Understanding the bioremediative potential of FRC microbial communities

[1]Mary Beth Leigh, [1]Erick Cardenas, [1]Christina Harzman, [2]Weimin Gao, [2]Terry Gentry, [2]Jizhong Zhou, [1]Nathaniel Ostrom, [1]Terence Marsh, and [1]James M. Tiedje

[1] Michigan State University, East Lansing, MI [2] Oak Ridge National Laboratory, Oak Ridge, TN.

ABSTRACT

We are using stable isotope probing (SIP), supporting ecophysiological studies and metagenomic approaches to understand the ecology and physiology of microorganisms and communities important for the reduction of U, Cr, Tc and nitrate at the NABIR-FRC, and to identify those that respond under field implementation of bioremediation.

Stable-isotope probing methods are being applied to identify denitrifying microorganisms that incorporate the biostimulatory substrate, ethanol, in the Criddle-Jardine denitrifying reactor. Sequencing of a 16S rRNA clone library from the reactor indicated that the community was dominated by Beta-Proteobacteria (primarily *Thauera* and *Hydrogenophaga*) and Bacteriodes (*Flavobacterium).* SIP results indicate that *Thauera, Acidovorax, Dechloromonas, Hydrogenophaga* and *Sterolibacterium* rapidly incorporated carbon from ethanol. SIP is currently being combined with functional gene analyses to detect a variety of denitrification genes in biostimulated organisms using targeted PCR primers and a functional gene array. Future SIP experiments will be conducted on FRC sediment samples to identify bacteria that respond to the provision of biostimulatory substrates (ethanol, lactate, acetate).

The metal-reducing bacterium, *Desulfitobacterium hafniense* (DCB-2), was screened for reduction of Fe(III), Cu(II), U(VI), and Se(VI). Bacterial growth and metal reduction under metallorespiratory conditions was observed for Fe(III), Cu(II), and U(VI), but not Se(VI), although Se(VI) was reduced when grown fermentatively with Se(VI) as an electron sink. SEM of fermentatively grown *D. hafniense* revealed multiple small polyps on the surface of cells when grown in the presence of Se(VI). Backscatter imaging and EDS analysis suggested that polyps were of high density and contained selenium concentrations above background. Growth and biofilm formation of *D. hafniense* were observed under both fermentation and respiration conditions using two different surfaces (Dupont and Siran™ beads), although the biofilm consistency and abundance as well as cell morphology varied with media and substrata.

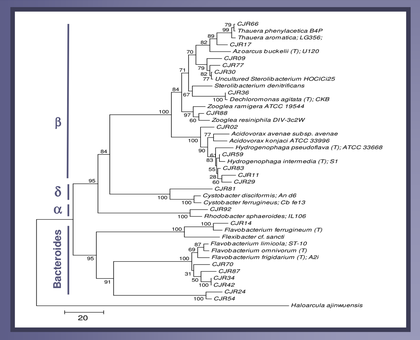
Direct and indirect DNA extraction methods have been evaluated and improved for the recovery of high quality, large fragment DNA from FRC sediments and groundwater for use in metagenomic library construction. The direct DNA extraction yielded up to 200kb DNA fragments that were successfully used to generate metagenomic libraries with an average insert size of 30-40kb. A new indirect DNA extraction was also developed, in which soil particles were flocculated and precipitated with Ca(II) or Mg(II) and suspended bacterial cells extracted, yielding DNA fragments larger than 300kb. This DNA is currently being used to construct a metagenomic library, and preliminary results suggest that it can generate libraries with > 50kb insert size. Approximately 120 L of groundwater has been collected from FRC well FW111 (up to 500 L will ultimately be collected) and extracted for a JGI community sequencing project.

Stable isotope probing of the Criddle-Jardine reactor

Introduction and Objectives: Since nitrate reduction is more energetically favorable than uranium reduction, high nitrate levels in FRC grounwater must be reduced to facilitate microbial uranium immobilization *in situ*. The Criddle-Jardine reactor performs microbial denitrification of FRC groundwater aboveground prior to reinjection and biostimulation of uranium (VI) reduction in the aquifer. The fluidized bed reactor (pictured right) is biostimulated with ethanol. Community sequence analyses and stable isotope probing (SIP) methods are being applied to:

- Investigate phylogenetic diversity and presence of denitrification genes (*nirS, nirK, narG*, etc.)
- Determine what organisms and what functional genetic potential are directly biostimulated by ethanol.

Community analyses



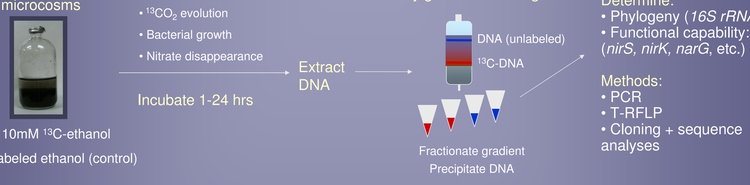
Phylogenetic tree of the Criddle-Jardine reactor community (Sampled 1/20/04) based on partial 16S rRNA gene sequence.

Results of BLAST searches of *nirS* sequences from a 35-clone library constructed from the Criddle-Jardine reactor

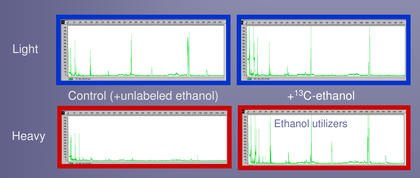
Group	Abundance	Length (bp)	Nearest match	Accession	% Similarity	Nearest named match	Accession	% Similarity
D-G3	6	904	Uncultured bacterium clone E04_0604 nitrite reductase (nirS) gene	AY939842	99.4	*Thauera aromatica* strain T1 putative dissimilatory nitrite reductase (nirS) gene	AY078257	92.5
B-F2	2	914	Uncultured bacterium clone G08_121903 nitrite reductase (nirS) gene	AY939837	97.1	*Thauera chlorobenzoica* strain 4FB1 putative dissimilatory nitrite reductase (nirS) gene	AY078262	80.5
E-E7	23	923	Uncultured bacterium clone G08_121903 nitrite reductase (nirS) gene	AY939837	92.6	*Thauera chlorobenzoica* strain 4FB1 putative dissimilatory nitrite reductase (nirS) gene	AY078262	80.3
C-F6	3	921	Uncultured bacterium clone G08_121903 nitrite reductase (nirS) gene	AY939837	90.2	*Thauera aromatica* strain T1 putative dissimilatory nitrite reductase (nirS) gene	AY078257	81.8
A-G2	1	887	Uncultured bacterium nirS gene for nitrite reductase, clone:NR1-87548.	AB164117	93.2	*Thauera chlorobenzoica* strain 4FB1 putative dissimilatory nitrite reductase (nirS) gene	AY078262	82.3

A *nirS* clone library was generated and 35 clones sequenced for preliminary study of *nirS* diversity (primers by Braker et al., 2000). Five groups of sequences were identified, bearing closest resemblance to *Thauera* spp., also found to be abundant in the 16S library (left).

Stable Isotope Probing (SIP)

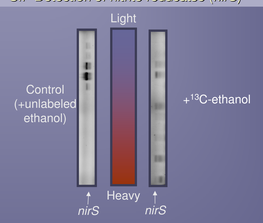


SIP Detection of ethanol utilizing bacteria



T-RFLPs of 16S rRNA genes amplicons in heavy and light density gradient fractions indicating ethanol utilization by the majority of the community following 24-hr incubation.

SIP Detection of nitrite reducatse (*nirS*)



Agarose gel detection of *nirS* amplified from density gradient fractions showing that ethanol-utilizers possess nitrite reducing capabilities (*nirS* primers from Braker et al., 2000).

Conclusions

SIP revealed that ethanol directly biostimulates the reactor community, including *Thauera, Acidovorax, Dechloromonas, Hydrogenophaga* and *Sterolibacterium*, and that the ethanol-utilizing community possesses genes important to the denitrification process. This work provides proof-of-concept that SIP can provide functional genetic information specifically from microbial populations that respond to biostimulatory substrates.

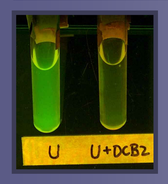
Future work:

- SIP of FRC sediments to determine which bacteria (in terms of phylogeny and genetic potential) are biostimulated by various substrates
- Link SIP with functional gene array (Wu et al., 2001) to detect numerous environmentally important genes in biostimulated FRC communities

Heavy metal reduction by *Desulfitobacterium hafniense*

Introduction and Objectives: Knowing that heavy metals bioaccumulate to toxic levels within organisms, *in situ* bioremediation has the potential to avoid human health risks associated with other remediation techniques. *Desulfitobacterium hafniense* is an anaerobic, sulfur-reducing, spore former with a proven ability to dehalogenate a variety of compounds. The ability of *D. hafniense* to reduce four heavy metals under fermentative and respiratory conditions was investigated. Metals investigated were: iron as Fe(III), copper as Cu(II), uranium as U(VI), and selenium as Se(VI).

Uranium: U(VI)

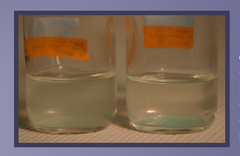


- **Uranium reduction is occurring:** U(VI) to U(IV): fluorescent yellow=> fluorescent white/ no fluorescence
- Left (U): uninoculated medium
- Right (U+DCB2): almost fully reduced inoculated medium
- Medium: uranyl acetate with lactate, acetate, pyruvate and vitamins (control)

Iron: Fe(III)

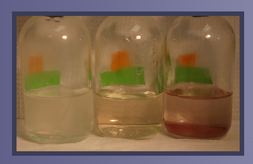
- **Iron reduction is occurring:** Fe(III) to Fe(II): orange-red=> gray-brown=> yellow
- Left: uninoculated medium
- Middle: partially reduced inoculated medium
- Right: almost fully reduced inoculated medium
- Medium: ferric citrate with lactate and vitamins (control)

Copper: Cu(II)



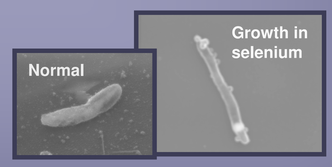
- **Copper reduction is occurring:** Cu(II) to Cu(I): bright blue=> light green=> colorless solid
- Left: partially reduced inoculated medium
- Right: uninoculated medium
- Medium: cupric sulfate with lactate, acetate, and vitamins (control)

Selenium: Se(VI)



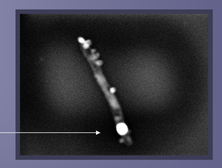
- **Selenium reduction is occurring:** Se(VI) to Se(IV): clear/ pale pink (soluble)=> red precipitate
- Left: inoculated medium fermentation control -no selenate
- Middle: uninoculated medium with sodium selenate
- Right: red precipitate in inoculated medium with sodium selenate
- Medium: DCB-1 media with pyruvate and vitamins

SEM of *D. hafniense* in selenium



The formation of selenium-containing polyps suggests possible mechanisms for protection from metal toxicity, metal reduction, or both

Backscatter using EDS
Brighter signal = higher atomic weight



Conclusions

- *Desulfitobacterium hafniense* was capable of reducing Fe(III) in the form of ferric citrate. Growth was observed with Fe(III) as the sole electron acceptor.
- *D. hafniense* can reduce Cu(II) when administered as cupric sulfate. Growth was detected when Cu(II) was the sole electron acceptor.
- *D. hafniense* was able to reduce U(VI) in the form of uranyl acetate. Growth was robust when U(VI) was the sole electron acceptor.
- *D. hafniense* can reduce Se(VI) as sodium selenate. Growth did not occur using Se(VI) as the sole electron acceptor under the conditions presented for metalorespiration. Growth did occur while reducing Se(VI) under conditions of fermentative growth.
- *D. hafniense* is a good candidate for bioremediation of heavy metals via reduction and metalorespiration.

DNA Extraction Methods for Metagenomic Applications

Objective

To develop and optimize methods for the extraction of high-quality, high-molecular-weight DNA for use in metagenomic applications.

Introduction

Unlike PCR-based approaches, metagenomic analyses require relatively large amounts of high-quality, high-molecular-weight (HMW) DNA. This is especially challenging for samples containing low levels of biomass and/or high amounts of humic substances. Based on previous studies, we developed and optimized two protocols which can be used for HMW DNA extraction.

Direct DNA extraction:

Methods: The method of Zhou et al. (1996) was modified to remove additional humic material from DNA extracts. Several different buffers, (Tris [pH = 8.0], PIPES [pH = 6.5], and sodium phosphate [pH = 6.4 & 8.0], were tested as components of the extraction solution. Soil microbial DNA was extracted by grinding with freezing/thawing according to methods modified from Zhou et al. (1996).

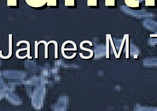
Results: Up to 200 kb sized DNA fragments were obtained by this direct extraction approach. The PIPES buffer removed the most humic materials and yet did not substantially decrease DNA yield. Soil microbial DNA extracted with the new buffer was relatively clean: *A*260/280 ratio = 1.5-1.7, *A*230/260 ratio = 0.9-1.2. However, this DNA still could not be directly used for PCR or enzyme digestion. DNA purified by either DEAE-Sephacel or gel purification could be used for enzyme digestion and PCR. After partially digesting the DNA, clone libraries were generated using an Epicentre BAC vector with an average insert size of 30-40 kb. Approximately 10,000 clones were generated from one ligation reaction (100ul) with ~95% containing inserts.

Indirect DNA extraction:

Methods: For each sample, 10 g of soil was mixed with 200 ml of ddH2O in a Waring blender at high speed for three minutes. Either AlNH4(SO4)2, CaCl2, or MgCl2 was then added (50 mM final concentration) and the solids permitted to settle. Bacteria were recovered from the supernatant by centrifuge or filtration.

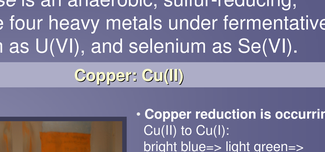
Results: After flocculation for 2-4 hrs, the soil solution became relatively clear and the microbial biomass could be collected by filtration or centrifugation. Three to four dispersion-flocculation cycles were typically sufficient to recover about 30 to 50 % of bacterial cells from surface soils. Ca(II) and Mg(II) were the best ions for flocculation. The DNA extracted from the obtained bacterial cells was sufficiently clean for PCR and enzyme digestion without further purification. Using freeze/thaw grinding combined with in-gel lysis, DNA fragments larger than 300 kb have been obtained from these bacterial cells. This DNA is currently being used to construct a clone library. Preliminary results suggest that the DNA can generate a library with > 50 kb insert size.

Buffer Impact on Extraction of Humic Material from Soil



1. Tris buffer (pH = 8.0)
2. PIPES buffer (pH = 6.5)
3. Sodium phosphate buffer (pH = 6.4)
4. Sodium phosphate buffer (pH = 8.0)

Buffer Impact on Recovery of Soil Microbial DNA



1. Sodium phosphate buffer (pH = 8.0)
2. Sodium phosphate buffer (pH = 6.4)
3. Tris buffer (pH = 8.0)
4. PIPES buffer (pH = 6.5)

Conclusions

By modifying the buffer in the DNA extraction solution, the humic substances recovered from direct soil extraction were substantially decreased, and this greatly improved subsequent purification procedures. Both the direct and indirect methods produced high-quality, high-molecular-weight DNA capable of generating the large insert clone libraries necessary for metagenomic analyses.

FRC Community Sequencing Project

Objective

To sequence the microbial community indigenous to contaminated FRC groundwater

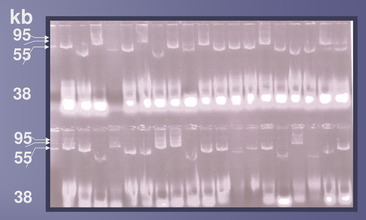
Methods

Biomass was collected from approximately 1,700 L of FRC well FW106 groundwater. This groundwater had a pH of 3.4 and contained approximately 50 mg of uranium, 4,000 mg of nitrate, and 2,000 mg of sulfate/L. Direct bacterial counts were between 10^4 and 10^5 cells/ml. Groundwater was collected using peristaltic pumps and passed through sintered metal (T. J. Phelps, unpublished) or 0.2 µm Supor® (Pall Corporation) filters to collect the biomass. Cells were recovered from the filters by shaking and/or brief sonication and were then pelleted by ultracentrifugation prior to DNA extraction according to the methods of Zhou et al. (1996). Recovered DNA was then treated with RNAse and sent to JGI for BAC/fosmid library construction and whole genome shotgun sequencing.

Results

Approximately 300 µg of DNA was obtained from the 1,700 L of FW106 groundwater. A 40 kb fosmid library has been constructed and is currently being sequenced.

Clone Profile of BAC Library



Conclusions

These results will reveal the genetic capabilities of microorganisms indigenous to highly contaminated FRC groundwater and will potentially allow researchers to more effectively utilize the microorganisms' genetic potential to remediate the FRC site.

Conclusions: Through combined molecular and ecophysiolocial approaches, we are gaining new insights into the bioremediative potential of organisms important to reduction of U, Cr, Tc and nitrate at the NABIR-FRC.

References

Braker, G., J. Zhou, L. Wu, A. H. Devol and J. M. Tiedje. 2000. *Appl. Environ. Microbiol.* 66:2096-2104.

Zhou, J. Z., M. A. Bruns, and J. M. Tiedje. 1996. *Appl. Environ. Microbiol.* 62:316-322.

Wu, L., D. K. Thompson, G. li, R. A. Hurt, J. M. Tiedje and J. Zhou. 2001. *Appl. Environ. Microbiol.* 67:5780-5790.

Acknowledgements

- DOE NABIR program
- Craig Criddle and Phil Jardine