Biodiversity of Sulfate Reducing Bacteria in Mercury-Contaminated Beaver Ponds at Avery Brook

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Beaver ponds act as mercury sinks, exhibiting favorable conditions for the production of neurotoxic methylmercury by sulfate reducing bacteria (SRBs), a group of bacteria that are characterized by function (sulfate reduction) rather than phylogeny. This study was conducted to explore the diversity of SRB communities inhabiting microenvironments at the sediment-water interface in one beaver pond located on the Avery Brook stream system in the Conway State Forest. Clone libraries were constructed in order to capture some of the diversity of sulfate-reducing bacteria as well as a biogeographic pattern. Though the clone libraries did not reveal a biogeographic pattern, the results showed an unprecedented amount of SRB diversity in all sampled microenvironments.

Keywords: Beaver Ponds, Sulfate Reduction, Mercury, Methylmercury, Dissimilatory Sulfate Reductase

Introduction

The release of mercury into the atmosphere through anthropogenic and natural processes is a matter of global concern, due to its capacity for long-distance transport in the atmosphere and its ability to bioaccumulate in local ecosystems. These properties cause significant negative health effects in fish, birds and humans (U.S. Geological Survey, 2000). Inorganic mercury is emitted as a result of the activities of coal-fired power plants, gold purification and volcanic eruptions (Morel et al, 1998). After deposition, mercury enters a cycle in which it is modified by microbes on land and in water. In order to understand the mercury cycle and its effects, it is also necessary to understand these microbes and the environments that facilitate mercury modification. This study addresses this process as it occurs in beaver ponds.

Beavers are known as "ecosystem engineers" due to their ability to influence stream dynamics by building dams to create their habitats (Naiman et al, 1988). Consequently, beaver ponds are hotspots for biogeochemical cycling, because of their ability to retain sediment and organic material (Naiman et al, 1994). As such, beaver ponds act as mercury sinks, sequestering inorganic mercury from a variety of sources (Roy et al, 2009). Beaver ponds exhibit favorable conditions for the production of methylmercury (Roy et al, 2009), a neurotoxin that biomagnifies in successive trophic levels, reaching high concentrations in the tissues of various fish and birds.

The transformation of inorganic mercury to methylmercury is mediated by sulfate reducing bacteria (SRBs) (Morel et al, 1998). SRBs comprise a polyphyletic group of bacteria, most of which are members of the Gram-negative Deltaproteobacteria (eg. *Desulfovibrio)* and the Gram-positive Firmicutes (eg. *Desulfotomaculum*). SRBs are characterized by their physiology, using sulfate as the terminal electron acceptor in anaerobic respiration (Wagner et al, 1998). In addition to the mercury cycle, SRBs are important contributors to the carbon and sulfur cycles, regulating a variety of processes in anaerobic environments (Gibson et al, 1990).

We investigated SRB composition of the sediment-water interface of three sites in a single beaver pond using culture-independent methods to build clone libraries from these sites by amplifying the protein-coding genes for the dissimilatory sulfate reductase enzyme (*dsrAB*). While we sought to characterize biogeographic patterns of SRB communities in different microenvironments, these analyses revealed a hyper-diverse system, underscoring the complexity of soil environments (Mocali et al, 2010).

Results

Capturing dsrAB by constructing clone libraries

Clone libraries were constructed using amplified DNA from *dsr* partial gene targets. Because sequencing was performed in a single direction, sequences were 500 to 1000 base pairs in length. This represents a large but incomplete fragment of the *dsrAB* genes, since the full length is approximately 1.9 kilobases. This means that only the alpha subunit (*dsrA*) of the *dsrAB* gene was captured.

Sequence analysis of clones

In total, 133 clones were generated from four samples. Sequences derived from these clones were assembled into contiguous sequences ("contigs") at decreasingly conservative percent similarities between 100% and 80% using Seqman. The number of contigs decreased roughly linearly with decreasing percentage, meaning there is no change in pattern that would signify a percent at which the sequences should be assembled into an OTU (Figure 1). Unlike the *16S* rRNA gene, for which an OTU is defined as containing sequences sharing at least 97% nucleotide sequence identity (Konstantinidis, et al, 2006), the sequence cutoff to define an OTU for *dsrAB* is less well defined; many studies do not even cite a cutoff percentage. Studies that elect to cite a cutoff percentage normally choose 90% or 93% to define an OTU (Hansel et al, 2008; Elshahed et al, 2003). Since *dsrAB* sequences from two cells share a smaller percentage of nucleotides than *16S* sequences from the same cells (Kjeldsen

et al, 2007), We chose a 95% cutoff to define an OTU, a percentage that falls between the 97% *16S* and 93% *dsrAB* cutoffs. Using these parameters, the 133 sequences were aligned into 84 OTUs. The majority of OTUs consisted of only one clone, fifteen OTUs had two to three clones and only four OTUs had 5 or more clones, indicating a high level of rare lineages in the sampled microenvironments (Table 2). Out of the 133 sequences, 81% are novel at 95% sequence similarity and 25% are novel at 85% sequence similarity.

Grouping of sequenced clones into bacterial taxa and statistical analysis

While no sequence had a close hit to named bacterial species (Table 3), most appear to be members of the Firmicutes, the Deltaproteobacteria and the Nitrospira (*Thermodesulfovibrio)* (Table 1). The sequences from this study generally had <70% nucleotide identity with cultured, named taxa and many of them had <90% nucleotide identity with currently available sequences from environmental clones. These sequences may represent novel taxa, however, further exploration of the full 1.9 kb of the *dsrAB* genes is necessary to confirm this.

We used Chao1 and ACE (abundance-based coverage estimate) richness estimators to quantify the diversity and composition of these data. According to both Chao1 and ACE, site 2 had the highest predicted bacterial species richness, and site 1a had the lowest (Table 2). However, Chao1 estimates tend to correlate with the number of clones at low sampling sizes, necessitating a greater sampling effort to strengthen these numbers. In addition, the rarefaction curves generated in this study are almost linear, indicating that sampling did not reach saturation for any of the microenvironments (Figure 2)

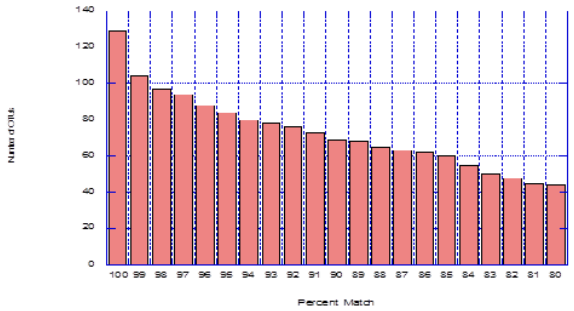

Figure 1: Number of contiguous dsrA sequences at decreasingly conservative assembly percentages. The drops in contiguous sequence number between 100% and 99% are due mostly to experimental error, while the subsequent drops reflect sequence content. Note that the decrease is step-wise, without a clear assembly percentage at which a distinct change in contiguous sequence number occurs.

Discussion

The four samples examined in this study constitute a preliminary survey of the spatial and temporal heterogeneity of SRB communities that reside in beaver ponds. The presence of *dsrA* genes in all four samples indicates that SRB are widespread in beaver ponds. Few lineages were observed more than once or in more than one sample. The level of rare and novel lineages indicates that current knowledge of SRB lineages is deficient.

In addition to detecting diversity and rare lineages, the sequences from the clone libraries were analyzed using the Chao1 and ACE species richness estimators. OTUs were also estimated and represented in a rarefaction curve. These analyses indicated that our sampling effort was not thorough enough given the hyperdiversity of the beaver ponds. At low sampling sizes, Chao1 and ACE are known to underestimate true species richness. Communities whose diversity has been thoroughly sampled generate rarefaction curves that exhibit asymptotic behavior (Hughes, et al, 2001). Since this is not the case in our study, the observed OTUs do not comprise a comprehensive representation of the SRB diversity in the sampled microenvironments. Additionally, studying the dsr operon to better understand how the genetic diversity observed in this study relates to variation (or lack thereof) on the *dsrA* protein level will contribute to overall understanding of SRB community dynamics.

A biogeographic pattern could not be established for the data in this study. As in most analyses of soil microbial communities, the difficulty lies in our ability to distinguish one microenvironment from another and sample each effectively enough to visualize the breadth of diversity and elucidate biogeographic patterns (Mocali et al, 2010). The findings presented here provide a glimpse into the exceptional SRB diversity in beaver pond sediments and underscore the need for more comprehensive sampling to further elucidate patterns associated with these hyper-diverse communities.

Experimental Procedures

Site description and sampling

Samples were obtained from the sediment-water interface of the southernmost pond in the Avery Brook series, which is the farthest pond from the headwaters. Avery Brook is a pristine subcatchment that lies almost completely inside the Conway State Forest in Conway, MA and forms part of the Mill River watershed (Fletcher, 1998, unpublished). Three sites in the pond were sampled for this study to explore the heterogeneity of microenvironments with differing characteristics (Figure 3): Site 1 is at the outflow from the pond and is characterized by relatively shallow (~10 cm), fast-moving water that receives both sun and shade throughout the day. Site 2 is in the middle of the pond, characterized by deep, still water that receives full sun. Site 3 is approximately 1 m from the northeastern bank of the pond, in dense shade of an overhanging hemlock canopy where the water is slow and shallow (~10 cm). Three samples were taken from each site on June 30, 2010 and a fourth was taken on September 29, 2010 to observe spatial and temporal differences in the microenvironments. All samples were taken by vertically coring into the sediment using a 50 mL syringe with the tip cut off, holding the plunger in place and pushing the syringe barrel down to preserve the soil profile. The core was sectioned off one centimeter at a time and stored in 1.5 mL tubes on dry ice during transportation back to the laboratory where they were stored at -80 °C.

Table 1: Distribution of dsrA clones across bacterial taxa from all four samples.

Taxon	% Detected in all samples
Firmicutes	72
Deltaproteobacteria	23
Nitrospira	5

Table 2: Table of OTUs and richness estimators by site.

Sample	Chao1[a]	ACE [b]
1a	52.9 (32.56, 121.54)	50.59
1b	104 (37.99, 196.5)	104.62
2	268 (68.76, 1395.55)	151.37
3	59.25 (26.2, 211.07)	62.4

[a] The mean value is reported with lower and upper 95% confidence intervals given in parentheses.

[b] The mean value is reported.

Table 3: Table of novelty parameters by site.

Sample	Number of novel clones at <95%*	Number of novel clones at <85%*
1a	26	11
1b	28	7
2	33	11
3	21	5

* A clone was defined as "novel" at <95% or 85% if the top hit from BLAST had a maximum identity of <95% or < 85% to the clone.

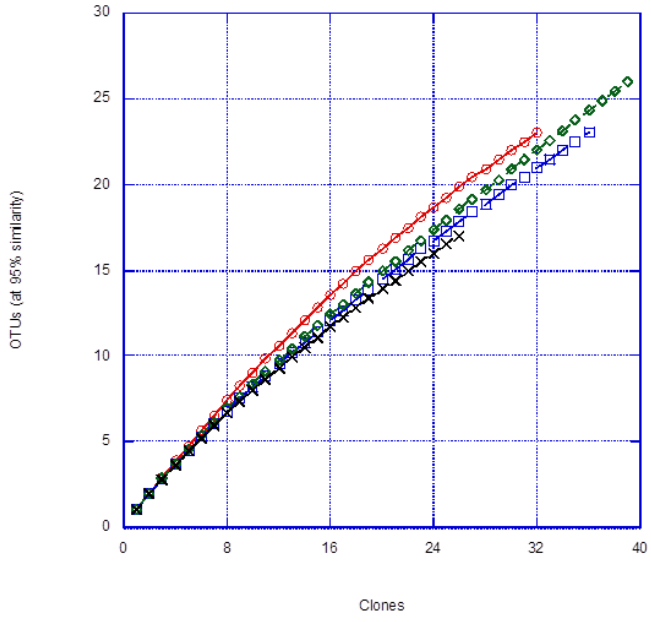

Figure 2: Rarefaction curves indicating *dsrA* species richness for clone libraries derived from samples 1a, 1b, 2 and 3. Rarefaction curves were generated using EstimateS by random sampling. The curves are almost linear in all cases, indicating that the samples did not reach saturation.

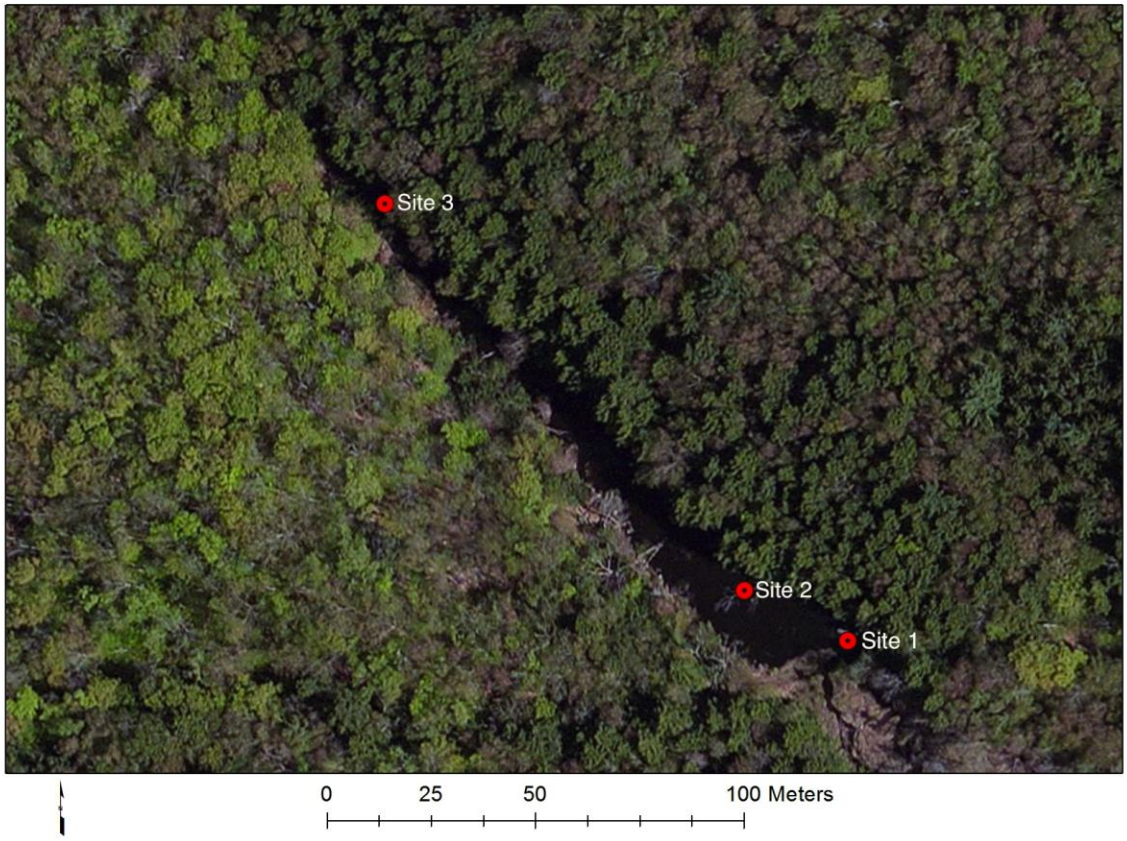

Figure 3: Air photo of the southernmost pond in the Avery Brook series showing sampling sites 1, 2 and 3. Sites were chosen at the outlet (site 1), at the middle of the pond (site 2) and at the inlet (site 3) to capture the heterogeneity present in beaver ponds (provided by R. Newton, Smith College Dept. of Geosciences).

DNA extraction

DNA was extracted from 0.25 grams (wet weight) of the top centimeter of the sediment cores using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Extracted genomic DNA was stored at -80 °C, and diluted aliquots were stored at -20 °C.

PCR amplification and construction of clone libraries

PCR amplification of *dsrAB* fragments (~1.9 kb) was performed in 20 µL reactions including 13.01 µL nuclease-free water, 4.58 µL 5X HF buffer (Finnzymes, Finland), 2.29 µL bovine serum albumin (New England Biolabs, Inc., Ipswich, MA), 0.69 µL MgCl, 0.46 µL dNTPs, 1 µL Phusion polymerase (Finnzymes, Finland), 0.5-1 µL DNA template and 0.46 µL each of primers DSR1F and DSR4R from Wagner, et al, 1998, which cover a 1.9 kb region of the dsrAB genes. PCR reactions were performed in a BioRad thermocycler using a protocol of 98 °C for 3 minutes followed by 30-35 cycles at 98 °C for 30 seconds, 58 °C for 1 minute and 72 °C for 1 minute. Cycling was completed after a final elongation step of 72 °C for 10 minutes followed by refrigeration at 4 °C.

For each DNA template, at least two PCR reactions were performed and pooled to mitigate PCR bias. Artifacts below 1.9 kb were avoided by excising the desired band from a 1% agarose gel using the CleanSpin Gel DNA Recovery Kit (Gel Company, Inc San Francisco, CA) according to the manufacturer's instructions. The PCR product was cloned into *E. coli* using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen Corp, Carlsbad, CA) as specified by the manufacturer. Colonies were transferred to 96-well plates containing LB broth and 50µg/mL kanamyacin, grown overnight at 37 °C and PCR screened for the presence of the correct insert using M13F and M13R vector primers. Colonies that contained the insert were miniprepped using the PureLink Quick Plasmid Miniprep Kit (Invitrogen Corp, Carlsbad, CA). Miniprepped DNA was sent to the Penn State Genomics Core Facility at University Park, PA and the Smith College Center for Molecular Biology in Northampton, MA for sequencing with primer M13F.

Statistical Analysis:

Operational taxonomic units (OTUs) were defined as clones sharing 95% dsrA nucleotide similarity. A predicted OTU count and the Chao1 and ACE richness estimators were computed using the EstimateS program (Colwell, 2013). ACE incorporates data from all species it defines as rare, which includes species that encompass fewer than 10 individuals.

The Chao1 estimator was calculated using the bias-corrected form, where S_{obs} represents the number of species

observed and F_1 and F_2 refer to singletons (unique sequences) and doubletons (sequences that are found twice), respectively (Colwell, 2013). The equation for the Chao1 estimator is shown below:

$$\hat{S}_{Chao1} = S_{obs} + \frac{F_1(F_1 - 1)}{2(F_2 + 1)}$$

Rarefaction curves were assembled from the predicted OTU count, which was also calculated using the EstimateS program. All graphs were generated using KaleidaGraph 4.0.

Sequence analysis

Primer and vector sequences were trimmed and sequences were assembled using the program SeqMan at decreasingly conservative assembly percentages. Sequences were compared to the GenBank database using BLAST (Altschul et al, 1990). Operational taxonomic units (OTUs) were defined as clones sharing 95% dsrA nucleotide similarity. Diversity statistics and rarefaction curves were computed using the EstimateS program (Colwell, 2011). All graphing was performed in Kaleidagraph 4.0.

Acknowledgments

This study was funded by undergraduate fellowship grants from the Howard Hughes Medical Institute to Rachel Kaminsky and Sara Sirois and the Smith College Blakeslee Fund in the Biological Sciences. The authors acknowledge Robert Newton for aiding in sample collection.

References

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. 1990. Basic local alignment search tool. J. Mol. Biol. **215**: 403-410.

Brigham, M., Krabbenhoft, D. Mercury in Aquatic Ecosystems—Recent findings from the National Water-Quality Assesment (NAWQA) and Toxic Substances Hydrology Programs (as presented to the NAWQA National Liaison Committee August 21, 2009). U.S. GeologicalSurvey. http://water.usgs.gov/nawqa/mercury/mercury.handout.final.08172009.pdf

Colwell R. K. 2011. *EstimateS*, Version 8.2: Statistical Estimation of Species Richness and Shared Species from Samples.

Elshahed, M.S., Senko J.M., Najar F.Z., Kenton S.M., Roe B.A., Dewers T.A., Spear J.R., Krumholz L.R. 2003. Bacterial Diversity and Sulfur Cycling in a Mesophilic Sulfide-Rich Spring. Appl. Environ. Microbiol. **69**(9): 5609-5621. http://aem.asm.org/content/69/9/5609.full

Gibson, G.R. 1990. Physiology and ecology of the sulphate-reducing bacteria. J. Appl. Bacteriol. **69**: 769-797. http://onlinelibrary.wiley.com/store/10.1111/j.1365-2672.1990.tb01575.x/asset/j.1365-2672.1990.tb01575.x.pdf?v=1&t=hi3a2syo&s=19110df3a83ecfc1976a639c79ae3ad9653c12dd

Hansel C.M., Fendorf F., Jardine, P.M., Francis, C.A. 2008. Changes in Bacterial and Archaeal Community Structure and Functional Diversity along a Geochemically Variable Soil Profile. Appl. Environ. Microbiol. **74:**1620-1633. http://aem.asm.org/content/74/5/1620.full

Hughes J.B., Hellmann, J.J., Ricketts, T.H., Bohannan, B.J.M. 2001. Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. Appl. Environ. Microbiol.**67:**4399-4406. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC93182/

Mocali S, Benedetti A. 2010. Exploring research frontiers in microbiology: the challenge of metagenomics in soil microbiology. Research in Microbiology. **161**: 497-505. http://www.sciencedirect.com/science/article/pii/S0923250810000926

Morel, F.M.M., Kraepiel, A.M.L., Amyot, M. 1998. The Chemical Cycle and Bioaccumulation of Mercury. Annu. Rev. Ecol. Syst. **29**: 543-566. water.usgs.gov/nawqa/mercury/mercury.handout.final.08172009.pdf

Naiman, R.J., Johnston, C.A., Kelley, J.C. 1988. Alteration of North American Streams by Beaver. BioScience. **38**: 753-762. http://www.uri.edu/cels/nrs/whl/Teaching/nrs592/2009/Class%209%20Stream%20Legacies%20and%20disturbance/Naiman%20beaver%20streams.pdf

Naiman, R.J., Pinay, G., Johnson, C.A., Pastor, J. 1994. Beaver-induced influences on the long-term biogeochemical characteristics of boreal forest drainage networks.Ecology.**75**:905-921. http://www.uvm.edu/~pbierman/classes/gradsem/2005/papers/naiman1994.pdf

Roy, V., Amyot, M., Carignan, R. 2009. Beaver ponds increase methylmercury concentrations in Canadian shield streams along vegetation and pond-age gradients. Environ. Sci. Technol. **43**: 5605-5611. http://ejournals.ebsco.com/Direct.asp?AccessToken=8UWP3PNV0OT3JWX11FF400YU909NVYU4NU&Show=Object&msid=-427809127

Wagner, M., Roger, A.J., Flax, J.L., Brusseau, G.A., Stahl, D.A. 1998. Phylogeny of Dissimilatory Sulfite Reductases Supports an Early Origin of Sulfate Respiration. J. Bacteriol. **180**: 2975-2982. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC107267/