Book Chapter

2. Energetic and molecular constraints on the mechanism of environmental Fe(III) reduction by *Geobacter*

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2.1 Introduction

Representatives of multiple ∂-Proteobacterial genera are (i) consistently isolated from Fe(III)-reducing subsurface habitats (14-17, 61, 63, 79, 103), (ii) found to be significant members of communities in molecular studies of stimulated Fe(III)-reducing zones and bioremediation sites (4, 5, 10, 12, 26, 65, 84, 95, 100, 111, 113, 118) and (iii) are regularly enriched on electrodes poised as electron acceptors (6, 11, 21, 29, 33, 36, 46, 49, 114, 115). These bacteria are primarily known for their ability to couple complete oxidative metabolism to respiratory growth with Fe(III) oxyhydroxides, and are represented by isolates from the genera *Desulfuromonas, Geobacter, Desulfuromusa, Malonomonas, Trichlorobacter, Geopsychrobacter,* and *Geothermobacter*. The available genomes of metal-reducing *Geobacter* and *Desulfuromonas* strains all contain a conserved core of genes enabling complete acetate oxidation, accompanied by hundreds of poorly conserved multiheme *c*-type cytochromes, most of which are predicted to be localized to the outer membrane or beyond the outer surface (2, 3, 9, 40, 64, 76, 78, 109). Based on these observations, these bacteria are considered to have evolved to compete in anoxic habitats where simple fermentation end products are the electron donors, and the electron acceptors are primarily available outside the cell.

Gene phylogenies suggest that significant divergence within this group has occurred to take advantage of different environments. Marine habitats typically contain bacteria related to *Desulfuromonas* and *Desulfuromusa*, while *Geobacter spp.* are normally found in freshwater environments (9, 38). The *Geobacter* genus forms at least three distinct clades that also appear to correlate with habitat; relatives of *G. metallireducens* and *G. sulfurreducens* are associated with surficial sediments, and relatives of the more recently isolated *Geobacter psychrophilus* and *Geobacter uraniireducens* each represent separate clades usually found in subsurface aquifers

(38, 41). An extreme example of specialization are the non metal-reducing *Pelobacter* isolates, which share a common genus name due to their fermentative physiology, but are phylogenetically scattered throughout the ∂-Proteobacteria, with some related to *Geobacter* and others being close relatives of *Desulfuromonas* (8). This pattern suggests multiple independent evolutionary events have occurred in which metal reduction inherited from the common ancestor was lost (8).

Such diversity means that this collection describes a group which diverges over 10% at the 16S rRNA level, demonstrates growth between 4° and 65°C (39, 47, 79), and shows high variability in salt tolerance, substrate utilization range, and ability to transfer electrons to various acceptors in the laboratory. Given this diversity, it is perhaps no surprise that genomic and genetic analyses have failed to identify well-conserved cytochromes or putative metal-reducing proteins by comparing the genomes of these metal-reducing bacteria. However, this lack of an obvious conserved electron transfer system is in contrast to the solution recently described for the γ-Proteobacterial genus *Shewanella*, which encompasses isolates obtained from a range of ocean sediments, oxic, and fermentative environments. Despite the fact that *Shewanella* strains also display high phylogenetic and phenotypic diversity, they only retain a single conserved cytochrome conduit for electron transfer out of the cell, and largely depend on soluble flavins to move electrons beyond the cell surface (19, 20, 35, 92).

This review aims to discuss how *Geobacter* and its relatives are shaped by the nature of their electron donor and acceptor, where electrons liberated during complete cytoplasmic oxidation of organics must travel far beyond the cell to reduce extracellular metals without the aid of soluble shuttles. This sequence of reactions must occur in permanently anoxic habitats where reactant concentrations lower the ΔG of respiration to only tens of kJ/mol, severely

limiting the energy available. This review will discuss the thermodynamic constraints on environmental metal reduction, and briefly mention aspects of the molecular mechanism of electron transfer by *Geobacter spp*. when viewed through this lens.

2.2 The energetic challenge of coupling complete oxidation to Fe(III) reduction

The importance of the acetate oxidation phenotype is underscored by the enrichment of the first *Desulfuromonas* by Prennig and Biebl (85). While numerous sulfur- and sulfate-reducing bacteria capable of incomplete lactate oxidation were already known, anaerobic sulfate- or sulfur-reducing bacteria able to completely oxidize the copious amounts of acetate produced by incomplete oxidizers were lacking. *Desulfuromonas acetoxidans* provided the first answer to this mystery. Subsequent biochemical tests revealed that *D. acetooxidans* used the citric acid cycle for acetate oxidation when sulfur was the electron acceptor. This was surprising, considering the fact that the formal potentials of some steps in the citric acid cycle (such as fumarate/succinate, E°'=-32 mV) have E°' values slightly more positive than reduction of menaquinone (E°'=-74 mV), and much more positive than the terminal electron acceptor (S^0/H_2S E°'=-240 mV) (108). While changes in intracellular concentrations of reactants could help solve some of these issues, subsequent bioenergetic experiments showed the need for membrane potential to drive 'uphill' succinate oxidation, consistent with inward flux of protons being used during some steps to catalyze complete oxidation (83). Such reverse electron transport reduces the total amount of energy remaining for bacterial ATP synthesis, but ensures unfavorable reactions operate in the oxidative direction (86, 97).

The poor $\Delta G°'$ of acetate/sulfur respiration (approximately -39 kJ/mol acetate, under standard conditions), coupled with this price of reverse electron transport and the need to use at

least one ATP equivalent in activation of acetate to acetyl-CoA, leaves little free energy for respiratory ATP generation. Consistent with these findings, when committed to acetate oxidation, *D. acetoxidans* acheives less than 0.5 ATP per acetate oxidized, and respires nearly 95% of acetate to CO_2 to generate enough ATP to produce biomass from this two-carbon precursor (30, 69, 112). Despite the low apparent value of acetate under such conditions, both calculations and sediment labeling studies have shown that nearly 70% of anaerobic organic matter oxidation in sediments ultimately proceeds via anaerobic oxidation of acetate (54, 66, 81, 108).

The reduction of Fe(III) presents a thermodynamic challenge similar to that of the reduction of S^0. While the redox potential of freshly precipitated Fe(III), such as ferrihydrite, is estimated to be in the range of -100 to +100 mV (102), this window represents a best-case upper boundary of the energy available to Fe(III)-reducing organisms. More crystalline Fe(III) forms such as goethite, lepidocrocite, and hematite will have much lower formal redox potentials. With this in mind, one of the most valuable findings from recent electrochemical measurements with *Geobacter spp*, is the observation that acetate oxidation can proceed down to an electron acceptor potential of approximately -220 mV (72, 73). This value reveals that *Geobacter* conserves very little energy, around 6 kJ per electron respired, when using Fe(III) as an external electron acceptor. The advantage of such a strategy is that, in taking so little for itself, *Geobacter* guarantees that electron transfer from the cell surface will always be downhill, even to more crystalline minerals or in environments where acetate concentrations are low (sub-μM).

The final consideration that makes extracellular Fe(III) reduction difficult, from a bioenergetic perspective, is the need to perform the oxidation of organic matter (releasing protons and electrons) in the cell interior, but transfer only the negatively charged electrons to

the outside of the cell. The net effect of this reaction is accumulation of protons (and positive charge) inside the cell, acidifying the interior and cancelling out many of the later proton-pumping events occurring during respiration (69, 70). This additional cost of Fe(III) reduction appears to diminish the yield of *Geobacter* more than 50% compared to what would be predicted from standard ΔG calculations. An illustration of this phenomenon is the comparison of growth with fumarate vs. growth with Fe(III) as the terminal electron acceptor (69, 70); when expressed as biomass per electron respired, *G. sulfurreducens* produces nearly three times more cells when grown with the intracellular acceptor fumarate (E°'=-32 mV) compared to growth with the extracellular acceptor Fe(III)-citrate (E°'= +350 mV), even though fumarate supplies less potential energy according to standard calculations. Similar yields have been found for *Geobacter* grown with high-potential Fe(III)-citrate acceptors as with lower-potential electrode acceptors (E°'=0 to +200 mV), and there is no evidence *Geobacter* is able to modify the amount ATP captured from external electron acceptors based on potential. The implications of this very low energy yield impose important constraints on the possible mechanisms of metal reduction.

2.3 Moving electrons beyond the cell must require multiple attachment and redox proteins

Once electrons are released from the quinone pool to the periplasm, all energy generation steps have been completed. However, electrons must still overcome multiple independent barriers to escape. Electrons first cross the insulating outer membrane, then hop across a protein-mineral interface to the terminal electron acceptor. Decades of work with electron transfer proteins has shown that electrons require a continuous path of redox centers or sites for multistep tunneling, which must be no more than 15-18 Å apart (31, 32) . While a bacterium can ensure tight protein-protein interactions within membranes, the surface of a metal (oxyhydr)oxide

electron acceptor is highly variable and uncontrollable in terms of charge, shape, and crystal structure. A single protein complex can achieve rapid and predictable transmembrane electron flow within or across a membrane, but should we expect a single protein to exist which is able to interface with all environmental metal acceptor surfaces?

An elegant illustration of this 'surface interfacing' problem was shown in molecular simulations by Kerisit et al. (48), who found that electron transfer rates from a cytochrome to a hematite surface could vary by over six orders of magnitude, simply depending on the orientation of the exposed heme colliding with the hematite surface. Though it may be theoretically simple to occasionally bring redox centers close enough to make physical contact with a particle, even tiny differences at the interface, or defects in the attachment process can mean a ten to hundredfold difference in interfacial transfer rates. Given the variability in environmental metal oxides, this argues for some diversity in the extracellular redox proteins of non-shuttle producing bacteria.

The discovery that many Fe(III)-reducing bacteria will also attach to electrodes poised to act as electron acceptors has provided a new tool for their study, as electrochemistry can probe the relationship between interfacial electron transfer rate and driving force under highly controlled conditions (44, 72, 73, 90, 101, 117). In particular, electrochemistry has solidified three key aspects of the *Geobacter* electron transfer phenotype; First, there have been no soluble electron shuttles reported to be secreted by these bacteria. Removing the medium surrounding active *Geobacter* biofilms growing on electrodes has no effect on the rate of electron transfer at any stage of growth. Second, the interfacial electron transfer reaction, from cell surfaces to electrodes, is not rate-limiting. *Geobacter* cultures using electrodes as electron acceptors double as fast on electrodes (~ every 6 h) as they do with dissolved Fe(III)-citrate as electron acceptors,

and electrode respiration is not accelerated by addition of dissolved redox shuttles. A more formal derivation of the argument for interfacial electron transfer being non-limiting can be found in the electrochemical modeling of Strycharz et al (104). Interestingly, growth with Fe(III) oxides is always slower (doubling times ~12-24 h), but can be accelerated by dissolved electron shuttles, suggesting that a rate-limiting step with more environmentally relevant Fe(III) acceptors is related to the availability of a nearby electron acceptor surface, or travelling to the new surface, not electron transfer per se. Third, the unlimited nature of the electrode electron acceptor enables growth of thick biofilms, which has provided the proof that many *Geobacter* strains possess a between-cell conductivity able to transfer electrons between cells over distances as great as 10-20 μm.

2.4 Cytochromes and pili; often more questions than answers

If a list of proteins implicated in *Geobacter* metal reduction is made, over 15 *c*-type cytochromes (1, 50-52, 57-59, 62, 75, 99), as well as pili (45, 90), multicopper proteins (37, 74, 87), porins (1), secretion systems (74) and polysaccharide synthesis enzymes (93, 94) could be described. This has led to some confusion, and an array of sometimes conflicting hypotheses aimed at describing electron transfer. The source of this confusion is likely twofold; as mentioned previously, there is little conservation of cytochromes or other redox proteins across *Geobacter* genomes. High diversity in cytochromes involved in extracellular metal respiration has also been reported in the genomes of natural Fe(II)-oxidizing communities (22, 23), suggesting that proteins at the interface between bacteria and metals are under constant selection in response to metal structure or potential. Thus, any discussion of data derived from the most

commonly studied strain (*G. sulfurreducens*) may not necessarily apply to members of other *Geobacter* clades.

The second consideration is that, for an organism not producing a soluble shuttle, there are many discrete electron transfer challenges, related to proteins bringing electrons to the outer membrane vs. those required to interface with surfaces. The different proteins implicated in metal reduction do not need to all be involved in electron transfer, but could contribute via adhesion, localization, or secretion.

2.4.1 Escaping the cell : the example of OmcB. The best example of this confusion, and the need for caution when conducting deletion experiments, is the outer membrane dodecaheme *c*-type cytochrome OmcB. First identified via biochemical enrichment of outer membrane proteins (67, 68), immunogold labeling has confirmed that OmcB is tightly associated with the outer membrane (87). Genetic experiments showed an Δ*omcB* mutant was unable to reduce both soluble and insoluble Fe(III) (58, 87). Expression of OmcB increases when Fe(III) is the electron acceptor, especially under Fe(III)-limiting conditions (13, 116), and when cells are grown in current-producing biofilms (80).

The location of OmcB, its expression pattern, and the initial behavior of a deletion mutant is consistent with this cytochrome playing a key role in electron transfer at the outer membrane. What makes interpretation of these experiments difficult, however, is the fact that an Δ*omcB* mutant is able to easily adapt to grow using soluble Fe(III), via outgrowth of suppressor strains that appear to express homologs (such as a parologus dodecaheme *omc*C located downstream), or alternate cytochromes encoded on the genome (57, 59). Experiments such as these show that

while OmcB is important, there also may be parallel pathways, or cryptic cytochromes not normally expressed under laboratory conditions which are easily selected for in mutants.

Another example of complexity is provided by the diheme peroxidase MacA (7, 51, 82, 99). Deletion of this protein was reported to severely decrease the ability of *Geobacter* to reduce soluble and insoluble Fe(III), leading to its inclusion in some models of electron transfer out of the cytoplasmic membrane. However later studies found that transcript and protein levels of OmcB were also diminished in a Δ*macA* strain, and expression of *omcB in trans* restored Fe(III) reduction to a *macA*-deficient mutant (51). Thus, MacA was not critical for Fe(III) reduction in an electron carrying capacity, but was rather intertwined with some mechanism of *omcB* expression. Recent work has confirmed that MacA has all the characteristics of a classic diheme peroxidase, and is unlikely to be involved in electron transfer, although it is still drawn in some cartoons of *Geobacter* respiration (98).

OmcB expression, translation, or post-translational stability is further influenced by at least four other proteins. Deletion of the small monoheme cytochrome OmcF eliminates the ability of *G. sulfurreducens* to reduce Fe(III), but also prevents expression of *omcB* (50, 52). Like the MacA mutant, Δ*omcF* mutants quickly evolve to select strains in which the expression of other compensatory *c*-type cytochromes is increased, showing that OmcF is not essential. Furthermore, when two homologous cytochromes, OmcG and OmcH are deleted in tandem, soluble Fe(III) reduction is again inhibited even though *omcB* mRNA is still detected (53). However, OmcB protein levels are depleted in this strain, indicating translational or post translational regulatory mechanisms have been disrupted (53). Finally, a mutant lacking the abundant porin OmpJ shows significantly decreased rates of Fe(III) reduction, but also has a

50% reduction in heme content, and lacks high molecular-weight membrane-associated cytochromes such as OmcB (1).

Thus, many phenotypes ascribed to single proteins in *Geobacter* are now known to be due to downstream effects on OmcB. In addition, the high redundancy of cytochromes in *G. sulfurreducens* often means mutants can quickly evolve to obscure the Δ*omc*B phenotype. These factors should be taken into consideration when evaluating any disruption in electron transfer proteins in *Geobacter.*

2.4.2 Interfacing with external acceptors: the examples of OmcS vs. OmcZ. Two other cytochromes, OmcS and OmcZ, warrant mention as they have consistently been linked to reduction of insoluble metals or electrodes, respectively. The hexaheme cytochrome OmcS was originally discovered by shearing of cells (75), an observation later explained by immunogold labeling that found at least some OmcS to be arranged along pili, which are also removed by shearing approaches (60). Deletion of OmcS eliminates reduction of insoluble Fe(III), with little effect on soluble Fe(III) reduction, further suggesting it is involved in processes beyond the cell membrane (75). Proteomic studies also found OmcS to be more abundant in cells grown with insoluble Fe(III) compared to cells grown with soluble Fe(III) (24, 25). However, it is less clear if OmcS is essential for growth on electrodes, as Δ*omcS* mutants are still able to colonize electrodes and use them as electron acceptors, but are initally defective in development of thicker biofilms requiring between-cell conductivity (80, 90).

In contrast, the octoheme cytochrome OmcZ (43) is more highly expressed when cells are grown as biofilms on electrodes, and an *omcZ*-deficient mutant is unable to transfer electrons to electrodes (80, 90). The OmcZ protein is not pili-associated, but has been found distributed

throughout a polymeric matrix between cells, and especially near the electrode in biofilms (42). Also, Δ*omcZ* mutants are not severely impacted in their ability to reduce Fe(III) (80). Data such as these support the hypothesis that different extracellular electron acceptors (Fe(III) oxides vs. electrodes) and/or modes of growth (suspended Fe(III) particles vs. attached as biofilms) may require different cytochromes, further indicating that there is no one master pathway that will emerge to explain all extracellular electron transfer by *G. sulfurreducens*.

2.4.3. Other matrix components; for attachment or cell-cell electron transfer? Because filaments sheared from the surface of *G. sulfurreducens* were shown to possess conductivity across their width when probed by conducting atomic force microscopy, and such filaments could not be found in a mutant lacking the Type IV pilin protein PilA, a hypothesis emerged that pili were involved in carrying electrons to electrode surfaces and other acceptors (88, 89). In addition, more recent measurements of conductivity through *Geobacter* biofilms placed across gaps in gold electrodes has provided support for unique conductivity between cells, which has again been attributed to pili.

In support of this theory, a Δ*pilA* mutant is partially defective in Fe(III) oxide reduction, and can barely attach to electrodes. Confounding this result, however, is the fact that pili are also involved in the attachment of cells to all surfaces, and to each other (88, 89). For example, Δ*pilA* mutants cannot form robust biofilms on glass, Fe(III) oxide-coated surfaces, or electrodes, even in the presence of additional dissolved energy sources such as fumarate (55, 56, 88-90). Mutants lacking PilA also lack the ability to bind to each other in cell-cell agglutination assays. These defects in attachment and biofilm formation mean that, to study issues such as conductivity of

biofilms, reactors must be incubated for up to two months to accumulate enough cells to perform measurements.

The pili of *Geobacter* have also proven difficult to solubilize and study via traditional biochemical techniques, leading to additional uncertainty in terms of amounts present outside the cell (18). As measurements have not been made on purified pili from Δ*omcS* strains, where pili-associated OmcS could not participate in conductivity, it is not yet known if the retractable Type IV *Geobacter* pili are actively involved in electron transfer *per se*, if they serve as scaffolds for other proteins, if they mediate attachment, or are essential for bringing cells in close enough contact for robust electron transfer. More recent work has shown that Δ*pil*A mutants show defects in cytochrome secretion, which is not surprising, as Type IV pili are evolved from the Type II secretion mechansim (91). Type IV pili have been shown to be required for the secretion of extracellular proteins in a number of other bacteria (93).

Similar to the role of pili in aspects of surface binding and cytochrome function, mutants in production of cell-surface polysaccharides are defective in attachment and cytochrome localization (93, 94). Mutants in a locus encoding a series of glycosyl transferases and sugar exporters demonstrate decreased affinity for Fe(III) oxides and electrode surfaces, lowering Fe(III) reduction rates and eliminating electrode biofilm formation. These mutants also possess significantly lower amounts of cytochromes outside the cell, particularly OmcZ, which is known to be involved in electrode colonization (93, 94). These results are consistent with labeling studies showing OmcZ to be located on polymers distant from the cell.

As with cytochromes, many single mutations in pili or polysaccharides show a pattern of more broadly affecting *Geobacter's* surface charge, extracellular sugar content, and secretion of cytochromes, producing an external surface very different from the wild type (91). As the Type

IV pili system is known to be used in secretion of extracellular proteins by other bacteria (34), attention should be paid to how the extracellular matrix of *Geobacter* is assembled, and if a cascade of downstream effects result from mutations in pili or pili-like structures. Mutations which manifest as the complete failure to attach to a surface are difficult to use as evidence for, or against, the larger concept of conductivity between cells.

2.5 A final word; energetic constraints for accessing Fe(III) beyond the cell.

The laboratory demonstration of *Geobacter* cells producing thick, 20 to 50 μm thick biofilms on electrodes suggests that *Geobacter* may form multicellular biofilms on Fe(III) oxide crusts which precipitate on sand grains. In the environment, could cells be surrounded by such dense suspensions of freshly precipitated Fe(III) oxide that they need to form thick microcolonies of cells connected by conductive pathways? The fact that extracellular attachment structures such as pili and polysaccharides, as well as cytochromes distributed between cells, are needed for efficient metal reduction, reinforces the idea that somewhere in nature, cells are growing as interconnected colonies. However, basic energetic calculations do not support this model. Instead, the low ATP yield of Fe(III) reduction, coupled with the high cost of protein synthesis, provides clues as to why *Geobacter* may possess strategies for moving electrons beyond the cell membrane.

The yield of *Geobacter*, in both Fe(III)-reducing chemostats and on electrodes, shows that acetate-oxidizing cells require at least 3.33 mol electrons to synthesize a gram of cell protein (28, 69, 73, 107). Based on an estimated value of 1×10^{-13} grams protein per cell (a range also consistent with chemostat measurements of *E. coli* cell doubling at similarly slow rates), 3.3×10^{-13} mol electrons are needed to produce a cell. From this basic yield value, one can ask the

question: if *G. sulfurreducens*, which is not motile in laboratory experiments, finds itself surrounded by Fe(III) oxyhydroxide particles that occupy 50% of the volume in all dimensions (using values from goethite, which has a MW of 88.8 g/mol and density of 4.26 g/cm^3), how many electrons can it transfer to the Fe(III) in contact with the cell membrane (i.e. forming a skin a few nm thick around the cell? The answer is, perhaps, surprising; this Fe(III) would not support synthesis of even a few percent of a new cell. In fact, we need to expand the volume a cell has access to outward in all dimensions to satisfy the needs of a single cell. Again, assuming 50% of the space around a cell is occupied with an Fe(III) oxyhydroxide, it would need to reduce all Fe(III) available in the space extending 2-4 μm *in all directions* beyond the outer membrane to access enough acceptor to even approach the ATP requirement for a single cell doubling.

In other words, the layer of Fe(III) that can make contact with the outer membrane of *Geobacter* is not sufficient to support growth, nor is the Fe(III) extending a cell length away. Instead, cells must access a space at least equal to 25-50 times their own biovolume in order to replicate, depending on the dimensions of the cell. Even if yield assumptions, or Fe(III) densities are off by a factor of two, there is no way to imagine dense microcolonies sitting still, reducing the Fe(III) they can access a few microns away, as a productive strategy.

Another way to approach this challenge is a cell residing on a sand grain, which is covered with a crust of Fe(III)-oxide. If a *Geobacter* cell is able to use only what it can directly touch beneath itself, effectively drilling a hole 1 μm in diameter, it needs to reduce into a crust over 10 μm deep in order to support a single doubling of itself. If that same cell sitting on a sand grain was able to also access all Fe(III) extending 2 μm in all directions on that same surface, enlarging its own 'footprint' and drilling a hole 5 μm in diamter, it could produce enough energy

to double by dissolving down into less than 1 μm of crust. While this would not produce a thick biofilm, it is at least in the realm of possibility for doubling.

Thus, in both planktonic and surface-attached situations, these calculations suggest the only viable strategy for Fe(III) reduction coupled to acetate oxidation is one in which a cell has access to the environment many microns beyond what would be considered 'direct contact' by surface-exposed, outer membrane embedded cytochromes.

Shuttle-producing bacteria (or bacteria using naturally present shuttles such as humic acids), partially solve this issue by secreting redox-active molecules at nanomolar concentrations that allow access to Fe(III) on the micron scale, as evidenced by stimulation of both current production and Fe(III) reduction by flavins in *Shewanella* incubations (19, 71, 96, 110). However, bacteria such as *Shewanella,* which partially oxidize lactate, obtain a 3 to 6-fold higher yield of ATP/electron, meaning they do not need to access as much Fe(III) to grow or recover the cost of shuttle production. Motility can also partially address this issue of accessing nearby Fe(III), although it also comes at a cost, and again, eliminates the need for conductive biofilms.

Geobacter's cytochromes, or 'mediators' that provide access to the Fe(III) beyond the cell membrane, or that provide conductivity between cells are not soluble, but are entrapped by structural proteins and polysaccharides. There are many ways to envision a conductive network of proteins outside the cell. For example, if redox or electron transfer proteins were randomly anchored outside the cell, creating a gel extending 2 μm in all dimensions from the outer membrane, they would need to be at a concentration high enough to randomly collide often enough to create conductivity. For a 50 kDa protein (which has a diameter of about 4.8 nm, (27)), filling a gel where each protein is on average 10 nm apart would require ~0.7 x 10^{-13} g protein, or over 70 % of a cell's total protein! As rapid electron transfer requires proteins to be

much closer than this, a highly conductive gel of proteins spaced 2 nm apart approaches 900% of a cell's total protein. Such calculations show that, while hydrogels containing high concentrations of randomly oriented redox-active mediators may work for enzyme electrodes, such three-dimensional randomness is prohibitively expensive for a single cell.

However, if these same 50 kDa proteins are imagined as being aligned in aggregates or chains, with an average distance of only 1 nm between each protein (a distance facilitating the conductivity observed in redox proteins) (105), roughly 345 proteins end to end would extend twice the cell's length (2 μm). A cell could construct 100 such chains to extend in 100 different directions, for a cost of less than 3% of the cell's total protein. Visualized differently, if proteins were arrayed akin to netting, with proteins spaced 1 nm from each other and intersecting every 10 proteins on average, a cell could produce over 20 square microns of conductive material for a similar cost. If other proteins are used to anchor or build these networks, the protein use could increase, but as polysaccharides cost about 25% as much as protein to produce, a conductive matrix extending widely in all directions, rather than a random gel, remains the only thermodynamically feasible approach.

In all permutations of these calculations, two facts become clear. First, no form of Fe(III) (oxyhydr)oxide appears to contain enough energy for an acetate-oxidizing *Geobacter* to form a classical, multilayer biofilm, just by touching it. This creates a requirement that cells are able to 'reach out and touch' Fe(III) in a dense suspension or crust over ~ 2-4 μm away *in all directions,* just to have a chance at making another cell. Lacking a dissolved shuttle, this rewards a single cell if it manufactures long-distance pathways which have the capacity to carry electrons, even if that cell is motile. Second, the enormous volume reaching 2 μm beyond the cell membrane (about 15-25 μm^3, depending on the cell size and shape) is prohibitively expensive to fill with

randomly oriented proteins. Regardless of the actual mechanism, any strategy must be organized in 2 dimensions, as this volume is much too big to fill randomly. Chains, nets, sheets and aggregations of proteins are very reasonable ways to solve this issue, and already existing extracellular structures may have been adapted to solve the challenge of Fe(III)'s low energy value.

Thus, the ability of cells to form conductive multicellular networks on electrodes may not be due to growth as Fe(III)-reducing biofilms in the environment. Rather, conductivity on the outside of the cell may be a response to the need to reach beyond the cell membrane just to obtain enough energy while in planktonic mode. Alternatively, conductive pathways may also reward cells growing syntrophically, where electrons are continuously shared between some cells able to oxidize a unique electron donor, and cells able to reduce soluble non-Fe(III) electron acceptor (8, 77, 106).

In this light, consider the observation that some proteins essential for Fe(III) reduction (such as OmcS) are not needed for direct electrode reduction, but are required for thicker biofilms. In contrast, some proteins required for direct electrode reduction (such as OmcZ) are not required for Fe(III) reduction. This further underscores the difference between reducing an acceptor that can reach the outer membrane, vs. building a conductive pathway to another cell or a distant Fe(III) particle. Polysaccharide fibrils, nonconductive proteins, and even pili may be essential components in metal reduction because of their ability to organize electron transfer proteins in two dimensions efficiently.

From these calculations, it also emerges that planktonic growth of *Geobacter* may actually be a sign of active metal reduction, since there is so little to gain from forming a biofilm on a single particle, and little evidence there is enough energy to support biofilm growth on

particulate Fe(III). In every case, these energetic constraints show that the delicate, highly inconsistent space beyond the cell remains an important, relatively unexplored compartment. As it represents the crucial link between cells and their energy source, how this challenge is overcome in response to varying surfaces and electron acceptors may ultimately be what controls the competitiveness of *Geobacter* in the environment.

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Figure 1. Illustration of the difference between intracellular and extracellular electron acceptors. Intracellular reduction of fumarate consumes both protons and electrons produced during acetate oxidation, and all electron transfer can be devoted to proton translocation driving subsequent ATP synthesis (estimated at ~1.5 ATP/acetate). Extracellular reduction of electron acceptors consumes only electrons, which leave the cell, leading to accumulation of positive charge inside the cell which dissapates the proton motive force. From observed biomass yields and *in silico* modeling, subsequent energy-dependent disposal of proton equivalents decreases the net ATP production to ~0.5 ATP/acetate (68, 69).

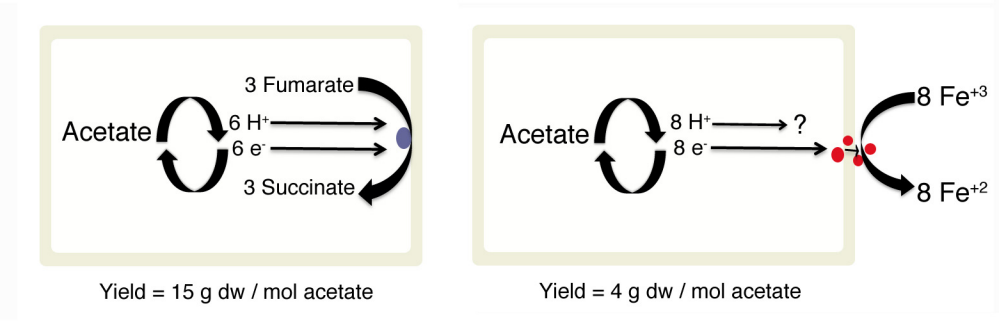

Figure 2: A) Illustration of the amount of energy available to a cell in a dense (50% by volume) Fe(III) (oxyhydr)oxide environment. If *Geobacter* could reduce all Fe(III) 1 µm away from its cell surface, it could not produce enough energy to make a second cell. The volume represented by extending outward 2 µm beyond the cell surface contains enough electrons to support one doubling, but daughter cells would have to move to a new location to find enough Fe(III) to continue respiration. In general, this shows growth in multicellular biofilms is unlikely when Fe(III) oxides are the electron acceptor. **B)** Comparison of two strategies for secreting proteins into the extracellular space. Producing a conductive hydrogel of randomly oriented proteins, even when spaced as wide as 10 nm apart on average, would consume nearly 900% of a cell's protein. However, if proteins are organized in chains or clusters, 100 such organized structures could be produced, extending outward in all directions, for less than 3% of the cell's protein budget.