**Plugging into Bacterial Nanowires: A Comparison of Model Electrogenic Organisms.**

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**Abstract**

Extracellular electron transport (EET) is an important metabolic process used by many bacteria to remove excess electrons generated through cellular metabolism. However, there is still limited understanding about how the molecular mechanisms used to export electrons impact cellular metabolism. Here the EET pathways of two of the best-studied electrogenic organisms, *Shewanella oneidensis* and *Geobacter sulferreducens,* are described. Both organisms have superficially similar overall EET routes, but differ in the mechanisms used to oxidise menaquinol, transfer electrons across the outer membrane and reduce extracellular substrates. These mechanistic differences substantially impact both substrate choice and bacterial lifestyle.

**Introduction**

An increasing number of bacteria have been revealed to be capable of extracellular electron transfer (EET). This allows for catabolically derived electrons to be removed from the cell, or for electrons to be imported to the cell interior for both catabolic and anabolic reactions [1,2]. These processes have potential applications in a wide range to technologies from microbial fuel cells and wastewater treatment to bioelectrosynthesis of valuable chemicals [3]. EET requires electrons to be carefully transported through electron transport networks between the cellular matrix and the extracellular environment. For Gram negative bacteria, the outer membrane represents an insulating barrier through which specific electron transfer mechanisms are required, while for Gram positive bacteria, EET must proceed through mediators through the insulative cell wall [1].

The best understood organisms for the study of EET are the bacterial families *Shewanella* and *Geobacter* spp*.* Both were discovered in 1980s when they attracted substantial interest due to their ability to respire on insoluble metal oxides [4]. Since then, there has been extensive research on both their molecular physiology and metabolism, allowing an understanding of their respiratory pathways to be developed [5]. In particular, the EET pathways of the model strains *Shewanella oneidensis* MR-1and *Geobacter sulfurreducens* PCA have been studied in detail. In both organisms’ electrons derived from oxidation of organic molecules are transferred to the menaquinone pool and then moved into periplasmic cytochrome shuttles. These electrons are then exchanged into transmembrane nanowires that bridge the gap between cell surface and environmental substrate.

While broadly similar, mechanisms by which both model organisms couple energy conservation to EET reveal significant differences. This review compares the electron transfer pathways used by both organisms to facilitate reduction of extracellular electron acceptors.

**EET by the model bacterium *Shewanella oneidensis* MR-1**

For *S. oneidensis*, the anaerobic EET pathway used to reduce terminal electron acceptors has been largely defined (Figure 1). Organic acids including lactate are oxidised in the cytoplasm during substrate-level phosphorylation and electrons released are transported to menaquinone in the cytoplasmic membrane [6]. Menaquinol is oxidised on the periplasmic side of the cytoplasmic membrane by CymA, a quinol dehydrogenase [7]. Unlike quinol dehydrogenases in other bacteria, which typically reduce a single electron acceptor, CymA functions as an electron transfer hub, transferring electrons to a range of different periplasmic cytochromes [8]. For EET the most significant are two tetraheme cytochromes: fumarate reductase (FccA) and Small Tetraheme Cytochrome (STC or CctA). These two cytochromes are abundant in the periplasm and transfer electrons from CymA to the decaheme cytochrome MtrA at the periplasmic side of the outer membrane [9,10]. NMR studies on interactions between MtrA, STC and FccA revealed electron transfer through these cytochromes are dominated by transient interactions instead of through stablised complexes [9,11].

MtrA is part of the heterotrimeric MtrCAB complex, a 17 nm nanowire responsible for translocation of electrons through the outer membrane [10,12]. MtrA is held across the membrane by a 26 β-strand porin, MtrB, that insulates the cytochrome from the outer membrane environment [1,10,13,14]. The MtrA component accepts electrons from both STC and FccA and moves them across the membrane to the surface of the cell into a second decaheme cytochrome, MtrC on the extracellular side of the outer membrane [11].

Recently a cellular turnover of 6.4 x 106 ATP s-1 was reported for *Escherichia coli* during exponential growth phase [15]. Based on this rate of turnover, it is interesting to consider whether the rate and amount of EET through MtrCAB would be able to support *S. oneidensis* with a similar metabolic requirement for ATP. Both *S. oneidensis* and *E. coli* are Gammaproteobacteria with comparable generation times, so the rate of ATP production may also be comparable.The electron transfer rates through MtrCAB have now been calculated to be 3 x 104 s-1, with the limiting step caused by the heme-edge distance between MtrC and MtrA [16]. In previous studies, *S. oneidensis* was estimated to contain approximately 70,000 MtrC molecules per cell [17]. As *mtrC* is in the same operon as *mtrA* and *mtrB* their expression is likely to be similar, suggesting that there could be a maximum of 70,000 MtrCAB complexes in the outer membrane. An individual *S. oneidensis* cell could therefore have a theoretical maximum electron flux of 2.1 x 109 e s-1. Assuming *S. oneidensis* has a similar ATP requirement to *E. coli* and that 2 e- are produced during for every ATP produced, then the rate of cellular e- generation during exponential growth would be 1.3 x 107 e s-1 approximately 160 times lower than the maximum rate of electron transfer through MtrCAB across the cell membrane. These metabolic rates are also comparable to maximal cellular *S. oneidensis*  currents of 1.2 x 10-18 A cell-1 s-1, which correspond to a cellular EET rate of 9.9 x 106 e s-1 [18] These values demonstrate that electron transfer through the hard-wired MtrCAB is not limiting during EET.

**EET by the model bacterium *Geobacter sulferreducens* PCA**

In contrast to *S. oneidensis,* the EET pathway through *G. sulferreducens* is substantially more complex (Figure 2). *G. sulferreducens* can use acetate as an energy source and ATP generation is completely dependent on EET [19]. Acetate is converted into acetyl-CoA and then oxidised to CO2 via the TCA cycle, reducing NAD+ to NADH that is used to transfer electrons to menaquinone via the proton pumping NADH dehydrogenase Nuo-1 [20]. In contrast to *Shewanella*, *G. sulferreducens* uses three different quinol dehydrogenases [21,22]. These quinol dehydrogenases, called CbcL, CbcBA and ImcH, are expressed at different extracellular potentials, presumably allowing for energy conservation between menaquinol and the extracellular substrate. The periplasm of *G. sulfurreducens* PCA contains a range of different multiheme cytochromes expressed under different conditions. Known and characterised cytochromes include the tri-heme families of PpcA and GSU0105 cytochromes [23-25]. In addition to these tri-heme cytochromes, a periplasmic dodeca-heme cytochrome GSU1996 has also been characterised. This 12 nm cytochrome has been proposed to act as a nanowire, allowing for rapid electron transfer across the periplasm, and is one of a number of periplasmic multi-heme cytochromes that are encoded by the genome of *G. sulferreducens* [26,27]*.* It is generally accepted that these periplasmic cytochromes facilitate electron transfer between quinol dehydrogenases and the outer membrane. The genome of *G. sulferreducens* contains gene clusters for five outer membrane porin-cytochrome complexes [28]. Deletion of all five gene clusters resulted in a *G. sulferreducens* strain unable to reduce extracellular substrates. The potentials and properties of most of the cytochromes that make up these complexes have not yet been defined, but their functions have been linked to a range of substrates with different potentials. The *Geobacter* porin-cytochrome gene clusters appear constitutively expressed and their deletion affects the reduction of specific extracellular substrates such as Fe(III)citrate or low-potential graphite electrodes [29]. These porin cytochrome complexes play an essential role in in controlling EET to the cell surface where electrons can be directly or indirectly transferred to a range of potential extracellular acceptors.

**Differences in *Shewanella* and *Geobacter* metabolic pathways are linked to bacterial lifestyles.**

The fundamental differences in the EET machinery between *S. oneidensis* MR-1 and *G. sulfurrenducens* PCA are observed in the electron transfer proteins of both membranes: in *Shewanella* menaquinol oxidation is through CymA, while *Geobacter* uses CbcL, CbcBA or ImcH. The porin-cytochrome complexes that transfer electrons across the membrane also vary, with *Shewanella* predominantly expressing the single MtrCAB complex, and *Geobacter* expressing multiple complexes that appear more effective at different potentials. These differences directly impact the lifestyles and mechanisms of EET of these two bacteria.

In the anoxic zone *S. oneidensis* makes most of its energy from substrate level phosphorylation during oxidation of lactate to acetate. The electrons released from this are not required for ATP generation, and so the cell has a simplified mechanism for disposing of electrons across a range of potentials. This allows the highly motile *Shewanella* to interact with different substrates across a range of potentials without having to significantly alter its respiratory pathways [30]. In contrast *G. sulfurreducens* generates ATP through the generation of a proton motive force and utilises different EET pathways from the quinol pool depending on the relative potential of the extracellular acceptor [22]. Switching pathways would require substantial energy for protein synthesis and is a likely strategy suitable for a bacterium in a constant environment. Interestingly these changes have significant impacts on the cells ability to grow and survive in these environments. *S. oneidensis* has been shown to grow on anodes poised between +0.74 and – 0.19 V, in agreement with flavin acting as a mediator between MtrC and the terminal electron acceptor [31], while *G. sulfurreducens* can grow on more negative anodes ranging in potential from +0.4 to −0.3 V. In addition the biomass of anodic *G. sulfurreducens* is approximately the same independent of potential [32] while the growth of biomass by a related *Shewanella* species (*Shewanella putrefaciens*) was dependent on anodic potential [33]. This may be linked to the different metabolic pathways, with *Geobacter* maintaining a constant growth by adjusting its metabolic pathways to balance energy conservation when using extracellular substrate with different potentials.

**Role of extracellular nanowires at the cell surface of *S. oneidensis* and *G. sulferreducens.***

Once electrons are transported to the cell surface, both *S. oneidensis and G. sulferreducens* use dedicated nanowire systems that exchange electrons with extracellular electron donors (Figure 3). For *S. oneidensis* the arrangement of the MtrCAB nanowire raises the MtrC domain approximately 100 Å out of the outer membrane [12]. MtrC is oriented to allow it to directly reduce small soluble metals, such as soluble metal chelates and radionuclides [34,35]. For insoluble metal oxides the mechanism is more complex. *S. oneidensis* has a dedicated transporter that secretes soluble flavins which enhance the rate of insoluble Fe(III)oxide reduction [36,37], however there is evidence that MtrC may also form a catalytic flavin-cytochrome complex that can directly reduce metal oxides [38,39]. A second extracellular cytochrome, OmcA, which is part of the gene cluster can also receive electrons from the MtrCAB complex [35]. OmcA interacts with MtrCAB transiently and facilitates the rapid exchange of electrons across the cell surface. Remarkably, *S. oneidensis* has been shown to generate µm length membrane tubules to interact with nearby cells substrates [40]. These tubules contain MtrCAB and OmcA, and electrons are transported via these cytochromes.

The cell surface of *G. sulfurreducens* is also covered by a range of cytochromes, some of these include the nanowires that are part of the five ExtD or OmbA/OmcB families of porin cytochrome complexes, while other extracellular cytochromes such as PgcA are important for the reduction of specific minerals [41]. These bacterial nanowires are likely to be important for *G. sulfurreducens* cellsthat are in close contact (~10 nm) with minerals. For longer distances *G. sulfurreducens* PCAgenerates conductive filamentous nanowires for electron transfer across intermediate distances (>100 nm) between cells and terminal electron acceptors [42]. Recent advances in Cryo-EM technologies have revealed the molecular structure of several of the filaments secreted by *G. sulfurreducens* PCA. Remarkably the first filament to be structurally characterised were shown to be composed of polymers of a hexaheme cytochrome, called OmcS [43,44]. Shortly afterwards a second, thinner filament was shown to be a polymer of the octaheme OmcZ [45]. These remarkable structural observations are hard to reconcile with earlier reports that indicate that the filaments are conductive pilin made from PilA-N monomers [46,47]. The potential conductive properties of pilin and cytochrome nanowires are consequently strongly debated, with *G. sulfurreducens* pilin conductivity occurring through aromatic groups, and OmcS/Z nanowire conductivity through the closely packed hemes in the protein core [48,49]. Potentially both filaments could be conductive, as recent studies suggest that the interior of proteins are more conductive than previously thought, with aromatic residues being the key [50]; this observation likely explains the conductivity of amyloid fibrils [51].

If proteins are inherently conductive then why would *G. sulferreducens* need tosynthesise cytochrome polymers? The intrinsic conductivities may not represent the conductivity *in vivo*, and cannot explain how electrons would enter and leave these structures. The closely packed OmcS/OmcZ heme core may also provide a highly conductive electron channel through the closely packed heme porphyrins within an insulative protein shell, while electron transfer through pili could be more uncontrolled. To further complicate matters, the first structure of a *G. sulfurreducens* PilA-N-PilA-C pilin structure was recently published [52] and showed that this conformation of pilin was unlikely to have a role in electron transfer. Gu and colleagues suggested the previous reports of EET mediated by PilA could be explained if PilA and PilC were involved in the transport of fully assembled OmcS or OmcZ [52]. Alternatively, the pili may facilitate the positioning of OmcS and OmcZ nanowires away from the cell and preventing them from adhering to the cell surface.

**Conclusion**

It is clear that EET can be coupled to both oxidative and substrate-level phosphorylation, with cellular clearance of reducing equivalents being potentially orders of magnitude greater than their formation. The limiting step of EET is therefore likely to be the accessibility and redox potential of the environmental substrates. For *S. oneidensis* the properties of extracellular cytochromes involved are well defined, although the exact mechanisms are still poorly understood. For *G. sulfurreducens* the extracellular mechanisms are much less understood, mainly due to the inherent complexity of the *G. sulfurreducens* EET system that scientists have had to grapple with over many years. The elucidation of the OmcS/OmcZ stuctures, and identification of the porin-cytochrome nanowires provide new information to further understand how electrons are transferred from the cell surface to different extracellular acceptors in this intriguing organism.

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Papers of particular interest, published within the period of review, have been highlighted as:

 • of special interest

 •• of outstanding interest

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Figure 1: Metabolic EET pathway of *Shewanella oneidensis* MR-1. Lactate is oxidised to acetate to generate ATP. Menaquinol (MQH2) is oxidised by CymA and electrons are then exchanged between cytochromes (red) to the cell surface, where they directly or indirectly reduce extracellular substrates. DLD, D-lactate dehydrogenase; PFL, pyruvate-formate lyase; PDH, pyruvate dehydrogenase; PTA, phospho-transacetylase; APK, acetate kinase; STC, small tetraheme cytochrome

Figure 2: Metabolic EET pathway of *Geobacter sulferreducens PCA*. Acetate is phosphorylated and enters the TCA cycle via Acetyl-CoA or Succinyl-CoA (not shown). NADH dehydrogenase, CbcA, CbCL and ImcH generate a proton gradient that is used to drive ATP production. Electrons are transported through periplasmic cytochromes and then to a series of Porin-cytochrome complexes in the outer membrane. Porins are represented by blue ovals. APK, acetate kinase; PTA, phosphotransacetylase; CS, citrate synthase; NDH, NADH dehydrogenase; ATPS, ATP synthase.

Figure 3: Structurally defined atomic models of nanowires involved in extracellular electron transport and substrate reduction. (A) The MtrCAB naowire (PDB ID. 6R2Q) receives electrons from the periplasm and electrons are transported using a 20 heme to the surface of the cell. OmcA (PDB ID. 4LMH) likely receives electrons from MtrC. (B) The OmcS (PDB ID. 6EF8) nanowire from *Geobacter sulfurreducens* is isolated the surface of the cell, it is proposed that OmcS is assembled inside the cell and passes through the PilA secretory system.