Recent advances in bacterial community involvement in mercury transformations in the environment

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**The Mercury SFA is led by L. Liang, Environmental Sciences Division, ORNL with University of Missouri as a partner
Mercury is an important pollutant

- Microbial transformations of mercury as it moves through the geochemical cycle are critical in its toxicity
Mercury sources range from natural emissions, to emissions from coal fired power plants, and releases from industrial sources.

- Mercury is a widespread pollutant at low levels due to atmospheric deposition.
- Hot spots are usually associated with industrial point sources.
Bioaccumulation can exacerbate the problem

Predator fish: 10-100 million x
Prey fish: 1-10 million x
Zooplankton: 20,000 – 100,000 x
Phytoplankton: 10,000 x
Water: 1x methylmercury
Hg concentrations in wildlife

* Denotes neotropical migrant species

Blood Hg Concentration (ppm, ww)

- **Very High Risk**
- **High Risk**
- **Moderate Risk**
- **Low Risk**

Species included:
- Louisiana Waterthrush (n = 20)*
- Wood Thrush (n = 100)*
- Traill’s Flycatcher (n = 6)*
- Yellow-throated Vireo (n = 2)*
- Seaside Sparrow (n = 6)
- Bicknell’s Thrush (n = 50)*
- Red-winged Blackbird (n = 40)
- Eastern Wood-pewee (n = 2)*
- Palm Warbler (n = 2)
- Indigo Bunting (n = 11)*
- Nelson’s Sparrow (n = 27)
- Rusty Blackbird (n = 23)
- Saltmarsh Sparrow (n = 472)
Hg cycling is complex and there is a microbial involvement in methylation and demethylation.
Methylmercury research timeline

1956 – Minamata disease discovered and in 1968 officially recognized that disease was caused by the consumption of seafood contaminated by methylmercury compounds.


1974 –Methyl-B12 compounds are likely the agent for transferring CH₃⁻ to Hg. (Wood, JM. Science, 183:1049.)

1994 – Researchers show that methylmercury formation can be an enzymatic process involving cobalt. (Choi et al, AEM, 60(4):1342)

2003 – Work contradicts the 1994 study, questioning the role of B12. (Ekstrom et al. AEM, 69(9):5414)
The ORNL/DOE Mercury Science Focus area examines a pollutant important to DOE

- Reductions of mercury input in EFPK have not led to decreases in Hg in Fish
- The US Department of Energy has been funding a research program at the Oak Ridge National Laboratory focused on increasing the understanding of mercury transformations in the environment.
The Hg SFA strategy for understanding contaminant transformation and behavior

Field biogeochemistry

Fundamental rates and mechanisms

Microbial and genetic controls

Molecular structure and simulations

Transformation in field

Speciation & mechanisms

Molecular dynamics

Sediment-water interface
Species/ abundance
Microbial communities

Coupled microbial and geochemical reactions

Molecular level understanding of contaminant association and reaction

Reaction mechanisms and kinetics at groundwater-surface water interface

Oak Ridge National Laboratory
U.S. Department of Energy
Site biogeochemical processes and microcosm studies

- **Site Investigations**
  - Chemical, physical, microbial data relevant to Hg transformations
  - Hg reactivity
    - Correlating Hg reactivity with methylation potential

- **Methylation Bioassay**
  - Hg speciation and methylation rate

- **Microcosm Studies**
  - Methylation/ demethylation in ecologically intact system

- **Enclosure Studies**
  - key variables on net methylation in-stream

- **Data Analysis, Geochemical Modeling, Site Conceptual and Numerical Model**

* Critical understanding of Hg flux, biogeochemical controls, and microbial determinants
Fundamental mechanisms and transformations

- Speciation and geochemical controls
  - Rates and mechanisms, oxidation/reduction
  - Single reactant to multi-component systems
  - Hg-microbial cell interactions and Hg uptake

- Roles of DOM and POM in Hg methylation, demethylation, and redox transformations
  - Specific moieties and functional groups
  - EXAFS analysis, speciation and coordination chemistry
  - Species, models, and effects on bioavailability

Catalyzed surface and photochemical reactions

- Roles in Hg reactivity and demethylation
- Sorbed species and reactions
- Labeled stable isotope studies

* Critical understanding of dominant Hg species, its bioavailability, and biogeochemical controls on rates and mechanisms of Hg methylation and demethylation
Microbial and genetic controls on Hg methylation

- Verification and validation of \textit{hgcAB} as a predictive measure of Hg-methylation potential
  - Testing of predicted methylators and non-methylators during growth
- Elucidate the native biochemical role and regulation of \textit{hgcAB}
  - Determine the effect of geochemical factors on gene regulatory networks for mercury methylation
  - Comparative gene expression, mutagenesis, and complementation
- Develop and utilize “universal primers” for determination of Hg-methylation potential in any environment.
- Examine relationships among community structure, geochemical conditions, and methyl Hg production in sediments globally
  - \textit{hgcAB} biomarker
  - Pyrosequencing and metagenomics

*Critical understanding of the genetic basis of the methylation and demethylation processes and the geochemical controls on microbial transformation.
Molecular structure, dynamics and simulations

- Establish biochemical pathways in bacterial demethylation
- Obtain structure of protein/protein and protein/DNA complexes
  - Apply small angle neutron scattering to reveal structure-function relationships
- Reveal enzymatic mechanisms to understand the processes of demethylation and reduction
  - Use quantum mechanical/ molecular mechanical simulations

*Critical understanding of the biochemical and biophysical mechanisms in Hg transformation (demethylation and methylation) by developing and validating subcellular models and investigation of structure-function relationships

Two parts of the puzzle: Community composition (Tasks 1 and 3) and Gene identity (Tasks 3 and 4)

Community Studies


Gene Identity


The study site included several streams in the Oak Ridge area including a control site.
There is a seasonal aspect to methylmercury concentrations that points to biological processes.
**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 RDP pyrosequencing pipeline. During this process the sequences were sorted by tag sequence, trimmed off the 16S primers and filtered out of low-quality sequences. From ~8,000 to ~12,000 high quality sequences by size 200-220 bp were obtained for each sample.
- Samples taken over three quarterly sampling periods (36 samples).
- The Deltaproteobacteria are the group of bacteria that are generally acknowledged to be involved in mercury methylation.
Relative numbers of Deltaproteobacteria in these surface sediments were low.
The most common Deltaproteobacteria was Desulfobulbus
There were seasonal trends in community abundance
Desulfobulbus is considered a logical candidate as a methylator in this stream.

- There was a statistically significant correlation of methyl-mercury concentrations with several Deltaproteobacteria including Desulfobulbus spp., Desulfonema spp., and Desulfobacca spp.

Triplot of the redundancy analysis (RDA) for bacterial phyla from the 20 stream sediments samples at five sites located (BCK samples excluded) on or near the Oak Ridge Reservation with forward selection of predictor variables followed by Monte Carlo permutations.
Identifying the genes involved in mercury methylation required an integrated omics and biochemical approach

• The Oak Ridge National Laboratory’s research team has used comparative genomics in the context of biochemical pathways to identify key genes involved in mercury methylation.

• The basis for this comparative analysis was the sequencing of several bacteria capable of methylation under the leadership of the Oak Ridge National Laboratory’s research program.

*Desulfovibrio africanus*  
(SEM by Dwayne Elias,)
Is there a genetic basis for bacterial Hg methylation

Geobacter chapellei, 172
Geobacter pelophilus, Dfr2
Geobacter brevis, Dfr1
Geobacter lovleyi, SZ
Geobacter metallireducens, GS5
Geobacter grbicai, TACP-2
Geobacter hydrogenophilus, strain H4
Geobacter sulfurreducens, PG9
Desulfuromonas palmitatis, SDY1
Desulfuromonas chloroethenica, TT4B
Desulfuromonas thio litha, NZ27 (DSMZ 8987)
Desulfuromonas acetoxidans, DSM 624
Desulfcapsa sulflexigen, SB164P1
Desulfotalea psychrophila, LSv54
Desulfobacillus propionicus, DSM 132
Desulfsarcina variabilis
Desulfococcus multivorans, DSM 132
Desulfobacterium sp., BG8
Coralloccus coralloides, DSM 2258
Myxococcus xanthus, DSM 435 (Mx x1)
Desulfobacterium chloracetivorans, ATCC 700912
Desulfobacterium desulfuricans, ND132
Desulfobacterium dechloracetivorans, DSM 11384
Desulfobacterium sp., X
Desulfobacterium sp., W
Desulfobacterium gigas
Desulfobacterium desulfuricans, DSM1926 El Agheila Z
Desulfobacterium desulfuricans, MB; ATCC27774
Desulfobacterium desulfuricans, Essex 6; ATCC 29577
Desulfobacterium africanum, NCIMB 13491
Desulfobacterium alaskensis, G2
Desulfobacterium alaskensis, NIM 13491
Desulfococcus buluus, DSM 4028T
Desulfomicrobium orale, DSM 12838

Shewanella alga, Bry
Shewanella oneidensis, M9-1
Shewanella putrefaciens, CM12

Non Hg methylator
Weak Hg methylator
Strong Hg methylator
Genomes plus chemical reasoning points to the genes

\[ \text{CH}_3^− + \text{Hg}^{2+} \rightarrow \text{CH}_3\text{Hg}^+ \]

The biochemical reasoning led to a focus on finding a corrinoid protein that could be required for mercury methylation.
Two Genes appear to be involved

The two proteins are present in the genomes of all known methylating bacteria that have been sequenced, but absent in all known non-methylators!
Deletion of \textit{hgcA} and \textit{hgcB}

\textbf{Diagram:}

\textbf{Wild type gDNA:}

- pUC ori
- SpecR
- \textbf{pMO4651}
- Up stream

\textbf{Mutant gDNA:}

- pKan
- KanR
- Upp

\textbf{Deletion of hgcA and hgcB:}

\textbf{D. desulfuricans ND132 and G. sulfurreducens PCA}

pMO4651 was electroporated into \textit{D. desulfuricans} ND132 (U. Missouri) and \textit{G. sulfurreducens} PCA (ORNL). Cells were plated for isolated colonies, picked and grown in liquid and checked for a "double cross-over event".

Once verified, cells were assayed for methylation at U. Missouri and ORNL (stable isotopes/ICP-MS).

\textbf{Southern verification:}

\textbf{Wild type gDNA:}

- 3\textsuperscript{2}P Southern Probe (PCR 1, ~0.7 Kb)

\textbf{Expected fragment = 3.8 Kb}

\textbf{Mutant construct:}

- pKan
- KanR
- Upp

\textbf{Expected fragment = 7.0 Kb}

1) Digest genomic DNA for wild-type and mutant with restriction enzyme (ZraI), probe with PCR product, which binds and yield a unique band for each strain.
Deletion studies confirm the importance of both genes in two methylators

![Graph showing methylmercury levels](image-url)

**Methylmercury (ng/L)**

- D. desulfuricans ND132
- G. sulfurreducens PCA
- DGSU1440
- DGSU1441

**Experiment**

- *G. sulfurreducens* PCA
- *G. sulfurreducens* ND132

**Y-axis:** Methylmercury (ng/L)

**X-axis:** Various conditions and strains.
Proposed Hg methylation pathway

- The diagrams show:
  - potential sources of C1 units entering the reductive acetyl-CoA pathway,
  - methyl group transfer from CH₃-H₄folate (CH₃-THF) to cob(I)alamin-HgcA, methylation of Hg(II) by HgcA, and
  - reduction to cob(I)alamin-HgcA by HgcB.

- In the absence of Hg, the methyl group is transferred to a different substrate, which may be a physiologically relevant metabolite.

Figure in Parks et al. (2013)
adapted from Choi et al
Critical points

• These analyses led to the discovery of a two-gene cluster which has been designated *hgcAB*.

• The involvement of these bacteria in mercury methylation has been confirmed using molecular techniques, which show that deletion of either gene halts mercury methylation.

• Also, this two gene cluster is present in some bacteria (e.g., specific methanogens and clostridia) that have not, as of yet, been shown to methylate mercury.

• Thus, the diversity of mercury methylators could be greater than previously known.
What are the implications? This discovery will change how mercury research is performed globally.

Immediate Microbiology team work based on the discovery

- Verify the predictability of methylating organisms.
  - Until now, methylators in 1 phylum. We predict at least 3 diverse phyla (and likely more). Do the others make more methylmercury and/or make it faster?

- Develop a **biomarker** for methylmercury generation
  - How prevalent are these genes in the environment?
  - We can correlate real methylation potential with gene expression and organism abundance.
  - We can directly determine geochemical factors influencing biological mercury methylation in any environment.

Implication: Correlating gene, protein and organism abundances with methylmercury formation rates and yields will lead to improved and more sensitive biogeochemical models!
### Known and Predicted Methylmercury-Producing Microorganisms in 4 Phyla

#### Proteobacteria
- *Desulfovibrio desulfuricans* ND132
- *Desulfovibrio aespoeensis* Aspo-2
- *Desulfovibrio africanus* str. Walvis Bay
- *Desulfovibrio baculatum* X
- *Desulfonatronospira thiodismutans* ASO3-1
- *Desulfovibrio oxyclinae* DSM 11498
- *Desulfovibrio africanus* PCS
- *Desulfovibrio longus* DSM 6739
- *Desulfovibrio putealis* DSM 16056
- *Desulfobulbus propionicus* DSM 2032
- *Desulfobulbus mediterraneus* DSM 13871
- *Desulfospira joergensenii* DSM 10085
- *Desulfotignum phosphitoxidans* FIPS-3
- uncultured *Desulfobacterium* sp.
- *Geobacter sulfurreducens* PCA
- *Geobacter sulfurreducens* DL-1 KN400
- *Geobacter metallireducens* GS-15
- *Geobacter metallireducens* RCH3
- *Geobacter sp*. daltonii FRC-32
- *Geobacter sp*. M18
- *Geobacter sp*. M21
- *Geobacter uraniireducens* R4
- *Geobacter bemidjiensis* Bem
- *Geopsychrobacter electrodiphilus* DSM 16401
- *Syntrophorhabdus aromaticivorans* UI
- *Desulfomonile tiedjei* DCB-1 DSM 6799
- *Syntrophus aciditrophicus* SB
- delta proteobacterium MLMS-1
- delta proteobacterium NaphS2
- *Deferrisoma camini* S3R1

#### Firmicutes
- *Acetivibrio cellulolyticus* CD2
- *Dehalobacter restrictus* DSM 9455
- *Dehalobacter sp.* CF
- *Dehalobacter sp.* DCA
- *Dehalobacter sp.* FTH1
- *Desulfotobacterium dehalogenans* ATCC 51507
- *Desulfotobacterium dichloroeliminans* LMG P-21439
- *Desulfotobacterium metallireducens* DSM 15288
- *Desulfotobacterium PCE1* DSM 10344
- *Desulfospirorosinus acidiphilus* SJ4
- *Desulfospirorosinus orientis* DSM 765
- *Desulfospirorosinus sp.* OT
- *Desulfospirorosinus youngiae* DSM 17734
- *Ethanoligenens harbinense* YUAN-3
- *Syntrophobutulus glycolicus* DSM 8271
- *Dethiobacter alkaliphilus* AHT 1
- *Clostridium termiditis* CT1112
- *Acetonomema longum* DSM 6540

#### Euryarchaeota
- *Methanofollis liminatans* GKZPZ
- *Methanoregula Boonei* 6A8
- *Methanoregula formicicum* SMSP
- *Methanomassiliicoccus luminyensis* B10
- *Methanosphaerula palustris* E1-9c
- *Methanospirillum hungatei* JF-1
- *Methanolobus tindarius* DSM 2278
- *Methanomethylovorans hollandica* DSM 15978
- *Methanolobus psychrophilus* R15
- *Methanocella arvoryzae* MRE50 RC-I
- *Methanocella paludicola* SANAE

#### Chloroflexi
- *Dehalococcoides mccartyi* DCMB5

This list is updated regularly by the ONRL team

Questions remain that will guide future research

Questions

• Are other genes involved?
• Are the predictions of other methylators robust?
• What determines rates of methylation and demethylation in the environment?
• What are the mercury update mechanisms and forms of mercury taken up?

Future Work

• Examine mercury methylation mechanism using \textit{in vivo and in vitro} studies
• Test predicted organisms for methylation activity
• Examine phylogenetic and functional relationships of mercury methylation in field samples with regard to geochemistry
What are the implications?

- Will likely change how mercury research is performed globally
- Mechanism is new chemistry, opening up new areas of research
- Biomarker for methylmercury generation
- Improved and more sensitive biogeochemical models
Summary

• These findings lay the groundwork for additional research that will increase our understanding of the transformation of mercury in the environment. An increased understanding could lead to new approaches in mercury remediation.
More than just another gene discovery

THE TEAM

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