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Molecular Basis for Electron Flow Within Metal-and Electrode-Reducing Biofilms

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Electrochemical, spectral, genetic, and biochemical techniques were developed to reveal that a diverse suite of redox proteins and structural macromolecules outside the cell work together to move electrons long distances between *Geobacter* cells to metals and electrodes. In this project, we greatly expanded the known participants in the electron transfer pathway of *Geobacter*. For example, in addition to well-studied pili, polysaccharides contribute to anchoring, different cytochromes are required under different conditions, strategies change with redox potential, and the localization of these components can change depending on where cells are located in a biofilm. By inventing new electrodes compatible with real-time spectral measurements, we were able to visualize the redox status of biofilms in action, leading to a hypothesis that long-distance electron transfer is ultimately limiting in these systems and redox potentials change within biofilms. The goals of this project were met, as we were able to 1) identify new elements crucial to the expression, assembly and function of the extracellular electron transfer phenotype 2) expand spectral and electrochemical techniques to define the mechanism and route of electron transfer through the matrix, and 3) combine this knowledge to build the next generation of genetic tools for study of this complex process. This work also contributed to at least 8 publications and 1 book chapter.

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. Yet a molecular understanding of how this electron transfer is accomplished, from reactions in the inner membrane, to the outer membrane, and to the biotic-abiotic interface is still lacking in the *Geobacteraceae.* This 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 a described pathway of conserved redox proteins, this prevents further engineering and prevents detection of these processes in metagenomic surveys.

A brief summary of progress in key areas is below:

Subtle changes in adherence created conflicting data in the past: Early in the project, we produced mutants in every well-studied cytochrome, pilus protein, and polysaccharide locus described to date. A preliminary finding from this survey was the fact that pili mutants were surprisingly defective in the quantity of extracellular sugars and cytochromes, cytochrome mutants were often defective in polysaccharides, and in general, phenotypes attributed to one protein were often much more complex. *This suggested that much data derived from mutants was confounded by the lack of other components.* Because of this, two choices were made- first to revisit the search for mutants defective in only electron transfer or adherence, and second, to develop a more robust genetic system. This work in deconvoluting adherence effects from electron transfer led to our collaboration with other laboratories to construct mutants (two 2014 publications) and report of a new genetic system in *Geobacter* in 2015.

Library screening: We then initiated the first genetic screening to find mutants specifically defective in Fe(III)-oxide reduction. We screened out mutants that had defects in cell-cell agglutination, dye binding, and adhesion, as these mutants could easily be in outer surface charge, pili, or well-known mechanisms. This produced over 30 new loci specifically involved in electron transfer to metals, none of which were identified in earlier studies using Fe(III)-citrate or other substrates. *Only one of these loci had been mentioned in the literature previously as being involved in metal reduction.* This work ultimately led to the discovery of the first route out of the inner membrane, via the cytochrome CbcL (published in 2016), and led us to invest in techniques using next-generation sequencing (Tn-Seq) to accelerate this kind of work (see below).

Proteomic cataloging of matrix: Because a catalog of outer surface proteins was lacking, matrix from the outer surface of wild type *G. sulfurreducens* was extracted by two different methods, subjected to trypsin digestion and major peptides identified by LC/MS/MS. Over 200 proteins were identified in this screen, and were compared to a collection obtained under more stringent conditions. Proteins present in both surveys were compared to bioinformatic predictions of secreted proteins and previous membrane proteomics. *Very few targets found in this survey had been identified in earlier work that had washed cells before analysis, suggesting a missing pool of loosely attached proteins.* These data led to a collaboration showing the localization of OmcB, and how it changed with distance from the electrode in 2014. Data were used with saturation mutagenesis work to identify proteins that were likely on the cell exterior.

Composition of the matrix: Using protocols developed in previous work, we extracted and purified sugars from the matrix of *G. sulfurreducens*, and submitted samples to a Carbohydrate Analysis Center. The composition was relatively simple, and was comprised of a sugar subunit of a consistent molecular weight under all conditions. *This chemical composition analysis was a key aim of our project and is being used to create artificial biofilm matrix material.*

Spectroelectrochemical analyses: By growing biofilms of *Geobacter* while continuously sampling spectral data, *we verified the hypothesis that electrons accumulate in some portion of the cytochrome pool during growth*. Further, by oxidizing and reducing biofilms at progressively faster rates, *we were able to make the first measurements of cytochrome kinetics within biofilms*. These experiments, detailed in our 2012 publication showed that a bottleneck exists within biofilms, limiting the rate at which electrons can travel. This bottleneck could not be overcome simply by applying electrical potential. *This simple result made under physiological conditions, also contradicts the hypothesis that long distance electron transfer can be overcome by increasing driving force, and shows there are other mechanisms limiting flux.* This work was complemented by comparisons with other bacteria, such as *Geothrix*, which were found to produce soluble shuttles and have vastly different rate-limiting steps (2012)

Genome-level screening via Tn-Seq: High-throughput sequencing-based saturation mutagenesis technologies were first developed in pathogenic bacteria in 2009. The approach, termed 'Tn-seq' can compare growth of >50,000 mutants simultaneously, allowing the phenotype of every non-lethal mutation to be measured. Compared to the labor required to screen a library of 10,000 mutants (in an anaerobe like *Geobacter*), this technique held considerable promise. Through collaboration with J. Gralnick at the University of Minnesota, we constructed the first mutant libraries to conduct saturation mutagenesis of genes required for growth with Fe(III), *a much more ambitious goal than what was originally outlined in our proposal, but at a fraction of the cost*. This led to discovery of ImcH, the second route for electrons out of the inner membrane, published in 2014, and forms the basis for all our current investigations into *Geobacter* electron transfer.

Key Publications

Liu Y, **Bond DR**. 2012. Long-Distance Electron Transfer by *G. sulfurreducens* Biofilms Results in Accumulation of Reduced *c*-Type Cytochromes. ChemSusChem **5**:1047–1053.

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Mehta-Kolte MG, **Bond DR**. 2012. *Geothrix fermentans* secretes two different redox-active compounds to utilize electron acceptors across a wide range of redox potentials. Appl Environ Microb **78**:6987–6995.

Stephen CS, **LaBelle EV**, **Brantley SL**, **Bond DR**. 2014. Abundance of the multiheme c-type cytochrome OmcB increases in outer biofilm layers of electrode-grown *Geobacter sulfurreducens.* PLoS ONE **9**:e104336.

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