144. The molecular basis for electron flow within metal-reducing biofilms: new insights from genome-scale genetics

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Project Goals: Electrochemical, spectral, genetic, and biochemical techniques have provided evidence that multiple redox proteins and structural macromolecules outside the cell work together to move electrons long distances between *Geobacter* cells and to metals. This extracellular matrix contains many proteins that contribute to conductivity, in addition to complex polysaccharides and other extracellular macromolecules. Many of these components were likely lost or ignored in previous proteomic and biochemical surveys, and many of these proteins are only present under specific growth conditions. We aim to define this matrix, develop new tools discover the basis for its synthesis and construction, and visualize it in action. The goals of this project are to 1) identify elements crucial to the expression, assembly and function of the extracellular conductive matrix, 2) expand spectral and electrochemical techniques to define the mechanism and route of electron transfer through the matrix, and 3 combine this knowledge of electron transfer proteins and their role in multicellular electron transfer to visualize redox and gene expression gradients in space over time.

When bacteria change the state of metals in the environment, they transport electrons unprecedented distances from intracellular metabolic reactions through their membranes, and ultimately to distant mineral surfaces or other bacteria. This electron movement drives subsurface bioremediation, controls aquifer chemistry, and powers new microbial energy generation applications. Yet a molecular understanding of how this electron transfer is accomplished both at the interface and across long distances by *Geobacteraceae,* who are among of the most predominant bacteria in such systems, remains one of the grand challenges in microbial environmental processes. Unlike key processes spanning anaerobic redox gradients, such as methanogenesis, sulfate reduction, and nitrate reduction, metal reduction still lacks conserved redox proteins that can be used as indicators of function in metagenomic surveys.

In past years of this project, direct measurements of living biofilms using electrochemistry have revealed redox-potential dependent exchange between *Geobacter* redox proteins to be a rate-controlling step at all stages of growth. Direct spectral analysis of living biofilms confirmed that *c*-type cytochromes are a major reservoir of charge in these films, and that these cytochromes experience a bottleneck to oxidation when electrons must be transferred longer distances. Fine-scale immunogold labeling has discovered gradients in cytochrome abundance throughout these films, further suggesting the presence of redox and/or nutrient gradients within this biofilms, where cells distant from the electrode experience different conditions than those close to the acceptor.

Genetics has discovered separate polysaccharide biosynthesis operons, and secretion systems essential for the attachment of *Geobacter* to metals and other cells in the biofilm, indicating that cells must sense their substrate (metals, electrodes, other cells) and utilize different attachment systems accordingly. Genome sequencing of new *Geobacteraceae* representatives, such as those from alkaline environments, has refined our concept of 'core' cytochrome and metabolic genes that may be most essential to this process, and most useful in metagenomic studies.

A key finding of transposon-based genetic screens combined with proteomic analysis has been the separation of conductive biofilm development into stages. For example, disruption of type II secretion (*gsp*) proteins in *G. sulfurreducens* does not affect the cell's ability to attach to surfaces, and mutants transfer electrons to electrodes at wild type rates. However, once the electrode surface is covered, *G. sulfurreducens gsp*$^-$ mutants are unable to attach to each other, or form conductive biofilms, showing that proteins required for interfacial electron transfer can be separated from those required for long-range interactions. Mutants in multiheme *c*-type cytochromes, such as *omcS* and *pgcA*, are able to attach to surfaces and transfer electrons to electrodes at wild-type rates, and are still able to form thick interconnected biofilms, but fail to exhibit the cell-cell electron transfer needed to support growth distant from the electron acceptor. This indicates that cell-cell adhesion can be separated from conductivity. To more accurately measure electron transfer between cells, we have fabricated Interdigitated Electrode Assemblies, containing 10 µm electrodes separated by 15 µm gaps, and measured electron transfer at known redox potentials defined by biopotentiostats. In general, over a hundred mutants have been characterized to have defects in one of these 4 stages of biofilm development (attachment, electron transfer, cell-cell attachment, and cell-cell electron transfer), supporting the hypothesis that electron transfer across distances requires multiple cooperative adaptations.

Recently, we have adapted saturation mutagenesis and adaptive evolution approaches to demonstrate that the pathway of electron transfer out of *G. sulfurreducens* is dependent upon the electron acceptor in unexpected ways. For example strains of *Shewanella* possess only one mechanism for electrons to exit the quinone pool and enter metal- reduction pathways. However, sequencing of Tn-seq libraries grown with a variety of electron acceptors, combined with analysis of new mutants in putative inner membrane quinone oxidases, has shown that different respiratory pathways are involved in reduction of soluble acceptors and electrodes than insoluble Fe(III) oxides. This data led to the discovery that mutants in the highly conserved multiheme inner membrane protein *imcH* are unable to respire any soluble metal acceptor, or electrodes, but can reduce solid metals. Thus, we have constructed new saturation mutagenesis libraries in markerless *imcH* backgrounds to identify this second pathway. Complementary analyses with parallel evolved *imcH* suppressor mutants have also been resequened and SNPs enabling this second pathway identified with breseq. As we untangle pathways to different metals, new targets for expression and metagenomic analyses are emerging which could for the first time indicate the type of metal acceptor being used by *Geobacteraceae* in the environment.

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