Final Report

Coupled In Silico Microbial and Geochemical Reactive Transport Models: Extension to Multi-Organism Communities, Upscaling, and Experimental Validation

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Principle Investigator:
Derek R. Lovley
Department of Microbiology
University of Massachusetts
Amherst, MA 01003
Phone: 413-545-9651
fax: 413-545-1578
email: dlovley@microbio.umass.edu

The project was highly successful in improving the understanding of physiological and ecological factors controlling the growth and interaction of subsurface microorganisms and in developing better strategies for *in silico* modeling of the interactions of subsurface microorganisms with other species and their environment. A summary of results leading to peer-reviewed publications follows.

A key parameter for assessing the predictive ability of *in silico* models is the growth rate of bacteria in the subsurface. Therefore, we developed a molecular technique for estimating the growth of *Geobacter* species. Whole-genome microarray analyses of the subsurface isolate, *Geobacter uraniireducens*, grown under a variety of conditions, identified a number of genes that were differentially expressed at different specific growth rates. Expression of two genes encoding ribosomal proteins, *rpsC* and *rplL*, was further evaluated with quantitative reverse transcription-PCR (qRT-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 specific growth rates. Therefore, expression patterns of *rpsC* were used to estimate specific 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 specific 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 and increased attachment of *Geobacter* species to solid phases. 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 changes in cell abundance. A

paper summarizing these results was published in *Applied and Environmental Microbiology*.

The findings from the growth rate studies led us to evaluate what factors might be limiting the net growth of *Geobacter* in the subsurface. It was hypothesized that a potentially significant factor influencing the growth of *Geobacter* species, and potentially other microorganisms in the subsurface, was protozoan grazing. Analysis of 18S rRNA gene sequences at the Rifle study site revealed a broad diversity of sequences closely related to known bacteriovorous protozoa in the groundwater before the addition of acetate. With the addition of acetate to the groundwater there was a bloom of *Geobacter* species, as had been observed in previous field studies. The bloom of *Geobacter* species was accompanied by a specific enrichment of the sequences most closely related to the ameboid flagellate, *Breviata anathema*, which at their peak accounted for over 80% of the 18S rRNA gene sequences recovered. The abundance of *Geobacter* species declined following the rapid emergence of *B. anathema*. Following the decline of *Geobacter* species there was a subsequent bloom of sulfate-reducing microorganisms in the family *Peptococcaceae*. This sulfate reducer bloom was accompanied by another specific enrichment of protozoa, but with sequences most similar to diplomonadid flagellates from the family Hexamitidae, which accounted for up to 100% of the18S rRNA gene sequences recovered during this phase of the bioremediation. These results suggest a prey–predator response with specific protozoa responding to increased availability of preferred prey bacteria. Thus, quantifying the influence of protozoan predation on the growth, activity, and composition of the subsurface bacterial community is essential for predictive modeling of in situ uranium bioremediation strategies. In the future, models for protozoa should also be developed. A manuscript summarizing these finding was published in *ISME Journal*.

In order to investigate other potential factors controlling the growth of *Geobacter* species during bioremediation the possibility of viruses attacking *Geobacter* species in the subsurface was evaluated. Transmission electron microscopy revealed an abundance of bacteriophage in groundwater at the Rifle study site after the growth of *Geobacter* had been stimulated with acetate to promote *in situ* uranium bioremediation. Analysis of the genomes of five different *Geobacter* species recovered from contaminated subsurface sites indicated that each of the isolates had been infected with phage. *Geobacter*-associated phage sequences were also detected in metagenomic and proteomic analysis of samples from the Rifle site. Transcript abundance for the genes for the *Geobacter*-associated phage structural proteins, tail tube gp19 and baseplate J, increased in the groundwater in response to the growth of *Geobacter* species when acetate was added, and then declined as the number of *Geobacter* decreased. Western blot analysis of a *Geobacter*-associated gp19 tail tube protein in the groundwater demonstrated that its abundance tracked with the abundance of *Geobacter* species. These results suggest that the enhanced growth of *Geobacter* species in the subsurface associated with *in situ* uranium bioremediation intensified the activity of *Geobacter*-associated phage. Phage mediated cell lysis is likely to not only decrease net growth rates of *Geobacter,* but also to release nutrients into the environment that will stimulate the growth of other organisms. These results demonstrate that the impact of phage should be considered

when attempting to model the growth and activity of microbial communities during subsurface bioremediation. A manuscript summarizing these results is under review at *ISME Journal*.

Key to modeling the reduction of U(VI) by *Geobacter* species in the subsurface is an understanding of the mechanisms by which cells transfer electrons to U(VI). Early studies with *Geobacter sulfurreducens* suggested that outer-surface c-type cytochromes might play a role in U(VI) reduction, but then a publication in PNAS suggested that there was substantial U(VI) reduction at the surface of the electrically conductive pili of *G. sulfurreducens* known as microbial nanowires. This phenomenon was further investigated. A strain of G. *sulfurreducens*, known as Aro-5, which produces pili with substantially reduced conductivity, reduced U(VI) nearly as well as wild-type, as did a strain in which the gene for PilA, the structural pilin protein, was deleted. In order to reduce rates of U(VI) reduction to levels less than 20% of wild-type it was necessary to delete the genes for the five most abundant outer surface c -type cytochromes of *G. sulfurreducens*. X-ray absorption near-edge structure spectroscopy demonstrated that whereas 83 ± 10% of the uranium associated with wild-type cells correspond to U(IV) after four hours of incubation, with the quintuple mutant 89 ± 10% of uranium was U(VI). Transmission electron microscopy and X-ray Energy Dispersion Spectroscopy revealed that wild-type cells did not precipitate uranium along pili as previously reported, but U(IV) was precipitated at the outer cell surface. These findings are consistent with previous studies which have suggested that *G. sulfurreducens* requires outer-surface *c*-type cytochromes, but not pili, for the reduction of soluble extracellular electron acceptors. These results validated our previous modeling approach, avoiding the need for a complete remodeling of the process. A paper summarizing these results was published in *Applied and Environmental Microbiology*.

It is important to be able to model the growth of *Geobacter* species with elemental sulfur as the electron acceptor because it is expected that elemental sulfur, produced by the interaction of sulfide with crystalline ferric iron, is an important electron acceptor for *Geobacter* species during later stages of *in situ* uranium bioremediation. In the last year the studies on this phenomenon were completed. The results definitely demonstrate that many of the outer-surface c-type cytochromes are capable of elemental sulfur reduction and that all the major outer surface cytochrome genes must be deleted before elemental sulfur reduction is inhibited. Furthermore, it was demonstrated that the capacity for elemental sulfur reduction is more widespread in *Geobacter* species than previously considered and that sulfide toxicity can be an important factor limiting growth with elemental sulfur as the electron acceptor. Completion of a manuscript summarizing these studies has been delayed due to personnel issues of the researcher conducting the studies, but completion is expected in the near future.

Stimulating U(VI) reduction with the addition of acetate has other impacts on the geochemistry of subsurface environments that must be considered in an overall evaluation of this approach. Therefore, the possibility of arsenic release and the potential role of *Geobacter* in arsenic biogeochemistry during *in situ* uranium bioremediation was investigated because increased availability of organic matter has been associated with

substantial releases of arsenic in other subsurface environments. In a field experiment conducted at the Rifle study site, groundwater arsenic concentrations increased when acetate was added. The number of transcripts from *arrA*, which codes for the a-subunit of dissimilatory As(V) reductase, and *acr3*, which codes for the arsenic pump protein Acr3, were determined with quantitative reverse transcription-PCR. Most of the *arrA* (ca. 60%) and *acr3-1* (ca. 90%) sequences that were recovered were most similar to *Geobacter* species, while the majority of *acr3-2* (ca. 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 the transcription of *arrA* in situ, even though the presence of As(V) increased the transcription of *arrA* in cultures of *Geobacter lovleyi*, which was capable of As(V) reduction. These results demonstrated that subsurface *Geobacter* species can tightly regulate their physiological response to changes in groundwater arsenic concentrations. The transcriptomic approach developed 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 paper summarizing these results was published in *ISME Journal* and was one of the journal's top cited papers for 2013.

Another consideration in modeling the metabolism of microorganisms in the subsurface is the possibility that the addition of organic compounds to groundwater in order to promote bioremediation may represent a new selective pressure on subsurface microorganisms. Therefore, the ability of *Geobacter sulfurreducens* to adapt for rapid metabolism of lactate, a common bioremediation amendment, was evaluated. Serial transfer of five parallel cultures in a medium with lactate as the sole electron donor yielded five strains that could metabolize lactate faster than the wild-type strain. Genome sequencing revealed that all five strains had non-synonymous single-nucleotide polymorphisms in the same gene, GSU0514, a putative transcriptional regulator. Introducing the single-base-pair mutation from one of the five strains into the wild-type strain conferred rapid growth on lactate. This strain and the five adaptively evolved strains had four to eight-fold higher transcript abundance than wild-type cells for genes for the two subunits of succinyl-CoA synthase, an enzyme required for growth on lactate. DNA-binding assays demonstrated that the protein encoded by GSU0514 bound to the putative promoter of the succinyl-CoA synthase operon. The binding sequence was not apparent elsewhere in the genome. These results demonstrate that a single-base-pair mutation in a transcriptional regulator can have a significant impact on the capacity for substrate utilization and suggest that adaptive evolution should be considered as a potential response of microorganisms to environmental change(s) imposed during bioremediation. A paper summarizing these results was published in *ISME Journal*.

Additional progress was made in the modeling of the competition between *Geobacter* species and sulfate reducers during *in situ* uranium bioremediation. The interaction of *Geobacter* and sulfate-reducing bacteria (SRB) was investigated both in sediment incubations that mimicked *in situ* bioremediation and with *in silico* metabolic modeling.

In sediment incubations, *Geobacter* grew quickly but then declined in numbers as the microbially reducible Fe(III) was depleted whereas the SRB grew more slowly and reached dominance after 30–40 days. Modeling predicted a similar outcome. Additional modeling in which the relative initial percentages of the *Geobacter* and SRB were varied indicated that there was little or no competitive interaction between *Geobacter* and SRB when acetate was abundant. Further simulations suggested that the addition of Fe(III) could revive the *Geobacter* , but have little to no effect on the SRB. This prediction was confirmed experimentally. The results demonstrate that it is possible to predict the impact of amendments on important components of the subsurface microbial community during groundwater bioremediation. The finding that Fe(III) availability, rather than competition with SRB, is the key factor limiting the activity of *Geobacter* during *in situ* uranium bioremediation will aid in the design of improved uranium bioremediation strategies. A paper summarizing these results was published in *Biogeosciences*.

In order to improve the representation of the environment that *Geobacter* species are actually likely to experience in the subsurface the genome-scale metabolic model of *Geobacter sulfurreducens* was coupled with a pore-scale simulation of microbial growth based on coupling of iron reduction to oxidation of acetate. Fluid flow and solute transport in the model was governed by a combination of the Navier–Stokes and advection–diffusion–reaction equations. Microbial growth was modeled to only take place on the surface of soil grains where solid-phase mineral iron oxides are available. Mass fluxes of chemical species associated with microbial growth were described by the genome-scale microbial model, implemented using a constraint-based metabolic model, and provide the Robin-type boundary condition for the advection–diffusion equation at soil grain surfaces. We used our pore-scale model to explore the relationship between genome-scale metabolic models and more traditional Monod-type formulations, and to assess the manifestation of pore-scale variability (microenvironments) in terms of apparent Darcyscale microbial reaction rates. The genome-scale model predicted lower biomass yield, and different stoichiometry for iron consumption, in comparison to prior Monod formulations based on energetics considerations. We were able to fit an equivalent Monod model, by modifying the reaction stoichiometry and biomass yield coefficient, that could effectively match results of the genome-scale simulation of microbial behaviors under excess nutrient conditions, but predictions of the fitted Monod model deviated from those of the genome-scale model under conditions in which one or more nutrients were limiting. The fitted Monod kinetic model was also applied at the Darcy scale; that is, to simulate average reaction processes at the scale of the entire pore-scale model domain. As we expected, even under excess nutrient conditions for which the Monod and genome-scale models predicted equal reaction rates at the pore scale, the Monod model over-predicted the rates of biomass growth and iron and acetate utilization when applied at the Darcy scale. This discrepancy is caused by an inherent assumption of perfect mixing over the Darcy-scale domain, which is clearly violated in the pore-scale models. These results help to explain the previously identified need to modify the flux constraint parameters in order to match observations in applications of the genome-scale model at larger scales. A paper summarizing these findings was published in *Advances in Water Resources*.

Anaeromyxobacter species have been detected at some subsurface sites undergoing *in situ* bioremediation. As the first step in predictively modeling the growth and activity of *Anaeromyxobacter* species in the subsurface a genome-scale metabolic network was reconstructed for *Anaeromyxobacter dehalogenans* by combining the Model SEED web resource with a novel algorithm for resolving bioenergetic inconsistencies. The optimized network consists of 665 metabolites involved in 1048 intracellular reactions, and 117 transport/exchange reactions. The model was validated by comparing the measured growth yields and transfer of electron equivalents with predictions, showing that the model can accurately predict the metabolism during growth with ferric iron and nitrate as electron acceptors, and with acetate as the electron donor. A rough draft of a manuscript summarizing these results has been completed.

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