Mercury Methylation: Genes and Communities (Hg SFA at ORNL, Task 3)

Oak Ridge National Laboratory – ERSP Science Focus Area

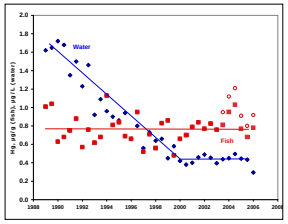
Introduction

- Discharges and spills of mercury associated with DOE facilities in Oak Ridge, TN, (e.g., Y-12) have contaminated groundwater and streams (e.g., East Fork Poplar Creek – EFK).
- The ratio of methyl Hg to total Hg in the streams is low.
- Total Hg in the streams has declined over time since the early 1980s but mercury in fish has not declined.

The ERSP Hg SFA at Oak Ridge address these issues.

- The Goal of Task 3 of the SFA is to elucidate the genetic basis of mercury methylation and to understand the geochemical controls on expression of the genes and rates of microbial mercury methylation in collaboration with the other tasks.
- The primary tasks are
  - Task 3.1 Identify the genes involved in Hg methylation.
  - Task 3.2 Elucidate the regulatory mechanisms of Hg methylation at the transcriptional level.
  - Task 3.3 Use our growing knowledge of the genes related to Hg methylation to upgrade the functional gene array (FGA) to gain insight about gene distribution and ultimately expression in microbial communities.

Figure 1. Mercury in stream and fish.



- Two major aspects of the task are presented here
  - Addressing Task 3.1 we have comparative genomics studies under way and experimental studies of specific genes identified as candidates for involvement in methylation
  - Task 3.3. and 3.2 As a step in identifying the geochemical controls on mercury methylation, we have collected samples in a survey of mercury contaminated sites in Oak Ridge and a control site to obtain the geochemical conditions in which mercury methylation occurs and to characterize the microbial community present at the site.

Methods

Sampling Procedure for Stream Sediments

- Sediment Samples
  - Sediment samples were taken a) in the middle of the creek and b) adjacent to one of the stream banks.
  - The samples were collected by scooping sediment and bailing water in a large bucket and pouring into an autoclaved nalgene container.
  - 1-2 L of the water with fine sediments was centrifuged and ~0.5 g of the sediments was taken for DNA extraction.
  - The total community genomic DNA (cgDNA) was extracted from approximately 0.5-1 g of sediment using the PowerSoil™ DNA Isolation Kit (Mo Bio Labs, Inc., Carlsbad, CA).

Sampling - Water Samples

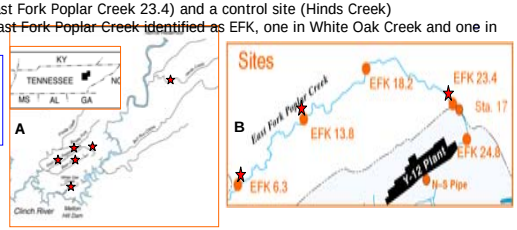
- Water was split into several sets for different analyses geochemical analysis. Including SRP and DOC.
- Uranium was measured using either a Kpa or an ICP-MS.
- Centrifuged water samples were analyzed for major anions and cations using ICP and IC.

Methods, continued...

Sampling Locations

- Seven sets of samples (water and sediment) were collected at a total of 6 sites (Figure 2) from October of 2007 to September of 2008.
- At each sampling time we either sampled
  - Two sites representing a highly contaminated (East Fork Poplar Creek 23.4) and a control site (Hinds Creek)
  - Six sites (including those above, 2 more sites in East Fork Poplar Creek identified as EFK, one in White Oak Creek and one in Bear Creek)

Figure 2. Sampling sites in creeks in the Oak Ridge Vicinity (★). **A**. Large scale view of 6 sites. **B**. Detail of EFK sites indicated by star.



Phylogenetic Analysis

- The primary method of phylogenetic analysis was via PCR amplification and 454 Pyrosequencing
  - The hypervariable V4 region (~290 bp) of the 16S rRNA gene was amplified from the total community genomic DNA using the high fidelity AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, CA) and specially designed primers.
  - The primers, in addition to the 16S rRNA priming region, contain sequences (adaptors) required for 454 FLX pyrosequencing, and the forward primer contains an additional short key (tag) sequence, so that 40 samples could be analyzed in one sequencing run.
  - Raw 454 data (~180,000 Mb from whole plate: region A and B) were initially processed through the RDP pyrosequencing pipeline. During this process the sequences were sorted by tag sequence, the 16S primers were trimmed off and low-quality sequences were filtered out. From ~8,000 to ~12,000 high quality sequences in the range of 200-220 bp were obtained for each sample.

Results: Site Geochemistry and Phylogeny

- There was a wide range in many geochemical parameters observed over the sampling sites (e.g., see Table 2 for range from March, May, July, September 2008 sampling dates).
- For the sampling dates with the most complete geochemical data (March to September of 2008) there were high correlations among water and sediment Hg (r=0.72), chloride and nitrate (r=0.976), uranium and both nitrate and chloride (r= 0.92, and 0.96).

	pH	Hgw	Hgs	Cl	NO_3	SO_4	DOC	U	SRP
		ng/L	ug/g	mg/L	mg/L	mg/L	mg/L	ug/L	ug/L
Max	8.87	107.7	46.8	2.7	18.3	4.3	59.7	267.0	767.1
Min	7.25	0.0	0.0	0.2	0.0	0.8	2.0	0.0	1.6

Table 1. Range of select geochemical parameters observed in the March to September time period at the six sites (average of replicate samples from each sampling time and location).

454 for Phylogenetic Characterization

A detailed look at just the most contaminated site and the control site at the phylum level indicate some commonalities

Deltaproteobacteria make up a very small proportion of the total but are the only bacteria currently implicated in mercury methylation

- Cyanobacteria are a very prominent group in the March Samples (Fig. 3), but do not persist at the same levels through the year (Fig 4).
- Betaproteobacteria, Gammaproteobacteria, and Verrucomicrobia are present thought the year

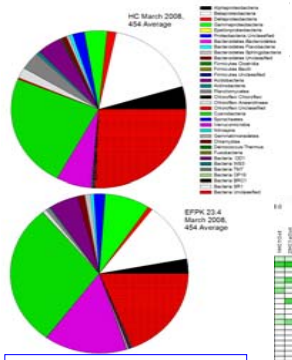


Figure 3. Phylogenetic representation of control and contaminated site for March.

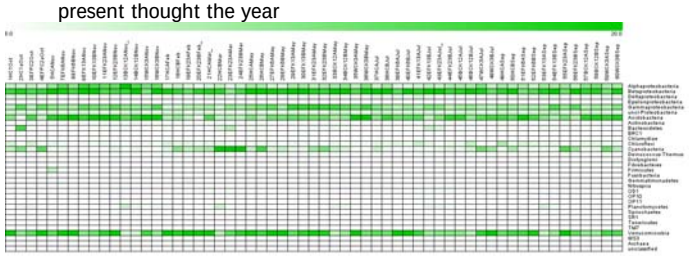


Figure 4. Heat map representation of phylogenetic data from all sites & months.

Betaproteobacteria, Gammaproteobacteria, and Verrucomicrobia are present though the year

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Environmental Remediation Sciences

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Results: Phylogeny by 454 Analysis

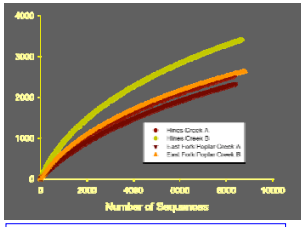
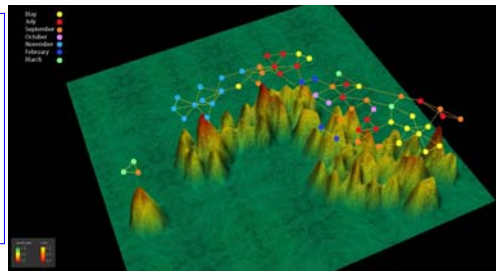


Figure 5. Example refraction curves.

Figure 6. Clustering of the samples using terrain mapping indicates that the sites cluster more closely in cold weather months (e.g., November) and are very divergent in warmer months (e.g., May and July)



- There is very high diversity in the samples
- Seasonal changes may dominate (also seen in FGA results)
- Thus analysis for relationships among the community structure and the geochemistry at the sites should be done using the samples from the warmer months.

Results: *Deltaproteobacteria* spp.

Oak Ridge Area Stream Samples (59 454 libraries)

Table 2. Two methods of identifying deltaproteobacteria with a range of confidence or similarity in the results.

Method 1. RDP Classifier, delta-Proteobacteria		Method 2. Sequence alignment and clustering with selected delta-Proteobacteria	
Confidence level, %	Number of sequences	Percentage similarity, %	Number of sequences
99	3016	97	511
95	4238	90	1374
90	5242	85	1588
80	7331	80	2943
70	10339	70	41077

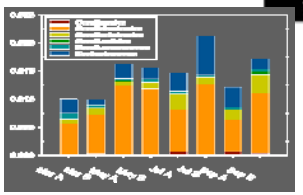
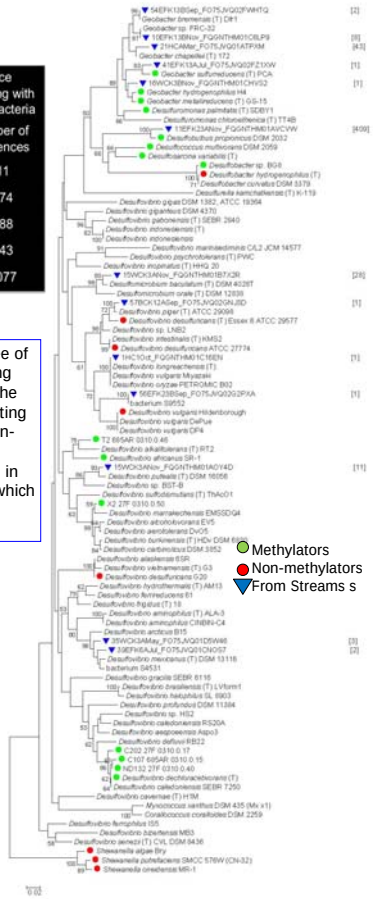


Figure 7. Histograms showing the presence of deltaproteobacteria (using method 1 in table 2) in EFPK 23.4 sample.

Figure 8. Phylogenetic tree of deltaproteobacteria showing sequences obtained from the stream samples and indicating known methylators and non-methylators. The tree was made from the 2nd method in Table 2 at 97% similarity which yielded the 511 sequences used to construct the tree



- Groups of deltaproteobacteria that include mercury methylators are Desulfovibrionales, Desulfobacteriales and Geobacteraceae
- Most major groups (Fig 4) are present in all samples (except for Desulfurellaceae)
- There appears to be within-site variability at EFK 23.4 in the presence of the Deltaproteobacteria that is as pronounced as the seasonal trend from March though September
- Found Deltaproteobacteria including close relatives (e.g., 97 % 16s sequence similarity or better) of those know to methylate including *Desulfobulbus propionicus*, *G. sulfurreducens*, *Desulfovibrio africanus* (500,000 sequences in 59 samples)

Results: Gene Discovery

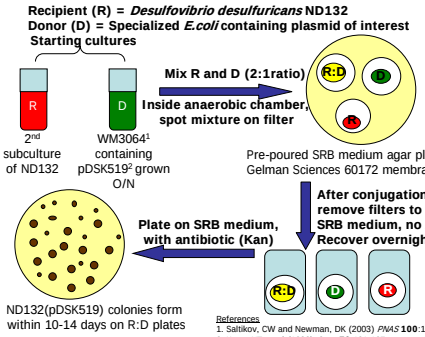
- Hg methylation appears constitutive
- Few gene expression (and no toxicity) changes at realistic mercury levels
- Comparative genomics underway (will now include *D. propionicus*)
- Genetic systems developed and applied in non-methylators and have tested numerous genes for involvement in methylation by transferring to non-methlyators with no positive results
- Random mutagenesis approach will be tried in methylators as well as targeted deletions

D. africanus	Gene/operon	Progress	Methylation
SAM-dependent methyltransferase	Gene	Completed	No
hypothetical protein	Gene	Completed	No
Methionine synthase I	Gene	Completed	No
D. desulfuricans ND132			
methionine biosynthesis (*metW*)	Gene	Completed	No
5-methylTHF-homocysteine methyltransferase	Gene	In progress	
ABC-type uncharacterized transporter	Operon	Completed	No
transcriptional regulator (*mar R*)	Operon		
homoserine O-acetyltransferase	Operon		
methionine biosysthesis (*met W*)	Operon		
ABC-type Fe^{3+}/colbalamin sideophores	Operon	Proposed	
ABC-type Fe^{3+}-hydroxamate transport	Operon		
Predicted O-methyltransferase	Operon		
Methylase (ubiquione/menaquinone biosynthesis)	Operon		
ABC-type Fe^{3+} siderophore transport permease	Operon		
ABC-type Fe^{3+}/colbalamin sideophores, ATPase	Operon	Proposed	
ABC-type Fe^{3+}-hydroxamate transport	Operon		
precorrin-2 C_{20}-methyltransferase	Operon		
cobalamin biosynthesis (*cbiK*)	Operon		

Protocols under development for SRBs

Conjugation Protocol:

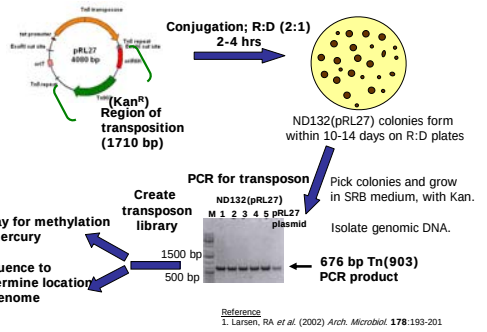
Recipient (R) = *Desulfovibrio desulfuricans* ND132
Donor (D) = Specialized *E.coli* containing plasmid of interest
Starting cultures



References
1. Saltikov, CW and Newman, DK (2003) *PNAS* **100**:10983-10988
2. Keen, NT *et al.* (1988) *Gene* **70**:191-197

Transposon mutagenesis:

Follow conjugation protocol; Donor (D) = WM3064 containing pRL27[1]

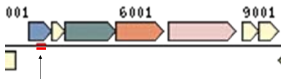


Reference
1. Larsen, RA *et al.* (2002) *Arch. Microbiol.* **178**:193-201

Summary

- Genes involved in methylation are likely to be constitutive
- Few changes in gene expression with mercury and activity seen under a number of growth conditions
- Targeted mutagenesis of putative Hg methylase genes underway
- Random mutagenesis protocols for Hg methylators under development
- Close relatives to known methylating deltaproteobacteria in stream samples
  - The most prevalent was related to *Desulfobulbus propionicus*
  - Sequences close to G. *sulfurreducens, Desulfovibrio africanus* were also found
- Site to site differences in the community were damped during cold months

First ND132 Transposon Mutant Confirmed by Sequence Analysis!



Region of sequence recognition (red)

Acknowledgments

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