FINAL TECHNICAL REPORT

TITLE: IDENTIFYING BIOMARKERS AND MECHANISMS OF TOXIC METAL STRESS WITH GLOBAL PROTEOMICS

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Funding

Institution	*FY 2007*	*FY 2008*	*FY 2009*	*Total*
University of Georgia	200,000	200,000	200,000	600,000*
University of California-San Francisco	125,000	125,000	125,000	375,000
Pacific Northwest National Laboratory	125,000	125,000	125,000	375,000
Total	$450,000	$450,000	$450,000	$1,350,000

*No-cost extensions granted through May 2012

PROJECT OBJECTIVES

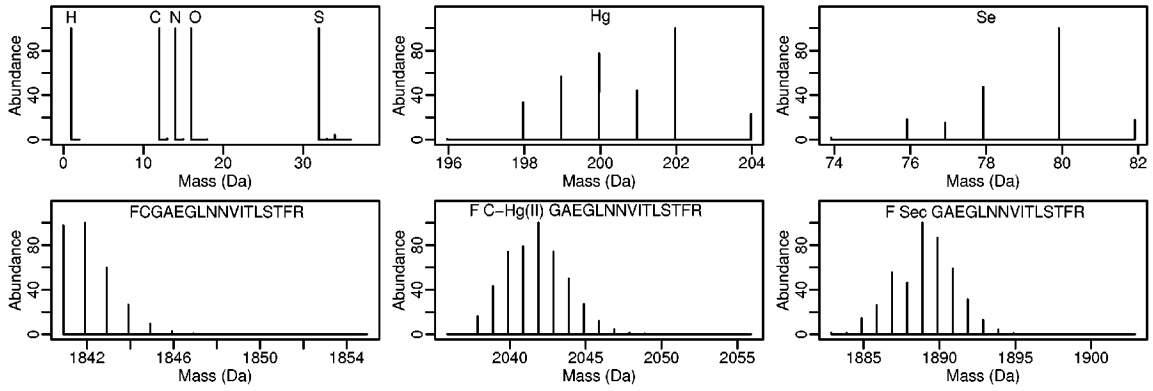
1.0 PROJECT OBJECTIVES

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 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).

SPECIFIC AIMS OF THE SUMMERS' LAB

1. DETECTING PROTEIN-Hg-ADDUCTS IN Hg-EXPOSED *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. The work is based on the change made in a peptide by the 7 stable isotopes of Hg. The method also allowed detection of selenoproteins which are also targets of Hg binding (Figure 1).



OUTCOME:

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). 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). We 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 also did not find selenoproteins with or without Hg adducts; in *E.coli* selenoproteins are typically made only in anaerobic growth or in late stationary aerobic growth.

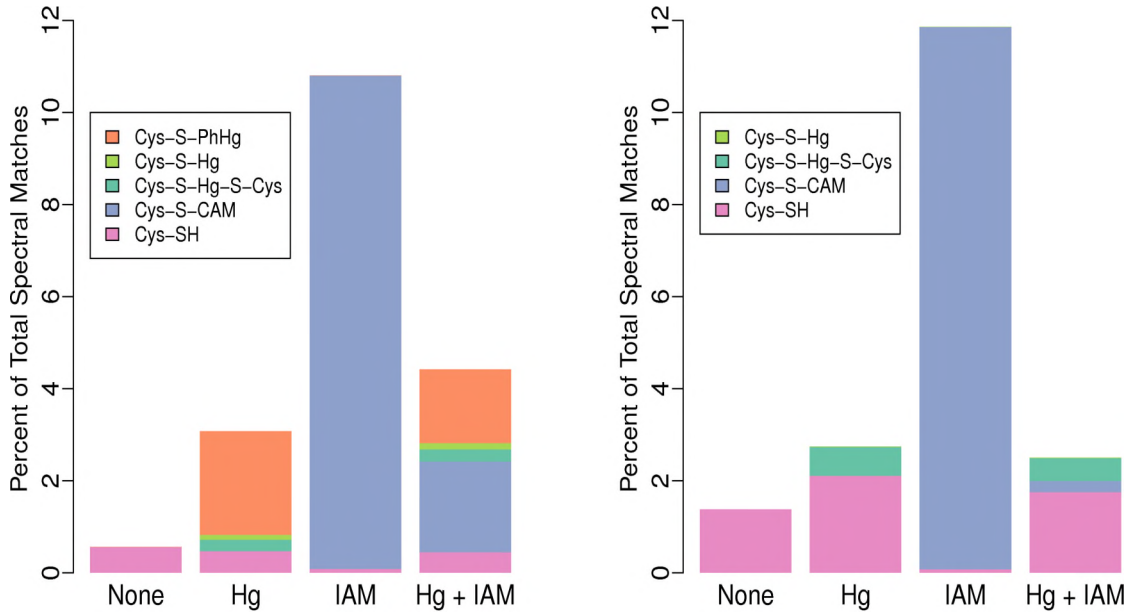


Figure 2. Comparison of *E.coli* protein adducts after exposure to PMA (*left*) or to inorganic Hg(II) (*right*).

2. ASSESSING DAMAGE TO CELLULAR THIOL AND METAL HOMEOSTASES RESULTING FROM MERCURY EXPOSURE

Damage to proteins will cause changes in bulk properties of the cell, Specifically we chose to measure total thiol content, total essential metal content, and status of labile iron pool, to test the hyposthesis that part of mercurial damage results from causing a cascade of reactive oxygen species.

OUTCOME

Brief treatment of late-log phase E.coli cells with Hg(II),phenylmercury acetate (PMA) or merthiolate (ethylmercuri-thiosalicylate, MT) revealed that Hg arrests growth at >10-fold lower levelsthan PMA or MT. Untreated cells averaged ~4x10e6 molecules of reduced thiols (RSH) per cell (~10 mM). MT(160 uM) decreased this to about 0.5 mM, but just 40 uM PMA or 16 uM HgCl2 obliterated bulk RSH. Similarly effects were seen on protein thiols by fluorimaging of SDS-PAGE of proteins tagged with BODIPY-iodoacetamide. The hierarchy of damage (Hg(II)>>PMA>MT) corresponds to cell uptake of each compound. Only 0.042% of 160 uM MT was absorbed, 1:1 ratio to cellular RSH; the 0.35% of 40 uM PMA absorbed was 2:1 over cellular RSH. The 0.60% of 16 uM HgCl2 absorbed yielded a 1:1 for Hg to total RSH.

Surprisingly, 0.42% of 80 uM HgCl2 was absorbed resulting in a 4:1 excess over total RSH, suggesting that with a large excess of Hg in the cell, it takes other ligands than sulfur. Indeed, XAFS analysis of the treated cells showed that for PMA and MT the Hg coordination was dominated by S1 and C1 ligands, with a small shoulder of N or O. In sharp contrast, spectra of HgCl2-treated cells had a smaller and broader S2 peak, indicating a very heterogeneous ligand mix, most likely including nitrogen and/or oxygen.

In a direct biophysical test of the ROS-cascade hypothesis using EPR quantification of redox active free Fe(II)/Fe(III) in intact cells (with UGA Chemistry colleague, Mike Johnson), we found that 16 uM Hg(II) increased free Fe(II)/(III) two-fold and 80 uM Hg(II)provoked a 3-fold increase, equivalent to ~80% of total cellular iron based on ICP-MS; neither of these levels of free Fe was achieved with standard ROS-stress compounds such as 40 mM H2O2. So, Hg(II) blocked formation of and/or directly destabilized Fe-S metalloproteins.

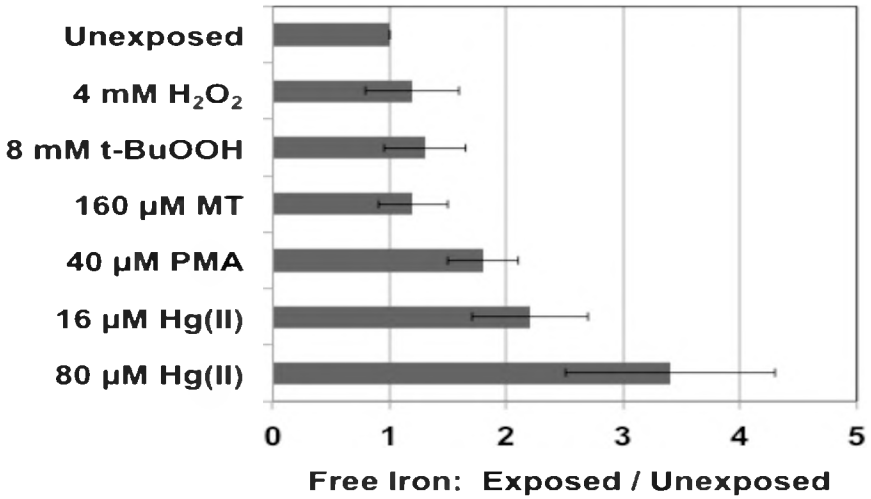


Figure 3: Increases in free intracellular iron levels in cells exposed to mercurials and common organic oxidants. The increase in intracellular free iron is represented as the average X-fold increase in the Fe(III):DF complex EPR signal at g = 4.3 for each stress condition relative to the unexposed control whose average free iron concentration was 24.4 µM (+/- 4.9 µM). Error bars are standard deviation of biological replicates. Replicates for each condition were: Unexposed (6): H_2O_2 (2); t-BuOOH (2); MT (3); PMA (3); 16µM $HgCl_2$ (3); and 80 µM $HgCl_2$ (5)

Lastly, regarding metal homeostasis, we found that the free Fe was not lost from the cell, nor was there any change in cellular content of seven essential metals (Mg, Mn, Fe, Co, Ni, Cu, and Zn). However, Hg significantly altered the electrolyte balance, causing a ~3-fold drop in K and a corresponding increase in Na.

PUBLISHED PEER-REVIEWED :

1. Polacco, BJ, Purvine, SO, Zink, EM, Lavoie, SP, Lipton, MS, Summers, AO, Miller, SM (2011) *Discovering mercury protein modifications in whole proteomes using natural isotope distributions observed in liquid chromatography-tandem mass spectrometry* Mol Cell Proteomics **10:**M110 004853.

2. LaVoie, SP, Mapolelo, DT, Cowart, DM, Polacco, BJ, Johnson, MK, Scott, RA, Miller, SM, Summers, AO (2015) *Organic and inorganic mercurials have distinct effects on cellular thiols, metal homeostasis, and Fe-binding proteins in Escherichia coli.* J. Biol. Inorg. Chem. 20:1239-51

IN PREPARATION FOR PEER REVIEW:

1. Zink E, Polacco, B, LaVoie, SP, Purvine, SO, Miller, SM, Lipton, MS, and Summers, AO (2016)*The Organomercury Exposome of Escherichia coli.* Manuscript in preparation for Metallomics.

MEETING PRESENTATIONS:

1. Summers, et al. (2011, 2012) *Posters and Talks on Hg Biology* DOE SBR Annual PI Meetings.

2. Summers, AO, Lavoie, SP, Olliff, L, Polacco, BJ, Zink, EM, Purvine, SO, Lipton, MS, Miller, SM (2011) *The Mercury (Hg) Exposome: Identifying Biomarkers & Mechanisms of Toxic Metal Stress with Global Proteomics* Gordon Research Conference Cellular & Molecular Mechanisms of Toxicity, Proctor Academy, Andover, NH, July 2011.

3. Summers, AO (2011) *The Molecular Nitty-Gritty of Hg Intoxication: Our Most Vulnerable Cellular Targets.* International Academy of Oral Medicine and Toxicology Annual Meeting, St. Louis, MO, March 2011

4. Summers, AO (2011) *Who Needs Mercury Resistance Genes and How Do They Work So Well?* 10^{th}- International Congress on Mercury as a Global Pollutant (CGMP-10), Halifax, NS. August, 2011.

INVITED SEMINARS:

Summers, AO (2011) *The Mercury Exposome: A Global Proteomic Look at Highly Vulnerable Targets of Inorganic and Organic Mercurials.* Biological Sciences Department, University of Missouri-St. Louis. St. Louis, MO, March 2011.