LBNL Sustainable Systems SFA: Unraveling Biogeochemical Reaction Networks

Use of biomolecular signatures to unravel biogeochemical reaction networks underlying Cr(VI) reduction at the Hanford 100H site

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ABSTRACT

We are developing the use of biomolecular signatures as part of the LBNL SFA challenge entitled "Unraveling Biogeochemical Reaction Networks Mediating Sustained Chromium Reduction", which focuses on *in situ* reductive immobilization of Cr at DOE's Hanford 100H site. There are two primary research components of biomolecular signatures in this challenge: (a) biomolecular signature discovery, which involves development and testing of meta-transcriptome-based gene expression microarrays and (b) quantifying biomolecular signatures, which involves assessing relationships between microbial metabolic activity (e.g., nitrate reduction) and specific biomolecular signatures (e.g., *narG* gene or transcript copy number). The biomolecular signature work described here is being integrated into an interdisciplinary effort involving spectroscopic, isotopic, and reactive transport modeling approaches. Among the research hypotheses to be addressed in this challenge are the following: (1) Microbial processes mediate both direct (enzymatic) and indirect Cr(VI) reduction at Hanford 100H, but indirect pathways dominate sustained reduction; furthermore, sulfate reduction is the electron-accepting process ultimately driving sustained Cr(VI) reduction at Hanford 100H, and (2) Fermentative/acetogenic versus respiratory metabolism will promote retention of organic carbon in the aquifer.

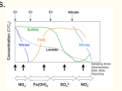
Meta-transcriptome-based gene expression microarrays: We are developing a novel high-throughput approach that uses the meta-transcriptome (cDNA representing the collective mRNA transcripts from the entire microbial community) to design high-density oligonucleotide microarrays, which can be used to identify highly expressed genes in a specific community under conditions of interest, without requiring any a priori hypotheses about which genes the community might be expressing or prior sequence information from data repositories. This approach represents an alternative to functional gene arrays, which entail a *priori* assumptions about key gene sequences, and has the potential to reveal novel and important molecular signatures for a specific community based on differential expression of genes represented in a customized array. To generate meta-transcriptome samples, we inoculated anaerobic microcosms with groundwater from the Cr-contaminated Hanford 100H site and supplemented them with lactate and electron acceptors present at the site, namely, nitrate, sulfate, and Fe(III). The microcosms progressed successively through nitrate-reducing, sulfate-reducing, and Fe(III)-reducing conditions, and after a second addition of nitrate, nitrate-dependent Fe(II)-oxidizing conditions. Cr(VI) was rapidly reduced initially and again upon further Cr(VI) amendments. Community DNA and RNA were harvested during each major biogeochemical phase and were subjected to PhyloChip analysis to characterize community composition under various electron-accepting conditions. Community mRNA was amplified and reverse transcribed using methods described elsewhere, which include microbial RNA polyadenylation and linear amplification with a T7 RNA polymerase (MessageAmp II-Bacteria Kit, Ambion). This cDNA preparation method was designed to reduce the amount of cDNAs representing rRNA. Samples representing several different biogeochemical regimes will be submitted for pyrosequencing using the 454 Titanium technology. Ultimately, arrays derived from the meta-transcriptomic sequence data will be used to interrogate 100H groundwater samples.

Microbial Activity-Signature Relationships: Initially, we are using well-characterized cultures isolated from the Hanford 100H site to establish whether quantitative relationships can be determined between relevant metabolic activity and (a) gene copy number (qPCR) and (b) transcript copy number (RT-qPCR). If consistent activity-signature relationships can be established, these should provide quantitative information (i.e., estimates of key *in situ* metabolic activities) that can be incorporated into the reactive transport modeling effort at Hanford 100H. Gene sequences have been determined for key functional genes (e.g., chromate reductase genes *chrR* and cytochrome *c3*, denitrification genes *narG* and *nirS*, and sulfate reduction genes *dsrA* and *aprB*) in two bacterial species isolated from Hanford 100H groundwater: strain RCH1 (a sulfate-reducing bacterium similar to *Desulfovibrio vulgaris* Hildenborough) and strain RCH2 (a denitrifying strain similar to *Pseudomonas stutzeri*). For Hanford bacterial strain RCH2, we have investigated some activity-signature relationships and Cr(VI) reduction kinetics in batch systems; some of this work has been transitioned to continuous culture (i.e., chemostat).

BIOMOLECULAR SIGNATURES APPROACH

SIGNATURE DISCOVERY - META-TRANSCRIPTOME-BASED MICROARRAYS

- Develop a novel and high-throughput approach that uses the meta-transcriptome to design high-density oligonucleotide microarrays, which can be used to identify highly expressed genes in a specific community under conditions of interest, without requiring any *a priori* hypotheses about which genes the community might be expressing or prior sequence information from data repositories.
- Meta-transcriptome samples derive from anaerobic microcosms inoculated with Hanford 100H groundwater and harvested at times representing a range of possible biogeochemical conditions (e.g., chromate reduction and denitrification, sulfate reduction, ferric iron reduction, etc.). (An idealized illustration is given on the right.)



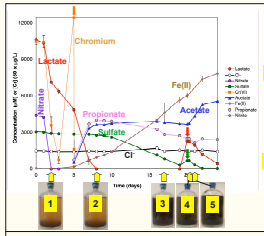
- Design high-density oligonucleotide arrays (NimbleGen platform) based on the collective meta-transcriptome sequences.
- Use the meta-transcriptome-based microarrays to interrogate Hanford 100H field samples to understand whole-community gene expression under conditions of interest.

SIGNATURE QUANTIFICATION - MICROBIAL ACTIVITY-SIGNATURE CORRELATIONS

- Use well-characterized bacterial cultures from Hanford 100H to assess whether quantitative constants can be established that correlate biogeochemical activities with molecular signatures based on DNA (gene copy number) or mRNA (transcript copy number).
- If robust correlations can be established, incorporate into reactive transport models qPCR or RT-qPCR data from groundwater samples to estimate *in situ* metabolic rates.

META-TRANSCRIPTOME-BASED MICROARRAYS

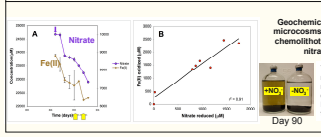
Microcosms inoculated with Hanford 100H groundwater were used to provide meta-transcriptome samples under various relevant TEAPs



Geochemical data for Hanford 100H anaerobic microcosms showing 5 harvesting time points (yellow arrows)

1	Denitrification, chromate reduction, and lactate mineralization;
2	Lactate fermentation to acetate and propionate, Fe(III) reduction, and minor sulfate reduction;
3	Sulfate reduction and Fe(III) reduction in the absence of lactate;
4,5	Lactate fermentation (to acetate) and sulfate reduction after re-spiking of sulfate and lactate.

The 3 downward arrows (color coded by chemical) represent re-spiking events. Error bars represent one standard deviation for all chemical variables.



Geochemical data for Hanford 100H anaerobic microcosms showing harvesting time points for chemolithotrophic Fe(II) oxidation coupled with nitrate reduction (yellow arrows)

These biogeochemical conditions may be relevant to anaerobic re-oxidation and re-mobilization of Cr(III) to Cr(VI), which can occur if Mn(II) is re-oxidized to Mn(III/IV). Error bars represent one standard deviation for both chemical variables.

Molecular analysis of microbial dynamics and expression over a range of relevant terminal electron-accepting conditions

Extraction optimization for FeS-rich samples

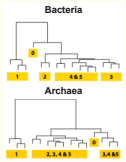
Chelex-100 optimized nucleic acid extraction

RNA - DNA

mRNA amplified for pyrosequencing

PhyloChip analysis

PhyloChip analysis



Cluster analysis of Bacterial and Archaeal community composition over Hanford 100H microcosm harvesting time points

Bacterial and Archaeal communities diverged rapidly from the initial composition but began to converge over time as electron acceptors were depleted.

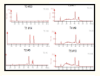
Bacterial communities transitioned from denitrifying populations (*Pseudomonadaceae*, *Rhodocyclaceae*, *Comamonadaceae*, *Flavobacteriaceae*), through fermenting (*Lachnospiraceae*, *Bacteroidetes*) and sulfate-reducing (*Desulfovibrionaceae*, *Desulfobacteraceae*) bacteria.

Archaeal communities did not display as distinct a transition over time as Bacteria. Methanogens were consistently detected by DNA but not by RNA analysis.

mRNA enrichment/amplification

Approach: Preferential polyadenylation of mRNA followed by oligo-dT-primed reverse transcription, second strand synthesis and *in vitro* transcription.
Requirements: High-quality RNA with minimal degradation. As preferential mRNA amplification occurs due to inefficient amplification of molecules with greater 2° structure, such as rRNA, any fragmented rRNA will also be amplified.

BioAnalyzer - QC



Relatively good quality RNA from FeS-rich samples

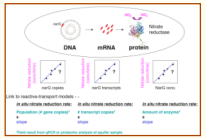
Preliminary mRNA amplification

No preferential mRNA amplification was found. This is probably due to the degree of fragmentation of the total RNA extracts. Even if RNA extraction protocols are optimal, complex populations will consistently contain active/dying/dead members with varying degrees of RNA integrity. Therefore any mRNA amplification/enrichment method that relies on intact rRNA may be impacted by the time point sampled and the microbial community composition.

We are currently developing a novel rRNA removal procedure that we hope will be independent of sample type and robust to varying degrees of RNA integrity.

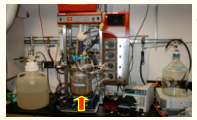
ACTIVITY-MOLECULAR SIGNATURE RELATIONSHIPS

Approach



- Use well-characterized bacterial cultures from Hanford 100H to assess whether quantitative constants can be established that correlate biogeochemical activities with molecular signatures based on DNA (gene copy number) or mRNA (transcript copy number).
- If robust correlations can be established, incorporate into reactive transport models qPCR or RT-qPCR data from groundwater samples to estimate *in situ* metabolic rates.
- For results reported here, we explored activity-signature relationships for two denitrification genes (*narG* - nitrate reductase α subunit, and *nirS* - nitrite reductase) as well as the "housekeeping" gene *rpoB* (RNA polymerase, β subunit) in a denitrifying bacterium isolated from Hanford 100H groundwater, strain RCH2 (like *Pseudomonas stutzeri*).

Anaerobic chemostat studies run with Hanford denitrifying strain RCH2 (like *P. stutzeri*)

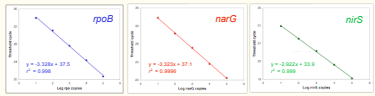


Four steady-state conditions were chosen for harvesting in a chemostat operated under the nitrate-limiting conditions below.

Dilution rate (D) (1/hr)	NO_3 in feed (S_o) (mM)	Lactate in feed (mM)	NO_2 in reactor (S) (mM)	Lactate in reactor (mM)	OD 600	$D*(S_o - S)$ (mM NO_3 per day)
0.084	3.6	5.7	<0.01	2.8	0.08	7.3
0.128	4.8	5.5	<0.01	1.6	0.13	14.8
0.192	6.5	5.5	<0.01	0.62	0.18	28.4
0.32	7.9	8.6	0.038	2.5	0.23	60.6

Molecular methods: 5-mL chemostat samples were harvested at the four conditions shown above, combined with 2 vol of RNALater (Ambion), and processed with the AllPrep DNA/RNA Mini Kit (QIAGEN). Total DNA and cDNA were quantified on a Nanodrop model ND-1000. Reverse transcription was performed with random hexamers and SuperScript II (Invitrogen). qPCR of DNA and cDNA was performed with a BioRad MyiQ real-time PCR system (SYBR Green).

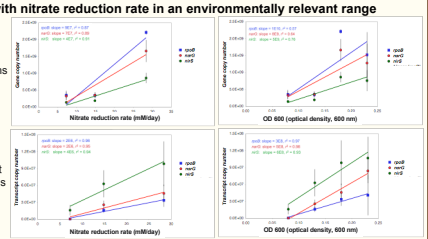
qPCR calibration



narG and *nirS* transcript and gene copy numbers correlated fairly well with nitrate reduction rate in an environmentally relevant range

Gene correlations

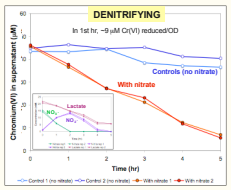
Transcript correlations



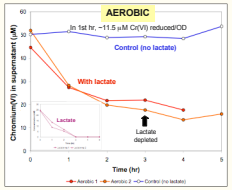
- In a nitrate reduction range between 8 and 28 mM nitrate/day, correlations to *narG* and *nirS* gene copy number yielded r^2 values of 0.89 - 0.91 and to transcript copy number, r^2 values of 0.94 - 0.95. The relationship did not hold between 28 and 61 mM nitrate/day, but such high rates are unlikely to occur in the subsurface.
- Correlations of *narG*, *nirS*, and *rpoB* to OD_{600} (a surrogate for cell number) were very strong for transcripts (r^2 values between 0.93 and 0.98) but were surprisingly poor for gene copy number (r^2 = 0.57 to 0.76). Since each of these genes occurs at a frequency of one copy per genome, the correlation of gene copy number to OD_{600} should be stronger.

ACTIVITY-SIGNATURE RELATIONSHIPS (CON'T)

Cell suspension studies conducted with Hanford strain RCH2 (like *Pseudomonas stutzeri*) showed considerable Cr(VI) reduction under both denitrifying and aerobic conditions



Methods: Cells were grown anaerobically with 20 mM lactate and 20 mM nitrate, harvested at mid log phase, washed with buffer, and resuspended in a bicarbonate (30 mM) and phosphate (10 mM) buffer containing 50 μM chromate, 20 mM lactate, 20 mM nitrate, and 40 μg/mL chloramphenicol (to prevent growth during resuspension). The suspension OD600 was ~ 0.95 (~1.85 E9 cells/mL). Growth and resuspension studies were conducted in an anaerobic glove box. At selected times, cells were spun down (14K rpm, 5 min) and the supernatant was analyzed by LC-ICP-MS [for Cr(VI) and Cr(III)] and by IC (for anions).



Methods: Cells were grown aerobically with 20 mM lactate (shaking at 200 rpm), harvested at mid log phase, washed with buffer, and resuspended in a bicarbonate (30 mM) and phosphate (10 mM) buffer containing 50 μM chromate, 20 mM lactate, and 40 μg/mL chloramphenicol (to prevent growth during resuspension). The suspension OD600 was ~ 1.8 (~3.4 E9 cells/mL); cells were shaken at 200 rpm. At selected times, cells were spun down (14K rpm, 5 min) and the supernatant was analyzed by LC-ICP-MS [for Cr(VI) and Cr(III)] and by IC (for anions).

Resuspension of aerobically grown cells under *anaerobic* conditions yielded negligible Cr(VI) reduction (not shown).

SUMMARY

- Meta-transcriptome samples have been generated under a range of relevant electron-accepting conditions (e.g., chromate-reducing, denitrifying, sulfate-reducing, iron-reducing, chemolithotrophic Fe(II)-oxidizing) using anaerobic microcosms inoculated with Hanford 100H groundwater
- In preparation for microarray design based on meta-transcriptome sequencing, RNA was extracted, reverse-transcribed and amplified from the Hanford microcosms using a method designed to preferentially enrich mRNA. However, rRNA was also amplified, so new methods to enrich cDNA representing mRNA are being developed.
- PhyloChip analyses have documented shifts in microbial community composition during the changes in electron-accepting regimes.
- Chemostat (continuous culture) experiments with denitrifying Hanford strain RCH2 (like *Pseudomonas stutzeri*) revealed a relatively strong correlation between transcript copy number of two denitrification genes (*narG* and *nirS*) and nitrate reduction rate. Less strong correlations were found between gene copy number and nitrate reduction rate. Such activity-signature correlations may ultimately be integrated into reactive transport models.
- Hanford bacterial strain RCH2 reduced Cr(VI) under both denitrifying and aerobic conditions at similar specific rates (lactate was the electron donor). In both cases, Cr reduction was negligible in the absence of the electron acceptor, possibly suggesting that Cr(VI) cannot act directly as an electron acceptor for this bacterium.

Task/Year	YR1	YR2	YR3	YR4	YR5
Field-relevant Hanford 100H, Cr-immobilizing microcosms for representative meta-transcriptome profiling	DONE				
mRNA enrichment and cDNA production for meta-transcriptome sequencing of Hanford 100H, Cr-immobilizing microbial communities	ONGOING				
PhyloChip analysis of microbial community dynamics in 100H microcosms	PARTLY DONE				
Design of meta-transcriptome-based microarrays and application to Hanford 100H microcosms					
Quantitative relationships between activity and molecular signatures for bacteria relevant to Hanford 100H	STRAIN RCH2 ($P.$ *stutzeri*)				
Application of meta-transcriptome-based microarrays to Hanford 100H push-pull study					
Cr isotopic fractionation by Cr(VI)-reducing bacteria relevant to Hanford 100H	ONGOING				
Assess performance of meta-transcriptomic microarray vs. pre-defined functional gene array					
Quantitative relationships between activity and molecular signatures for metabolic processes relevant to Hanford 100H					

ERSP ANNUAL PI MEETING APRIL 20-23, 2009

Funding for this study was provided by the U.S. Department of Energy, BER Program Contract DE-AC020SCH11231 to the LBNL Sustainable Systems Scientific Focus Area