Final Report

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Grant No. DE-FG02-97ER62475
Title: Molecular analysis of rates of metal reduction and metabolic state of *Geobacter* species
Project Period: 9/15/97-12/14/06

This project began with the simple goal of trying to understand the diversity of dissimilatory metal-reducing microorganisms that might be found in subsurface environments. It ended with a sophisticated understanding not only of what microorganisms are important for metal reduction in uranium-contaminated subsurface environments, but also their physiological status during *in situ* uranium bioremediation. These findings have provided unprecedented insight into uranium bioremediation and the methods by which this process might be optimized. A brief summary of the major accomplishments of the project follows.

Elucidation of the Diversity of Metal-Reducing Microorganisms and Physiological Properties

When this research project was started there was a very poor understanding of the potential diversity of dissimilatory metal-reducing microorganisms in subsurface environments and there was no information on what metal-reducing microorganisms were likely to be most important in metal reduction under different environmental conditions. A series of isolation and characterization studies were carried out in order to evaluate the diversity of metal-reducing microorganisms in subsurface environments of interest to DOE. A number of novel dissimilatory metal-reducing microorganisms were discovered. For example, a novel metal reducer, *Desulfitobacterium ferrieducens* was isolated from uranium-contaminated subsurface sediments from a site in Shiprock, NM (7). This organism is of interest because it has the novel ability to reduce both metals and chlorinated organic compounds. Metals and chlorinated organics are co-contaminants and many DOE sites. Another *Desulfitobacterium* species was isolated from the subsurface and shown to be able to use structural Fe(III) in clays as an electron acceptor (32).

Rhodoferax ferrireducens was isolated from subsurface sediments at the DOE study site in Oyster Bay, VA (6). This organism has the unusual feature that it is able to completely oxidize a wide diversity of organic compounds to carbon dioxide with the reduction of Fe(III), but is also able to grow with atmospheric oxygen as an electron acceptor (2, 6). Subsequent studies have suggested that *Rhodoferax* species may be important constituents of the subsurface metal-reducing community at several uranium-contaminated sites prior to the addition of electron donors to stimulate U(VI) reduction.

A novel species of *Salmonella*, *Salmonella subterranean*, was isolated from the field research center study site in Oak Ridge, TN (31). This organism is of interest because of its ability to grow at low pH and reduce U(VI). Low pH, uranium-contaminated subsurface environments are prevalent at Oak Ridge and other DOE uranium-processing sites and are difficult to remediate (30).

A number of novel *Geobacter* species were isolated and characterized, better defining the phylogenetic scope of subsurface *Geobacter* species as well as providing insights into novel physiological features, such as the ability to grow at low temperatures and with additional electron donors (4, 22, 23, 29, 33). Furthermore, it was demonstrated that *Geobacter* species are capable of reductive precipitation of vanadium, often a co-contaminant in uranium-contaminated subsurface environments (27), as well as technetium (14). Analysis of 16S rRNA gene sequences and other highly conserved genes (9) in isolates provided important information for the molecular analysis of subsurface communities described below.

Molecular Analysis to Determine the Predominant Metal-Reducing Microorganisms in Subsurface Environments

Culturing a diversity of dissimilatory metal-reducing microorganisms from the subsurface provided a better understanding of what metal-reducing microorganisms are present in the subsurface, but did not indicate which of these organisms were important in metal reduction. Therefore, the composition of the microbial communities associated with dissimilatory metal reduction in contaminated subsurface environments was investigated with molecular techniques that avoided potential culture bias. These studies unequivocally demonstrate that, with the exception of high-salinity environments (21), *Geobacteraceae* are the predominant metal-reducing microorganisms when microbial metal reduction in circumneutral pH subsurface environments is stimulated to promote reductive precipitation of contaminant metals (1, 8, 12, 34, 35). This surprising finding, based initially on analysis of 16S rRNA gene sequences, has subsequently been confirmed by a diversity of researchers using multiple techniques, including most recently, environmental proteomics.

The degree to which *Geobacter* species can dominate the subsurface microbial community during *in situ* bioremediation of uranium is stunning. At the study site in Rifle, CO *Geobacter* species can account for more than 90% of the microorganisms in the groundwater when *in situ* reduction of uranium is most active. Furthermore, the diversity of *Geobacter* species is quite limited, with species from a tight phylogenetic group, designated subsurface clade I, predominating (12). This is an important finding because it means that elucidation of the factors controlling the rate and extent of metal reduction of *Geobacter* species in the subsurface should provide the information necessary to optimize *in situ* uranium bioremediation. The abundance and low diversity of *Geobacter* species made it feasible to consider interrogating the physiological status of the subsurface metal-reducing microbial community with molecular techniques.

Elucidation of Mechanisms for Microbial Metal Reduction

As it became apparent that *Geobacter* species were the organisms catalyzing metal reduction in a diversity of subsurface environments in which this is an important process, studies were initiated to select the appropriate molecular targets to study the metabolism of *Geobacter* species directly in subsurface environments. Many of these early studies focused on biochemical approaches that identified putative metal reductases (13, 19) or cytochromes (15, 16) thought to be involved in electron transfer to metals. The realization that genetic verification of the role of these proteins in metal reduction was required due to the potential for nonspecific *in vitro* reduction of metals led to the

development of a genetic system for *Geobacter sulfurreducens* (5), which was used to evaluate potential genes that could be important for tracking U(VI) reduction (28).

Whole cell physiological studies demonstrated that whereas some other organisms relied on the production of soluble, electron-shuttling molecules and chelators to facilitate reduction of Fe(III) oxides (25), *Geobacter* species directly established contact with Fe(III) oxides in order to reduce them (24). This discovery is important because it is a likely explanation why *Geobacter* species are such effective competitors in subsurface environments. Electron shuttles or chelators released into subsurface environments are likely to be quickly lost from the site of release via advection and diffusion. Thus, microorganisms that produce these compounds face a metabolic energy drain that *Geobacter* species do not incur.

Diagnosing the In Situ *Physiological Status of Subsurface Metal Reducers*

The ultimate goal in studying the microbiology of contaminated subsurface environments is to be able to model how the activity of the subsurface microorganisms influences the fate and transport of contaminants of interest. This requires an understanding not only of what reactions the microorganisms are capable of catalyzing, but also knowing what processes they are actually carrying out in the subsurface. Therefore, we developed the concept of quantifying levels of transcripts for key genes to diagnose the physiological status of *Geobacter* species during *in situ* uranium bioremediation.

Pure culture studies demonstrated that transcript levels for key *Geobacter* genes could be related to rates of Fe(III) reduction (3), rates of central metabolism (11), or limitations for key nutrients (10, 20, 26). Methods for quantifying gene transcript levels in microorganisms living in the subsurface were perfected. Comparison of transcript levels for key genes versus transcript levels for housekeeping genes in the subsurface during *in situ* uranium bioremediation demonstrated that the metabolism of *Geobacter* species was linked to the availability of acetate in the subsurface (11) and that the activity of the *Geobacter* species may be suboptimal due to nutrient limitations (10, 20, 26).

Conclusions

These studies have demonstrated that an integrated combination of investigations with pure cultures and natural communities can lead to a deep understanding of important subsurface microbial processes. The molecular ecology studies at the Rifle site have set the stage for an in-depth genome-scale investigation of the physiological status of *Geobacter* species under different environmental conditions during *in situ* uranium bioremediation. This data is crucial for the ultimate goal of predictively modeling the *in situ* bioremediation process. The concepts and techniques developed in these studies are likely to have broad application to the study of both natural attenuation and engineered bioremediation of a diversity of contaminated subsurface environments (17, 18).

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