FINAL TECHNICAL REPORT

TITLE: Identifying Key Proteins in Hg Methylation Pathways of *Desulfovibrio* by Global Proteomics

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Funding

Institution	***FY 2011***	***FY 2012***	***FY 2013***	***Total***
University of Georgia	170,000	146,225	138,800	455,025*
University of California-San Francisco	141,000	135,000	135,000	411,000
Pacific Northwest National Laboratory	110,000	110,000	110,000	330,000
University of Missouri	29,000	58,775	66,200	153,975
Total	$450,000	$450,000	$450,000	$1,350,000

*No-cost extensions granted through Feb 2015

PROJECT OBJECTIVES

1.0 PROJECT OBJECTIVES

Elemental mercury, Hg(0) is a contaminant at many DOE sites, especially at Oak Ridge National Laboratory (ORNL) where the spread of spilled Hg and its effects on microbial populations have been monitored for decades. To explore the microbial interactions with Hg, we have devised a global proteomic approach capable of directly detecting Hg-adducts of proteins. This technique developed in the facultative anaerobe, *Escherichia coli*, allows us to identify the proteins most vulnerable to acute exposure to organomercurials phenyl- and ethyl-mercury (as surrogates for the highly neurotoxic methyl-Hg) (Polacco, et al, 2011). We have found >300 such proteins in all metabolic functional groups and cellular compartments; most are highly conserved and can serve as markers for acute Hg exposure (Zink, et al. 2016, in preparation). We have also discovered that acute Hg exposure severely disrupts thiol, iron and redox homeostases, and electrolyte balance (LaVoie, et al., 2015) Thus, we proposed to bring these techniques to bear on the central problem of identifying the cellular proteins involved in bacterial uptake and methylation of mercury and its release from the cell.

SPECIFIC AIMS OF THE SUMMERS' LAB

1. DETECTING PROTEIN-Hg-ADDUCTS IN Hg-EXPOSED *Desulfovibrio* AND *E. coli*

Our initial work on Hg-proteome mass spectrometry employed the monovalent organomercurial (Polacco, et al, 2011), phenylmercuric acetate, which can only make monothiol adducts and, thus, will not create confusing thiol-peptide crosslinks. However, this project had the two-fold aim of (a) detecting proteins in *Desulfovibrio* modified by the substrate for methylation which is inorganic Hg(ii) and (b) looking for protein crosslinks made by Hg(II) in our standard model, *E.coli.*

OUTCOME:

With our guidance *Desulfovibrio* expert, Judy Wall (U Missouri, funded co-PI), grew *Desulfovibrio desulfuricans ND132* (*Dd* ND132) facultatively in pyruvate/fumarate medium with 40 μM HgCl2. The cells did produce copious amounts of MeHg (J. Wall, pers. comm.). The harvested cells were prepared by the method we devised for *E. coli* (Polacco, et al, 2011) and sent to PNNL for proteomic analysis. The surprising results were that there were no Hg-protein adducts under any conditions (i.e. with or without IAM) in *Dd* ND132 (Figure 1).

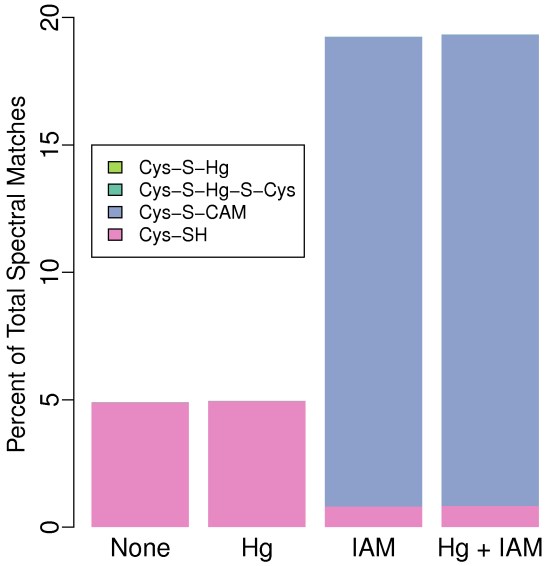

Figure 1. Although protein thiols are detectable by IAM-modification, no protein-Hg adducts are detectable in *Desulfovibrio desulfuricans ND132.*

In troubleshooting this curious result, we learned that the medium used for facultative growth of *Dd* ND132 contains 1 mM cysteine. Thus, the $HgCl_2$ substrate provided would have been immediately converted to bis-cysteine-Hg(II) which apparently was taken up by *Dd* ND132 because it did make MeHg. However, the large molar excess of cysteine may have protected cystsolic and membrane proteins. Another possibility is that *Dd* ND132 may have non-GSH

low molecular weight thiols that complex Hg(II) or an unrecognized “Hg chaperone” that sequesters Hg(II) for delivery to the HgcAB mercury methylase protein discovered by the ORNL SFA group during the course of this project. Collaborator Judy Wall used a genetic approach to pursue this question, but but was not able to confirm this hypothesis with limited funds and time remaining.

In contrast to *Dd* ND132, exposure of *E. coli* MG1655 to inorganic Hg(II), led to abundant modification of a very specific class of proteins: those that have peptides with two or more cysteines in close enough to each other to form a stable bis-coordinate (chelate) structure with Hg(II) (Figure 2, right and Figure 3, right). This was also a sharp contrast to phenylmercury and ethylmercury adducts which are stable to LC-MS/MS proteomic analysis even as mono-thiol adducts (Figure 2, left and Figure 3, left). We also found no evidence of Hg-mediated cross-links between peptides; i.e. inter-cysteine Hg-bonding appeared only when the cysteines were in the same peptide (i.e. intra-peptide), suggesting that inter-peptide Hg-crosslinking was not sufficiently stable to LC-MS/MS conditions to be detectable. We decided to use a bona fide Hg-binding protein, the metalloregulator, MerR, to refine our understanding of the protein-ligand preferences of inorganic and organic Hg compounds.

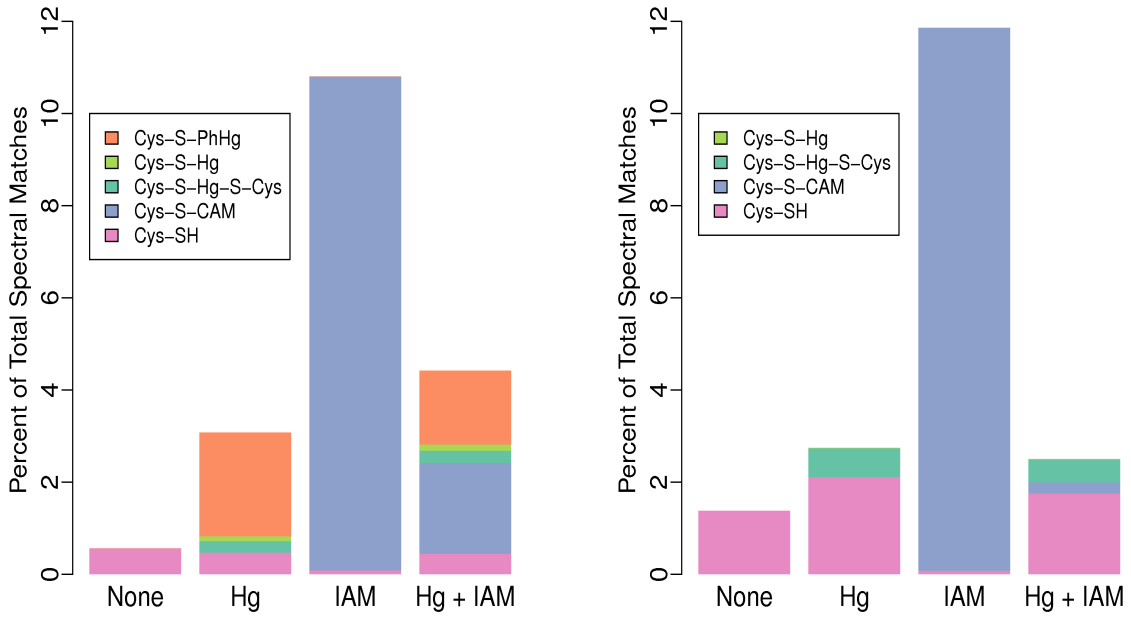

Figure 2. Comparison of *E.coli* protein adducts after exposure to PMA (*left*) or to inorganic Hg(II) (*right*).

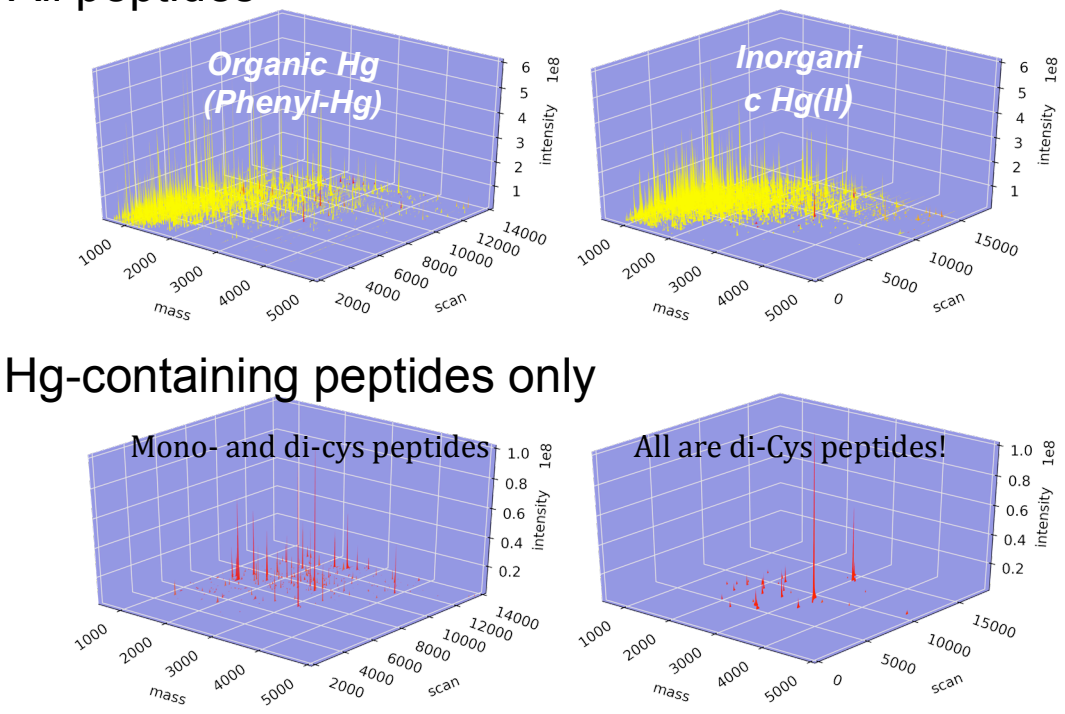

Figure 3. Peptide-PhHg adducts are more abundant and more diverse than peptide-Hg adducts which are limited to di-cysteine peptides.

2. PROTEOMIC ANALYSIS OF A NATURAL Hg-BINDING PROTEIN, MerR

A method for quantitative analysis of Hg-peptides in a proteomic experiment was developed by in collaborator Sue Miller's lab by postdoc Ben Polacco. We applied this computational pipeline to MerR, a homodimeric DNA-binding protein that has 4 cysteines per monomer and dimerizes via a 35-residue anti-parallel coiled coil. An inter-monomer Hg-binding site forms at each end of the coiled coil and involves 2 cysteines from one monomer and a 3rd cysteine from the other monomer (Figure 4). The 4th cysteine in each monomer is not conserved and plays no role in Hg-binding.

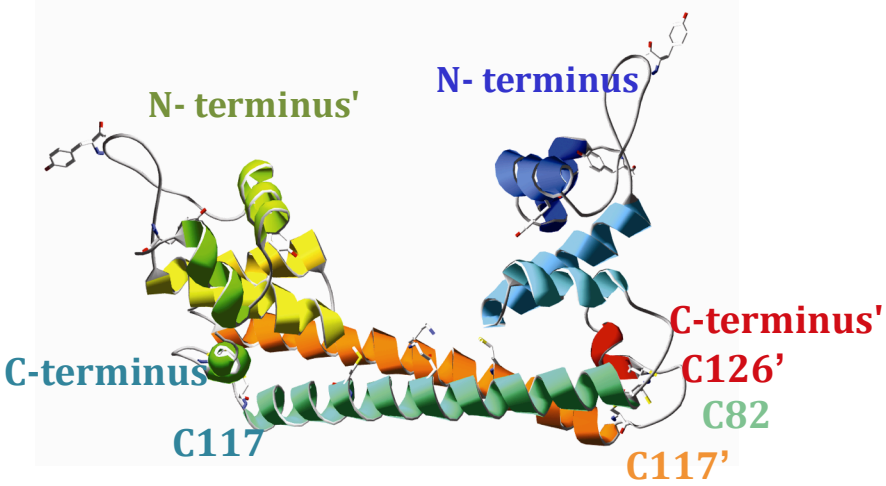

Figure 4. Structural model of MerR (Song, et al. J. Mol. Biol., 2007).

OUTCOME:

Using conditions identical to those used for the above proteomics experiments on the pure MerR protein, we also found that the most stable Hg-binding site is in the peptide that has a CysAlaCys motif (C115-C117) (Figure 5), consistent with the proteomic scale work (Wireman, et al, in preparation). Thus, for although monothiol Hg(II) adducts may be formed in the cell,as long as an excess of free low molecular weight thiols such as glutathione are present, such protein-single cysteine adducts are not sufficiently stable to be detected by LC-MS/MS. However, there chelation by two proximal cysteines in the same peptide is possible, inorganic Hg(II) adducts are very stable. Additional work with MerR has also shown that both phenylmercry and ethylmercury make more stable adducts to all of MerR's cysreines that are readily detectale by LC-MS/MS (Wireman, et al, in preparation).

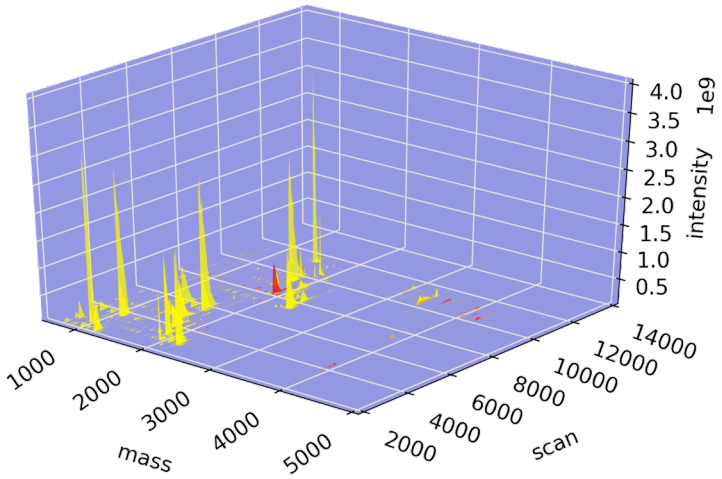

Figure 5a. LC-MS spectra of tryptic digest of Hg(II)-exposed-MerR. Yellow = isotope profile of unmodified peptide. Red = isotope profile of Hg-peptide.

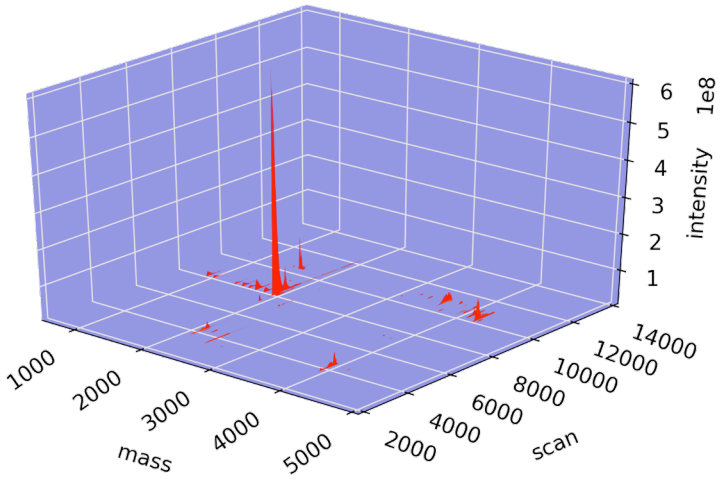

Figure 5b. As in Figure 5a, but filtered for peaks with Hg-peptide isotope profiles. Major peak is the C115-C117 peptide that can chelate Hg.

Figure 5. The MerR peptide which contains two adjacent cysteines makes the most stable Hg-adduct.

3. TRANSCRIPTIONAL ANALYSIS OF *E. coli* RECOVERY FROM EXPOSURE TO INORGANIC AND ORGANIC MERCURY COMPOUNDS

Because we were able to answer our major questions about Hg-cysteine crosslinking using MerR, the more laborious experiments we had originally proposed were not necessary and related experiments had by then been published by others. So, we used the balance of our funding to do a companion experiment to our proteomics work by assessing the transcriptional response and recovery of *E.coli* to exposure to sub-acute levels of mercuric chloride and of phenylmercuric acetate (PMA). Strain MG1655 was grown to mid-log phase and exposed to 3 µM HgCl2 or 3 µM PMA or not exposed. Samples for RNA-seq analysis were then taken at time zero and at 10 min, 30 min, and 60 min after exposure.

OUTCOME:
Consistent with our proteomics findings there are important similarities but also large differences in how the cell responds to inorganic and organic mercurials. The most dramatic overall finding was that cells recovered more quickly from PMA exposure than from Hg exposure. An overview of this phenomenon is depicted in the heatmap of Figure 6.

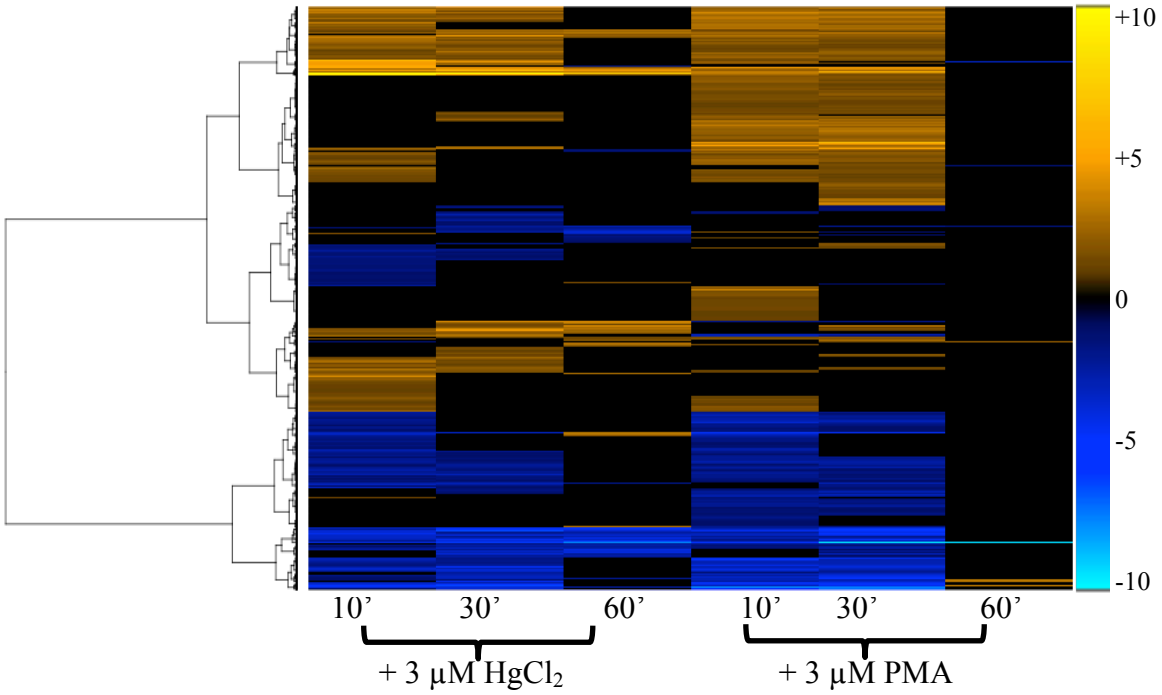

Figure 6. Heatmap of differential expression in response to and recovery from exposure to inorganic Hg(II) or organic mercury (PMA). Left axis is the 4320 encoded genes of *E.coli.* Signal intensity represents the increase (yellow) or decrease (blue) in expression of genes (log base 2 scale) compared to unexposed cells at the same time point (10, 30, or 50 min after exposure).

Heatmaps for other functional categories (data not shown) reveal that differentially expressed functional categories include up regulation by PMA (but not by Hg) of biofilm, fimbriae, flagella, aliphatic amino acid biosynthesis genes and a subset of anaerobic respiration genes. In contrast

Hg strikingly up-regulated of histidine biosynthesis and iron-sulfur cluster genes. PMA strongly down-regulated copper homeostasis and a subset of the Fli genes involved in flagellar assembly, whereas Hg down-regulated ribosomal proteins more broadly and strongly than PMA. Apart from these differences up and down regulation was generally similar for both toxicants and most notably included oxidative and heat stresses, inorganic ion homeostasis, and multidrug resistance. This first ever longitudinal transcriptional profiling of the response and recovery of living cells to Hg and RHg compounds reveals clear physiological differences in how a living cell 'sees' these compounds and how it recovers its viability. These differences undoubtedly underlie the different toxicological profiles of organic and inorganic Hg seen in metazoans and is the first time a global biochemical basis has been adduced for these differences in any model system.

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