Final Report

Diagnosis of In Situ Metabolic State and Rates of Microbial Metabolism During In Situ Uranium Bioremediation with Molecular Techniques

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This final report summarizes progress and findings for the grant entitled "Diagnosis of In Situ Metabolic State and Rates of Microbial Metabolism During In Situ Uranium Bioremediation with Molecular Techniques" funded under award numbers DE-FG02-07ER64377 and DE-SC0004814.

The goal of these projects was to develop molecule tools to tract the metabolic activity and physiological status of microorganisms during *in situ* uranium bioremediation. Such information is important in able to design improved bioremediation strategies. As summarized below, the research was highly successful with new strategies developed for estimating *in situ* rates of metabolism and diagnosing the physiological status of the predominant subsurface microorganisms. This is a first not only for groundwater bioremediation studies, but also for subsurface microbiology in general. The tools and approaches developed in these studies should be applicable to the study of microbial communities in a diversity of soils and sediments.

These studies built upon our previous extensive investigations into the composition of the microbial community that developed when acetate was added to groundwater at the study site in Rifle, CO. *Geobacter* species predominate shortly after the addition of acetate and are considered to be responsible for the U(VI) reduction that is observed during this time. In order to understand the physiological status of the *Geobacter* species and whether environmental conditions were optimal for their activity, expression levels of key genes diagnostic of important potential stresses were evaluated.

For example, it was determined that the expression the genes *feoB* and *ideR* were indicative of a limitation of iron for assimilatory purposes, during *in situ* uranium bioremediation. Expression of these genes was high during the initial phase of bioremediation, revealing the surprising result that growth and metabolism of *Geobacter* were limited for dissolved Fe(II) at this time. This was attributed to the fact that *Geobacter* species are only able to assimilate Fe(II) in its soluble form, whereas more

than 90% of the Fe(II) produced from Fe(III) reduction is in solid forms in the subsurface. The results of this study were published in *Environmental Microbiology*.

Our previous studies had demonstrated that multicopper proteins on the outer surface of *Geobacter* species play an important role in electron transfer to Fe(III) oxides. The expression the genes for two of these proteins, OmpB and OmpC, was evaluated under different conditions in the laboratory and in natural communities of *Geobacter* species during *in situ* uranium bioremediation. In pure culture, expression of the OmpB gene appeared to be constitutive whereas transcript levels of the ompC gene were higher at faster growth rates. Constant levels of *Geobacter* OmpB gene transcript levels were detected in groundwater during a field experiment in which acetate was added to the subsurface to promote *in situ* uranium bioremediation. In contrast, OmpC gene transcript levels increased during the rapid phase of growth of *Geobacter* species following addition of acetate to the groundwater and then rapidly declined. Furthermore, *Pelobacter* species contained the gene for OmpB, but not OmpC. These results suggest that quantifying OmpB/OmpC-related genes in the subsurface could help alleviate the problem that *Pelobacter* genes may be inadvertently quantified via quantitative analysis of 16S rRNA genes. Furthermore, comparison of differential expression of the genes for OmpB and OmpC may provide insight into the *in situ* metabolic state of *Geobacter* species in environments of interest. A manuscript summarizing these findings was published in *Microbiology*.

Our previous studies suggested that *Geobacter* species might be under oxidative stress during *in situ* uranium bioremediation. This was further investigated with *Geobacter uraniireducens,* which was isolated from the Rifle study site. Genes thought to be indicative of oxidative stress, such as the genes for cytochrome bd ubiquinol oxidase (*cydA*), superoxide dismutase (*sodA)*, and cytochrome c peroxidase (*ccp*), were expressed under strict anaerobic conditions, and the number of mRNA transcripts for these genes increased transiently in response to oxygen stress over a 24-hour period. Furthermore, fold changes in expression were related to both the type of culture media and level of oxidative stress. The mRNA expression levels of *cydA* and *sodA* relative to a housekeeping gene *proC* also increased with acetate concentrations in the *Geobacteraceae* community sampled from a uranium-contaminated groundwater aquifer during bioremediation efforts. These results suggest that expression levels of putative oxidative stress genes can be related to oxygen exposure in pure culture, but their *in situ* response may be indicative of other global environmental stresses, making their usefulness in environmental monitoring applications debatable. A manuscript summarizing these results was published in *ISME Journal*.

Phosphate is potentially important nutrient controlling rates of growth and metabolism. Analysis of groundwater phosphate concentrations at the Rifle study site demonstrated that dissolved phosphate concentrations are exceptionally low. Addition of phosphate to sediment/groundwater incubations did not increase dissolved phosphate. The *Geobacter* community in the subsurface during *in situ* uranium bioremediation expressed genes diagnostic of phosphate limitation. This finding that phosphate may be a key limiting nutrient during *in situ* uranium bioremediation was published in *ISME Journal*.

In addition of phosphate, ammonium can be an important nutrient limiting the growth of some subsurface organisms. Thus, the influence of ammonium availability on bacterial community structure and the physiological status of Geobacter species during in situ bioremediation of uranium-contaminated groundwater were evaluated. Ammonium concentrations varied by 2 orders of magnitude (<4 to 400 μM) across the study site. Analysis of 16S rRNA sequences suggested that ammonium may have been one factor influencing the community composition prior to acetate amendment with Rhodoferax species predominating over Geobacter species with higher ammonium and Dechloromonas species dominating at the site with lowest ammonium. However, once acetate was added and dissimilatory metal reduction was stimulated, Geobacter species became the predominant organisms at all locations. Rates of U(VI) reduction appeared to be more related to acetate concentrations rather than ammonium levels. In situ mRNA transcript abundance of the nitrogen fixation gene, nifD, and the ammonium transporter gene, amtB, in Geobacter species indicated that ammonium was the primary source of nitrogen during uranium reduction. The abundance of amtB was inversely correlated to ammonium levels, whereas nifD transcript levels were similar across all sites examined. These results suggested that nifD and amtB expression are closely regulated in response to ammonium availability to ensure an adequate supply of nitrogen while conserving cell resources. A manuscript summarizing these studies was published in *Environmental Science and Technology.*

It is known from the field studies at the Rifle site that acetate availability can limit the growth and activity of *Geobacter* species in the subsurface, but there was no indication of the minimum concentration of acetate that would be required in order to overcome this acetate limitation. We conducted a study to identify genes indicative of acetate limitation, which was published in *Microbiology*. We next used this information to diagnose acetate limitation during different phases of *in situ* uranium bioremediation. These results were published in *Microbial Ecology*.

The potential for assessing the activity of different clades of sulfate-reducing microorganisms from *in situ* abundance of gene transcripts was also evaluated. In order to achieve this we focused on the gene for dissimilatory sulfite reductase. Dissimilatory sulfite reductase (DSR) catalyzes a key step in the reduction of sulfate and the sequence for this gene is highly conserved across all known sulfate reducers. Analysis of DSR gene sequences at the Rifle site suggested that there were three major groups of sulfate reducers. These included sulfate reducers most closely related to Firmicutes, Desulfobulbaceae, and Desulfobacter. DSR genes for each of these groups were present prior to initiation of bioremediation and persisted throughout all phases of the bioremediation field experiment. In order to determine which of these predominant groups of sulfate reducers were metabolically active, primers specific for the DSR gene sequences of each of these groups were designed and the abundance of DSR gene transcripts were quantified. Transcript abundance was normalized to the abundance of DSR genes for each of these groups. The results clearly demonstrated that Firmicutes and Desulfobulbaceae species had low DSR gene transcript abundance that did not change significantly throughout the field experiment. The abundance of DSR gene

transcripts for Desulfobacter species was also low prior to the start of the field experiment and during the phase when Fe(III) reduction was the predominant terminal electron-accepting process. However, the initiation of sulfate reduction was accompanied by a substantial increase in transcript abundance of Desulfobacter DSR gene transcripts. The abundance of these transcripts declined after acetate additions to the groundwater were halted and sulfate reduction rates dropped. These results demonstrate that monitoring the expression of DSR may be a useful strategy for determining not only the overall activity of sulfate reducers in the subsurface, but also for determining which groups of sulfate reducers are contributing to sulfate reduction. The results suggest that *Deslufobacter* species are the predominant sulfate-reducing bacteria following acetate additions to the Rifle site and further investigation of these sulfate reducers is warranted in order to better understand the engineered bioremediation at the Rifle site. It is expected that monitoring DSR gene expression will be a very useful tool to study microbial activity in subsurface zones in which natural attenuation appears to be an important process for uranium removal. A manuscript summarizing these results was published in *Applied and Environmental Microbiology*.

Although quantifying transcript abundance for key metabolic genes was shown to be an excellent approach for diagnosing the in situ physiological state of subsurface microorganisms, environmental transcript analysis can be technically challenging. This has restricted the application of this approach to a few laboratories that have mastered this technique. An alternative strategy is to quantify the abundance of the protein products of these genes. In order to evaluate this strategy, an antibody-dependent quantification approach (AQA) for measuring protein abundance was developed. Initial AQA studies focused on the *Geobacter* species that predominate during the early phases of acetate-stimulated in situ uranium bioremediation at the Rifle study site. The abundance of the citrate synthase protein and the nitrogenase (NifD) was analyzed with *Geobacter*-specific antibodies. The abundance of *Geobacter* citrate synthase appeared to be correlated with the concentration of acetate and with the reduction of uranium (VI), suggesting that the amount of *Geobacter* citrate synthase protein reflects the metabolic activity of *Geobacter* species involved in the bioremediation. The abundance of the NifD protein from *Geobacter* species was dependent on ammonium availability. These results suggest that the physiological status of *Geobacter* species during the bioremediation can be monitored by quantifying key metabolic proteins with *Geobacter*-specific antibodies. This study shows promise for AQA as a new molecular tool for diagnosing the physiological status of microorganisms in natural environments and during bioremediation. A manuscript summarizing these studies was published in *Applied and Environmental Microbiology*.

Additional molecular tools to assess aspects of arsenic biogeochemistry were developed because of the potential for arsenic release when dissimilatory metal reduction is stimulated. In a field experiment at the Rifle study site, groundwater arsenic concentrations increased when acetate was added. The number of transcripts from *arrA*, which codes for the *alpha* subunit of dissimilatory As(V) reductase, and *acr3*, which codes for the arsenic pump protein Acr3, were determined with quantitative RT-PCR. Most of the *arrA* (> 60%) and *acr3-1* (> 90%) sequences that were recovered were most

similar to *Geobacter* species, while the majority of *acr3-2* (>50%) sequences were most closely related to *Rhodoferax ferrireducens.* Analysis of transcript abundance demonstrated that transcription of *acr3-1* by the subsurface *Geobacter* community was correlated with arsenic concentrations in the groundwater. In contrast, *Geobacter arrA* transcript numbers lagged behind the major arsenic release and remained high even after arsenic concentrations declined. This suggested that factors other than As(V) availability regulated transcription of *arrA in situ* even though the presence of As(V) increased transcription of *arrA* in cultures of *G. lovleyi,* which was capable of As(V) reduction. These results demonstrate that subsurface *Geobacter* species can tightly regulate their physiological response to changes in groundwater arsenic concentrations. The transcriptomic approach developed here should be useful for the study of a diversity of other environments in which *Geobacter* species are considered to have an important influence on arsenic biogeochemistry. A manuscript summarizing these studies was published in *ISME Journal*.

In our final study under this grant we examined the possibility that gene transcript abundance can be used to estimate the rate of microbial growth in the subsurface. Whole genome microarray analyses of the subsurface isolate, *Geobacter uraniireducens*, grown under a variety of conditions identified a number of genes that are differentially expressed at different growth rates. Expression of two genes encoding ribosomal proteins, *rpsC* and *rplL*, were further evaluated with quantitative RT-PCR in cells with doubling times ranging from 6.56 h to 89.28 h. Transcript abundance of *rpsC* correlated best (r^2= 0.90) with growth rates. Therefore, expression patterns of *rpsC* were used to estimate growth rates of *Geobacter* species during an *in situ* uranium bioremediation field experiment in which acetate was added to the groundwater to promote dissimilatory metal reduction. Initially, increased availability of acetate in the groundwater resulted in higher expression of *Geobacter rpsC* and the increase in the number of *Geobacter* cells estimated with fluorescent *in situ* hybridization compared well with growth rates estimated from levels of *in situ rpsC* expression. However, in later phases cell number increases were substantially lower than predicted from *rpsC* transcript abundance. This change coincided with a bloom of protozoa thought to prey on *Geobacter* species. Other factors such as increased attachment of *Geobacter* species to solid phases may also have contributed to the discrepancy. These results suggest that monitoring *rpsC* expression may better reflect the actual rate that *Geobacter* species are metabolizing and growing during *in situ* uranium bioremediation than monitoring cell numbers. A manuscript summarizing these studies received positive reviews at *Applied and Environmental Microbiology* and publication is expected soon.

Publications

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