U.S. Department of Energy

Office of Science

Office of Biological and Environmental Research

DOE DE-SC0006849- Final Report

Title: Mechanisms of Hg(II) uptake and methylation in methylating bacteria

Applicant/Institution: **The Trustees of Princeton University**
Princeton, NJ 08544

Principal Investigator: **François M. M. Morel**
Geosciences Department
153 Guyot Hall
Princeton, NJ 08544
Tel: 609-258 2416
Email: morel@princeton.edu

DOE/Technical Program Manager Contact: **Paul Bayer**
301-903-5324
Paul.bayer@science.doe.gov

Collaborator and Institution: **Jeffra K. Schaefer**
Rutgers University
schaefje@envsci.rutgers.edu
848-932-5779

Mechanisms of Hg(II) uptake and methylation in methylating bacteria

Introduction

The bioaccumulation of methylmercury (MeHg) in aquatic foodchains poses a threat to the health of top predators, including humans. Mercury uptake by methylating microorganisms is a key first step in the production of methylmercury. The goal of this project was to understand the critical factors that control the availability and transport of inorganic mercury, Hg(II), into methylating bacteria. Specifically, this research focused on understanding the mechanism of bacterial mercury uptake and how mercury speciation affects the specificity and kinetics of mercury transport.

The role of thiols in the uptake of mercury by methylating bacteria.

Our initial work focused on the uptake of Hg(II) by two important methylating strains, the iron reducer (FeRB) *Geobacter sulfurreducens* and the sulfate reducer (SRB) *Desulfovibrio desulfuricans* ND132 (**Schaefer *et al* 2011**). Our results demonstrated that in both organisms (1) Hg uptake is an active transport process requiring energy, (2) Hg(II) uptake

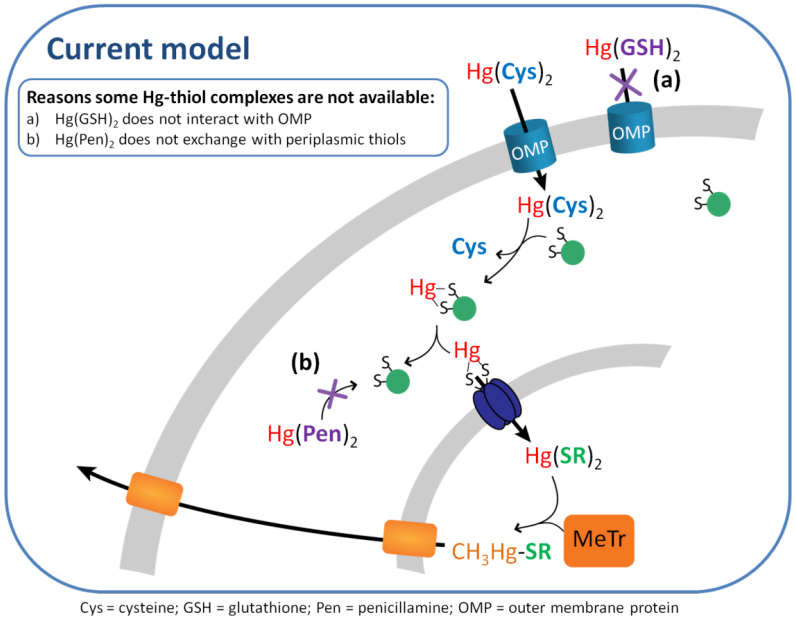

Fig. 1: Model of the mechanisms for Hg(II) uptake, ligand exchange in the periplasm, transport into the cytosol, followed by subsequent methylation and export of the newly formed methylmercury species.

is dependent upon the structure of the Hg binding ligand, and (3) methylmercury (MeHg) is rapidly exported out of the cell upon its synthesis. The Hg(II) uptake system of *D. desulfuricans* has a higher affinity than that of *G. sulfurreducens* and promotes Hg methylation in the presence of stronger complexing thiols. On the basis of these results we hypothesized that Hg(II) uptake in methylating bacteria occurs accidentally through heavy metal transporter(s) and developed the working model shown on Figure 1 (which is supported by subsequent work; see below): (1) Hg(II) is transported across the outer membrane, likely as an intact thiol complex, (2) Hg(II) undergoes ligand exchange with cellular thiols in the periplasm, and (3) Hg(II) is transported across the inner membrane through ligand exchange with thiol groups in the transporter. Due to differences in size, steric hindrance, and/or binding strengths, thiols such as penicillamine (Pen) and glutathione (GSH) do not support transport or ligand exchange with periplasmic or transporter thiols.

Inhibition of Hg uptake and methylation by divalent metals

If, as hypothesized, Hg(II) uptake is an accidental byproduct in the acquisition of essential trace metals by methylating bacteria, it should be affected by the concentration and chemical speciation of these metals. We thus studied mercury methylation in the presence of a variety of essential metals to act as possible competitive substrates for Hg(II) uptake (**Schaefer *et al.* 2014, a**). Hg(II) methylation in *G. sulfurreducens* and *Desulfovibrio* sp. ND132 was inhibited by Zn(II) and Cd(II), but not other divalent metals such as Ni(II), Co(II), or Fe(II). (Inhibition of Zn uptake by Cu(II) in the presence of cysteine was also observed as was expected since Cu(II) causes the dismutation of cysteine to cystine.) As little as 10 uM Zn, equivalent to 0.9 uM Zn' (where Zn' = Zn bound to inorganic ligands), was inhibitory to Hg methylation, a concentration below that is consistently present in the growth medium. This inhibitory effect by Zn(II) is due to decreased Hg(II) uptake as evidenced by the reduced uptake of Hg(II) in whole cells and spheroplasts when exposed to high concentrations of Zn(II).

These results imply that Hg(II) shares cellular binding sites with Zn(II) and Cd(II) for uptake into the bacteria, and that Hg(II) uptake occurs via a Zn transporter. The inhibitory effect of Zn(II) on Hg methylation was observed in the absence of cysteine when Hg exists almost exclusively as neutral Hg-chloro complexes, further arguing against the passive diffusion model for Hg uptake in our model bacteria since Zn(II) should have no effect on passive processes. The inhibitory effect of Zn(II) on Hg(II) uptake was competitive in nature, as the inhibition could be alleviated by decreasing the Zn':Hg(II) ratio by any of the following: (1) the addition of the Zn(II) chelator, NTA, which decreased the Zn' pool (2)

increasing the cell density which decreases Zn' through non-productive cellular Zn binding, and (3) increasing [Hg(II)].

Hg uptake in non-methylating bacteria:

Our experiments with Hg(II) methylating anaerobic bacteria indicate that Hg(II) uptake is accidental, likely occurring through Zn transporters. This raises the question of whether a similar uptake mechanism a may be common in other bacteria and whether anaerobic conditions are important for uptake. We investigated this question by studying Hg(II) uptake in the non-methylating facultative aerobe bacterium, *Shewanella oneidensis* MR-1 under aerobic and anaerobic conditions (Szczuka *et al* 2014). Cells demonstrated energy dependency for Hg(II) uptake under both conditions, as observed for the methylating strains, *G. sulfurreducens* and ND132. Further, *S. oneidensis* showed similar substrate specificity with regard to Hg-complexes available for uptake with *G. sulfurreducens*, as evidence by the preferential uptake of Hg-cysteine complexes over Hg-penicillamine or Hg-glutathione complexes. In addition, the uptake of Hg(II) was inhibited by Zn(II). Remarkably, initial cellular uptake rates in *S. oneidensis* were similar under aerobic and fumarate-reducing conditions. These results demonstrate a common Hg(II) uptake mechanism in a wide variety of Proteobacteria but differences in the ability to take up Hg bound to different thiols. These results also provide strong support for our model or an accidental uptake mechanism for Hg(II) transport through divalent metal transporters (Fig. 1), a process likely widespread in the Proteobacteria, and possibly in other phyla as well.

Mercury uptake in Firmicutes

Hg methylation has recently been observed in a variety of prokaryotes besides the *δ-Proteobacteria*, including methanogens and firmicutes (Gilmour et al., 2013, Parks et al., 2013). However, there are considerable differences in the rate and yield of MeHg produced by these different microorganisms. The highest rates and yields are typically observed for the sulfate and iron-reducing bacteria in the *δ-Proteobacteria*, and the lowest for the methanogens and *Firmicutes* (Gilmour et al., 2013, Yu et al., 2013). To understand the physiological basis for these differences, we studied Hg uptake and methylation in the iron-reducing *Firmicute*, *Desulfitobacterium metallireducens* (**Janssen *et al.* in preparation**). To determine whether differences in Hg methylation rates were due to Hg speciation or physiological differences, we performed experiments in which the speciation of mercury was controlled by cysteine complexation. Under these conditions, *D. metallireducens* failed to methylate Hg(II) significantly above abiotic controls (50 pM) even after 16 hours of

incubation despite active iron reduction. In contrast, under identical Hg(II) speciation conditions, the iron-reducing δ-Proteobacterium, *Geobacter sulfurreducens* methylated almost 100% of the total Hg (5 nM) within 4 hours. We attribute these low methylation potentials in *D. metallireducens* to physiological differences in the intracellular transport of Hg(II). Unlike members of the *Proteobacteria*, this *Firmicute* species appeared to take up Hg passively by facilitated diffusion rather than active transport. This is supported by the lack of inhibition by the protonophore, CCCP, and Zn(II) addition – suggesting a completely different Hg uptake mechanism in *Firmicutes*, as compared to the *Proteobacteria*. While the dominant mechanism may be passive, Hg is likely still being transported by facilitated diffusion through a protein porin or channel as Hg(II) uptake was highest for Hg(II)-cysteine zwitterionic complexes and less in the presence of neutral $HgCl_2^0$ or $Hg(HS)_2^0$ species. Preliminary experiments with a distantly related *Firmicute*, the obligate fermenter, *Ethanoligenes harbinense*, suggests that this may be a common trait within the *Firmicutes*. Since *Firmicutes* dominate some anaerobic habitats where MeHg is produced (Podar et al., 2015), how these physiological differences impact Hg uptake and bioavailability is critically important to predict the production of MeHg in complex microbial communities. These results highlight the complex differences in Hg uptake and bioavailability to diverse methylating microorganisms – and may partly explain the observed results from field studies suggesting the relative importance of the sulfate and iron-reducing bacteria within the *δ*-Proteobacteria in the methylation of Hg.

Distribution and diversity of the Hg methylation gene, *hgcA*, in wetland soils

We took advantage of collaborations with colleagues studying various microbial processes in wetlands to assess the diversity of the newly discovered Hg methylation gene, *hgcA.* We investigated the diversity of *hgcA* from temperate and tropical wetland soils where Hg methylation is demonstrated (**Schaefer et al. 2014, b**). Sequences obtained from both environments clustered with those from the d-*Proteobacteria*, *Chloroflexi*, and *Methanomicrobia* with significant overlap in *hgcA* phylogeny between libraries. Clear differences in *hgcA* distribution were observed between two highly contrasting sites within a tropical wetland in Everglades National Park, USA. *hgcA* sequences obtained from the northern site clustered primarily with those of methanogens, while sequences from the estuarine site clustered primarily with sulfate-reducing bacteria and syntrophs in the *d*-Proteobacteria. Libraries obtained from soils collected from a temperate swamp in Sweden were dominated by *hgcA* sequences within the δ-*Proteobacteria* with *hgcA* sequences clustering primarily with iron-reducers in the upstream portion of the swamp and with sulfate-reducers in the downstream portion of the swamp. Interestingly, enrichments prepared from the lower portion of this temperate wetland contained a high abundance of *hgcA* sequences clustering with methanogens. This first report on *hgcA* diversity in

environmental samples suggests a role in Hg methylation for various phenotypic groups in different portions of wetlands.

Supporting references

J. K. Schaefer, S. S. Rocks, W. Zheng, L. Liang, B. Gu, and F. M. M. Morel. 2011. Active transport, substrate specificity, and methylation of Hg(II) in anaerobic bacteria. *Proc. Nat. Acad. Sci.* 108: 8714-8719.

J. K. Schaefer, A. Szczuka, and F. M. M. Morel. 2014 a. Effect of divalent metals on Hg(II) uptake and methylation by bacteria. *Environ. Sci. Technol.* 48: 3007-3013.

Szczuka, F. M. M. Morel, and J. K. Schaefer. 2015. The effect of thiols, zinc, and redox conditions on Hg uptake in *Shewanella oneidensis*. *Environ. Sci. Technol.* 49: 7432-7438.

S. Janssen and J. K. Schaefer. Mercury uptake and methylation in firmicutes. *(in preparation) Submission planned to ES&T Lett.*

Gilmour, C.C., M. Podar, A.L. Bullock, A.M. Graham, S. Brown, A.C. Somenahally, A. Johs, R. Hurt, K.L. Bailey, D. Elias. Mercury methylation by novel microorganisms from new environments. *Environ. Sci. Technol.* 2013, **47**: 11810-11820.

Parks, J.M., A. Johs, M. Podar, R. Bridou, R.A. Hurt, S.D. Smith, S.J. Tomanicek, Y. Qian, S.D. Brown, C.C. Brandt, A.V. Palumbo, J.C. Smith, J.D. Wall, D.A. Elias, L. Liang. The genetic basis for bacterial mercury methylation. *Science* 2013, **339**: 1332-1335.

J. K. Schaefer, R.-M. Kronberg, F. M. M. Morel, U. Skyllberg. 2014 b.. Detection of a key Hg methylation gene, *hgcA*, in wetland soil. *Environ. Microbiol. Rep.* 6: 441-447.

Podar, M., C.C. Gilmour, C.C. Brandt, A. Soren, S.D. Brown, B.R. Crable, A.V. Palumbo, A.C. Somenahally, D.A. Elias. Global prevalence and distribution of genes and microorganisms involved in mercury methylation. *Science Advances* 2015, **1**: 1-12.

Yu, R.-Q., J.R. Reinfelder, M.E. Hines, T. Barkay. Mercury methylation by the methanogen *Methanospirillum hungatei*. *Appl. Environ. Microbiol.* 2013, **79**: 6325-6330.

Publications

J. K. Schaefer, S. S. Rocks, W. Zheng, L. Liang, B. Gu, and F. M. M. Morel. 2011. Active transport, substrate specificity, and methylation of Hg(II) in anaerobic bacteria. *Proc. Nat. Acad. Sci.* 108: 8714-8719.

J. K. Schaefer, A. Szczuka, and F. M. M. Morel. 2014 a. Effect of divalent metals on Hg(II) uptake and methylation by bacteria. *Environ. Sci. Technol.* 48: 3007-3013.

J. K. Schaefer, R.-M. Kronberg, F. M. M. Morel, U. Skyllberg. 2014 b.. Detection of a key Hg methylation gene, *hgcA*, in wetland soil. *Environ. Microbiol. Rep.* 6: 441-447.

Szczuka, F. M. M. Morel, and J. K. Schaefer. 2015. The effect of thiols, zinc, and redox conditions on Hg uptake in *Shewanella oneidensis*. *Environ. Sci. Technol.* 49: 7432-7438.

S. Janssen and J. K. Schaefer. Mercury uptake and methylation in firmicutes. *(in preparation) Submission planned to ES&T Lett.*

Presentations

J. K. Schaefer, S. Janssen, E. Gwiszcz, M. de Freitas, A. Szczuka, and F. M. M. Morel. Comparison of Hg(II) uptake and methylation across diverse anaerobic microorganisms. Geological Society of America Annual Meeting. Baltimore, MD. November 2015.

J. K. Schaefer. The role of low molecular weight thiols in Hg(II) uptake by methylating and non-methylating microorganisms. Swedish University of Agricultural Sciences, Umeå, Sweden. August 2015.

J. K. Schaefer. Molecular approaches for studying Hg methylation. Swedish University of Agricultural Sciences, Umeå, Sweden. August 2015.

J. K. Schaefer. Unraveling the mechanism of Hg(II) transport and methylation in bacteria. Department of Civil and Environmental Engineering, University of Delaware. May 2015. *Invited.*

J. K. Schaefer. A molecular approach for identifying the microbial guilds responsible for methylmercury cycling in wetlands. Uppsala University, Uppsala, Sweden. May 2014.

J. K. Schaefer. Identifying the bacteria responsible for methylmercury formation: detection of *hgcA* gene and transcripts in soils. Symposium on Mercury Cycling Related to Forestry Activities. Umeå, Sweden. February 2014. *Invited.*

J. K. Schaefer, A. Szczuka, and F. M. M. Morel. Active uptake of Hg(II) by mercury methylating bacteria: where and how? International Conference on Mercury as a Global Pollutant. Edinburgh. July 2013.

J. K. Schaefer, A. Szczuka, and F. M. M. Morel. Active uptake of Hg(II) by mercury methylating and non-methylating bacteria. Department of Marine Sciences and Chemistry Seminar. University of Connecticut, Groton, CT. April 2013. *Invited.*

J. K. Schaefer, A. Szczuka, and F. M. M. Morel. Unraveling the mechanism of Hg(II) transport and methylation in anaerobic bacteria. Department of Environmental Sciences Seminar, Rutgers University. April 2013. *Invited.*

J. K. Schaefer, O. Baars, R. Anwar, L. Liang, B. Gu, and F. M. M. Morel. Role of zinc transporters in the uptake of Hg(II) by *Geobacter sulfurreducens*. Subsurface Biogeochemical Research Annual Meeting. Washington DC. April 2012.
J. K. Schaefer, S. S. Rocks, W. Zheng, L. Liang, B. Gu, and F. M. M. Morel. 2011. Active transport and methylation of Hg(II) in anaerobic bacteria. Subsurface Biogeochemical Research Annual Meeting. Washington DC. April 2011.

J. K. Schaefer. Mechanisms of Hg(II) uptake and methylation in anaerobic bacteria. Theobold Smith Society, Rutgers University, New Brunswick, NJ. Dec. 2011. *Invited.*

J. K. Schaefer, S. S. Rocks, W. Zheng, L. Liang, B. Gu, and F. M. M. Morel. Active transport and methylation of Hg(II) in anaerobic bacteria. Subsurface Biogeochemical Research PI Meeting. Washington DC. April 2011.