Final Report

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The overall goal of this project was to better understand the mechanisms by which *Geobacter* species transfer electrons outside the cell onto Fe(III) oxides. The rationale for this study was that *Geobacter* species are often the predominant microorganisms involved in *in situ* uranium bioremediation and the growth and activity of the *Geobacter* species during bioremediation is primarily supported by electron transfer to Fe(III) oxides. These studies greatly expanded the understanding of electron transfer to Fe(III). Novel concepts developed included the potential role of microbial nanowires for long range electron transfer in *Geobacter* species and the importance of extracytoplasmic cytochromes functioning as capacitors to permit continued electron transfer during the hunt for Fe(III) oxide. Furthermore, these studies provided target sequences that were then used in other studies to tract the activity of *Geobacter* species in the subsurface through monitoring the abundance of gene transcripts of the target genes. A brief summary of the major accomplishments of the project follows.

Major Accomplishments

1. Identified OmpJ, the most abundant outer-membrane protein in *Geobacter sulfurreducens* and demonstrated its function through genetic and biochemical studies.
2. Discovered the role of a novel secretion system in exporting a multi-copper protein required for Fe(III) oxide reduction to the outer cell surface.
3. Identified the novel outer-surface cytochromes OmcS and OmcE and demonstrated their role in Fe(III) and Mn(IV) oxide reduction.
4. Demonstrated that the pili of *Geobacter sulfurreducens* are electricially conductive and provided genetic and physiological data which suggest that these 'microbial nanowires' are the final conduit for electron transfer from *G. sulfurreducens* to Fe(III) oxides.
5. Demonstrated that the abundant *c*-type cytochromes in *G. sulfurreducens* can act as capacitors, permitting continued electron transfer in the absence of an external electron acceptor.
6. Demonstrated that some outer surface cytochromes that are required for Fe(III) reduction have an indirect effect on Fe(III) reduction by influencing either the transcription or translation of OmcB, an outer-surface *c*-type cytochrome required for optimal Fe(III) reduction.

1. OmpJ

OmpJ was the most abundant protein band when outer membrane proteins of *G. sulfurreducens* were separated via SDS-PAGE (1). The gene for OmpJ was identified via mass spectrometry analysis of peptide fragments of the gel-purified protein. The *ompJ* gene is present in other *Geobacteraceae*, but not in organisms outside this family. Predictions of the structure of OmpJ suggested that is was comprised primarily of extended beta-chain fragments. Further analysis of the localization of OmpJ in the outer membrane with structure prediction programs and proteolytic reagents indicated that OmpJ is embedded in the membrane with little of the protein exposed on the outer surface. These findings suggested that OmpJ was a porin.

Deletion of *ompJ* in *G. sulfurreducens* produced a strain that grew as well as the wild-type strain with fumarate as the electron acceptor but could not grow with metals, such as soluble or insoluble Fe (III) and insoluble Mn (IV) oxide, as the electron acceptor (1). Further evaluation of the mutant revealed that the heme *c* content in the mutant strain was only ca. 50% of that of the wild-type and that there was a widespread loss of multiple cytochromes from soluble and membrane fractions. Transmission electron microscopy analyses of mutant cells revealed an unusually enlarged periplasm, which is likely to trigger extracytoplasmic stress response mechanisms leading to the degradation of periplasmic and/or outer membrane proteins. The substantial loss of cytochromes, some of which may be involved in electron transfer to Fe(III), the lack of any apparent moieties in OmpJ that could be involved in electron transfer, and the lack of exposure of the protein on the outside of the cell suggest that OmpJ is not directly involved in electron transfer to Fe(III) oxides. However, these findings did increase our understanding of the protein content of the outer membrane and emphasized that proteins other than those directly involved in electron transport may be essential for Fe(III) reduction.

2. Role of a Secretion System and a Copper-Containing Outer-Membrane Protein

Another approach to searching for outer-membrane proteins that might be important in Fe(III) oxide reduction also yielded information on non-cytochrome proteins that are required for Fe(III) oxide reduction in *G. sulfurreducens*. In this approach *oxpG*, a gene that is involved in a type II general secretion pathway in other gram-negative bacteria, was deleted in accordance with the hypothesis that deletion of this gene might prevent the secretion of some proteins required for Fe(III) oxide reduction to the outer membrane (14). When *oxpG* was deleted, *G. sulfurreducens* was unable to grow with insoluble Fe(III) oxide as the electron acceptor. Growth on soluble electron acceptors, including soluble, chelated Fe(III) was not affected. Furthermore, the OxpG-deficient mutant grew in Fe(III) oxide medium if the electron shuttle anthraquinone-2,6-disulfonate (AQDS) or the Fe(III) chelator nitrilotriacetic acid (NTA) was added to the medium. These amendments alleviate the need for direct electron transfer to the insoluble Fe(III) oxide. This was the first description of a mutant in which the reduction of Fe(III) oxide, but not soluble Fe(III) was specifically affected and demonstrates that reduction of Fe(III) oxide and soluble Fe(III) may not proceed via the same pathways.

A comparison of periplasmic proteins in the *oxpG* mutant and the wild-type revealed that a 140 kDa protein accumulated in the periplasm of the mutant (14). The gene for this protein was identified by mass spectrometry of the gel-purified protein.

Analysis of the gene sequence suggested that the protein, designated outer membrane protein B (OmpB), is a multicopper oxidase-like protein, with highest homology to the manganese oxidase, MofA, from *Leptothrix discophora*. OmpB, also contains a potential Fe(III)-binding site and a fibronectin type III domain, suggesting a possible role for this protein in accessing Fe(III) oxides. OmpB was localized to the membrane fraction of *G. sulfurreducens* and in the supernatant of growing cultures, consistent with the type II secretion system exporting OmpB. Homologues of OmpB are found in a diversity of *Geobacter* and *Desulfuromonas* species, but not in *Pelobacter* species or any organisms outside the *Geobacteraceae* whose genomes have been sequenced (8). A mutant in which the *ompB* gene was deleted had the same phenotype as the *oxpG* mutant suggesting that the failure to export OmpB was responsible, at least in part, for the inability of the *oxpG*-deficient mutant to reduce Fe(III) oxide. This is the first report of a physiological role for a multicopper oxidase-like protein in an anaerobic organism. These results also further emphasize the importance of outer-membrane proteins in Fe(III) oxide reduction and suggest that outer-membrane proteins other than *c*-type cytochromes are required for Fe(III) oxide reduction in *Geobacter* species.

3. OmcS and OmcE

In order to identify proteins highly exposed on the outer surface of the cell, proteins were physically sheared from the outer surface of intact cells of *G. sulfurreducens* grown with Mn(IV) oxide as the electron acceptor (15). Two of the most abundant proteins that were easily sheared from the outer surface of intact cells were *c*-type cytochromes. One, designated OmcS, had a molecular weight of ca. 50 kDa and is predicted to be an outer membrane hexaheme, *c*-type cytochrome. Transcripts for *omcS* could be detected during growth on Fe(III) oxide, but not on soluble Fe(III) citrate. The *omcS* mRNA consisted primarily of a monocistronic transcript and, to a lesser extent, a longer transcript that also contained the downstream gene *omcT*, which is predicted to encode a second hexaheme outer membrane cytochrome with 63 % amino acid sequence identity to OmcS. The other abundant *c*-type cytochrome sheared from the outer surface of *G. sulfurreducens*, designated OmcE, had a molecular weight of ca. 30 kDa, and was also predicted to be an outer membrane tetraheme, *c*-type cytochrome. When either *omcS* or *omcE* was deleted, *G. sulfurreducens* could no longer grow on Fe(III) oxide, but could still reduce soluble electron acceptors, including Fe(III) citrate. The mutants could reduce Fe(III) in Fe(III) oxide medium only if NTA or AQDS was added. Expressing *omcS* or *omcE in trans* restored the capacity for Fe(III) oxide reduction. OmcT was not detected among the proteins sheared from the outer surface of the cell, and genetic studies indicated that *G. sulfurreducens* could not reduce Fe(III) oxide when *omcT* was expressed but *omcS* was absent. In contrast, Fe(III) oxide was reduced when *omcS* was expressed in the absence of *omcT*. These results suggested that OmcS and OmcE are involved in electron transfer to Fe(III) oxides in *G. sulfurreducens*. They also further emphasize the importance of evaluating mechanisms for Fe(III) reduction with environmentally relevant Fe(III) oxide, rather than the more commonly utilized Fe(III) citrate, because additional electron transfer components are required for Fe(III) oxide reduction that are not required for the reduction of Fe(III) citrate.

4. Conductive Pili

One of the most obvious proteins exposed on the outer surface of cells that is encoded in all of the available *Geobacteraceae* genomes is PilA, the structural protein for pilin. Previous studies demonstrated that *G. metallireducens* specifically produced pili when growing on insoluble Fe(III) or Mn(IV) oxides, but not during growth on soluble, Fe(III) citrate (3). The pili have an unusual orientation, in that they are only displayed on one side of the cell, which suggested that their function might be different from that of pili in other organisms. Studies with *G. sulfurreducens* demonstrated that it also produced pili when growing on insoluble Fe(III) or Mn(IV) oxides, but not during growth on Fe(III) citrate (17).

Several lines of evidence suggest that the pili of *G. sulfurreducens* may be an important conduit for extracellular electron transfer to Fe(III) oxides (17). These include the findings that: 1) Fe(III) oxides appear to preferentially associate with the pili; 2) deletion of the gene for PilA, inhibits Fe(III) oxide reduction; 3) restoring the PilA gene restores the capacity for Fe(III) oxide reduction; 4) the pili appear to be electrically conductive. These considerations and the observation that the pili can extend up to 20 µm from the cell have suggested that the pili of *G. sulfurreducens* may aid in accessing Fe(III) oxides that are substantial distances from the cell. Other organisms may employ a similar strategy for long-range electron transfer, but with somewhat different extracellular appendages (7, 12). Subsequent studies demonstrated that pili may aid in long-range electron transfer through the biofilms that form on the anodes of microbial fuel cells (16, 18) in addition to serving a structural role in biofilm formation (19).

A substantial effort was placed on further evaluating the potential for electron transfer along pili. Attempts to query electrochemical characteristics of the pili by placing them between two interdigitated electrodes proved to be too technically complex. Therefore, less direct methods were evaluated. For example, *G. sulfurreducens* was grown in a system that contained two gold electrodes separated by a 50 µm, non-conducting gap (13). The gold electrodes served as the sole potential electron acceptor. As the cells grew on the two electrodes, biofilms developed, and the two biofilms became confluent, bridging the non-conducting gap. Analysis of the electrochemical properties of the biofilm bridging the gap with alternating current impedance and direct current measurements demonstrated that the *G. sulfurreducens* biofilm was highly conductive. If biofilms of *G. sulfurreducens* were grown under conditions that did not require long-range electron transfer, i.e. with fumarate as the electron acceptor, the biofilm was not conductive (13). This finding that *G. sulfurreducens* can form electrically conductive biofilms contrasts with the fact that all previous studies have suggested the microbial biofilms act as insulators, not conductors. Mathematical modeling suggested that the degree of biofilm conductivity matched well with the expected density and resistance of *G. sulfurreducens* pili. These results do not "prove" that pili are responsible for long-range electron transfer by *G. sulfurreducens,* but they are consistent with this concept.

As another approach, gold electrodes were masked with a non-conductive polymer that had a high density of pores that were 100 nm in diameter. In this manner, pili were the only potential electrical contact between the cell and the electrode. Preliminary results have indicated that *G. sulfurreducens* is capable of electron transfer through the 100 nm diameter pores, suggesting that long-range electron transfer, presumably via the pili, is possible.

5. Possible Alternative Role for c-type Cytochromes

Our studies have suggested that the abundant *c*-type cytochromes in *G. sulfurreducens* may play an important ecological role in addition to functioning as components in electron transfer chains to Fe(III) and U(VI) (6). This role was suggested in studies in which *G. sulfurreducens* was grown in chemostats with excess acetate as the electron donor and with growth-limiting Fe(III), the electron acceptor (4, 5). This growth condition was chosen to mimic the growth during *in situ* uranium bioremediation as Fe(III) oxides become depleted close to the acetate injection wells and *Geobacter* species must move further down gradient in the aquifer in order to locate Fe(III) oxides (2). *G. sulfurreducens* grown under electron-acceptor limiting conditions had a substantially higher *c*-type cytochrome content than cells grown under electron-donor limiting conditions (4). Furthermore, although there was a close 1:1 correspondence between electrons derived from acetate oxidation and electrons transferred to Fe(III) in chemostats in which acetate availability was the factor limiting growth, only ca. 60% of the electrons derived from acetate oxidation were transferred to Fe(III) in the chemostats which were electron-acceptor limited. These 'missing' electrons could not be completely accounted for in alternative sinks such as formate or hydrogen.

These results have led to the hypothesis that the substantial quantities of *c*-type cytochromes found in *Geobacter* species may permit continued oxidation of electron donor and continued electron transport and proton-pumping across the inner-membrane during periods when Fe(III) oxides are not immediately accessible. In this hypothesis the abundant extracytoplasmic *c*-type cytochromes, i.e. those in the periplasm and outer-membrane, can continue to accept electrons from inner-membrane electron transfer until they are completely reduced. As energy conservation results from proton-pumping across the inner membrane, ATP can continue to be generated from electron transfer to periplasmic and outer-membrane cytochromes, even if the subsequent electron transfer to Fe(III) can not be completed. If so, this would provide *Geobacter* species that have depleted a source of Fe(III) oxide with the ability to continue to generate energy to support motility and maintenance of cellular functions during the search for a new source of Fe(III) oxide. Once an Fe(III) oxide source is located, the electrons stored in the extracytoplasmic cytochromes can be transferred to the Fe(III). Thus, the extracytoplasmic cytochromes are viewed as acting as capacitors, accepting electrons and temporarily storing them for subsequent discharge. If true, this 'capacitor' hypothesis would help explain how *Geobacter* species can function in subsurface environments in which Fe(III) oxides are heterogeneously dispersed and is consistent with the highly planktonic nature of the most active *Geobacter* species during *in situ* uranium bioremediation at the Rifle site (9).

In order to investigate the capacitor hypothesis, we developed a novel fluorescent method for quantifying the abundance of *c*-type cytochromes in the reduced state in *Geobacter* species (6). Analysis of oxidation of the cytochromes with the external electron acceptors suggested that the extracytoplasmic cytochromes of *G. sulfurreducens* could store ca. 10^7 electrons per cell. This estimate was corroborated with an independent estimate of heme content quantified from the incorporation of ^{55}Fe into heme groups. It was calculated that this abundance of extracytoplasmic cytochromes can provide electron storage capacity sufficient for enough continued respiration in the

absence of external electron acceptors to supply maintenance energy requirements for 8 minutes or to permit the cells to swim hundreds of cell lengths.

Next the capacitance of the cells was evaluated electrochemically using the two electrode system described in the previous section (13). These measurements demonstrated that the biofilm had a significant capacitance and suggested that the *c*-type cytochromes in the cells could account for ca. 90% of the capacitance. Biofilms grown with fumarate as the electron acceptor had similar capacitance as biofilms grown with the electrodes serving as the electron acceptor, consistent with the concept that the cytochromes are the capacitors, because fumarate-grown cells are also cytochrome rich.

6. *Outer-Membrane Cytochromes that Influence Production of OmcB*

We discovered three additional outer-membrane cytochromes that are essential for optimal Fe(III) reduction, designated OmcF, OmcH, and OmcG (10, 11). However, these cytochromes do not appear to be directly invovled in electron transfer to Fe(III). Rather they influence either the transcription (10) or translation (11) of OmcB, which is essential for optimal Fe(III) reduction. It is likely that these cytochromes act as sensors. Further study into this novel sensing capability is warranted.

In summary, these studies have provided significant insight into the mechanisms of electron transfer to Fe(III) in *G. sulfurreducens*. With the tools and understanding developed in those studies and the availability of environmentally relevant subsurface clade I *Geobacter* species we are poised to make significant contributions to understanding factors controlling U(VI) reduction in subsurface environments.

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