extensions in experiments using single-molecule tracking (14). EET activity in *Shewanella* integrates electron transfer into Mtr conduits, interactions between conduit complexes and mobility of these complexes (MtrCAB), and individual redox-active proteins (OmcA) within the outer membrane architecture.

5. CONCLUDING REMARKS

In the last few years, structural biology has dramatically altered our understanding of electron transfer out of *Shewanella* and *Geobacter*. While these organisms share common porin-cytochrome strategies creating conductive pathways across their outer membranes, these proteins appear to be evolutionarily unrelated to each other. In addition, there remains no explanation for why these organisms encode many versions of the same homologous conduit, as in the case of *Shewanella*'s MtrCAB and MtrDEF conduits, and *Geobacter*'s OmcB and OmcC conduits. It is unclear what role(s) can be attributed to accessory outer membrane multiheme cytochromes such as OmcA, or why there is broad diversity for this specific component across the genus. An even higher diversity of cytochromes exists in the *Geobacter* membrane.

Beyond the cell, electron transfer by *Shewanella* is a blend of direct and indirect electron transfer, where flavins influence both processes in important ways. In *Geobacter*, electron transfer is primarily based on extracellular proteins and nanowire extensions, with little evidence for the use of soluble flavins. While we know the identities and structures of three nanowire filaments so far, how electrons enter and leave the filaments is not well understood, nor is how they are assembled or organized in three-dimensional space within a biofilm. A key finding from all these new structures is that, while at the primary sequence level many of these cytochromes may look very different or even unrelated, they can have highly similar heme arrangements and functions. This uniquely low level of sequence conservation has caused multiheme cytochromes to be overlooked in most metagenomic surveys, and ignored when inferring the metabolism of uncultured organisms, suggesting a large number of organisms and new mechanisms catalyzing extracellular electron transfer remain to be discovered.

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