FINAL TECHNICAL REPORT 04/25/2016

TITLE: Molecular Mechanisms of Bacterial Mercury Transformation

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Funding

Name	Institution	FY 2010	FY 2011	FY 2012	Total
Summers	**UGA**	$40,000	$40,000	$40,000	$120,000
	Total	$40,000	$40,000	$40,000	$120,000

Two no-cost extensions granted through November 2014

PROJECT OBJECTIVES

Hg is of special interest to DOE due to past intensive use in manufacture of nuclear weapons at the Oak Ridge Reservation (ORR). Because of its facile oxidation/reduction [Hg(II)/Hg(0)] chemistry, ability to bond to carbon [as in highly toxic methylmercury: MeHg(I)] and its unique physical properties [e.g., volatility of Hg(0)], Hg has a complex environmental cycle involving soils, sediments, waterways and the atmosphere and including biotic and abiotic chemical and physical transport and transformations.[1] Understanding such processes well enough to design stewardship plans that minimize negative impacts in diverse ecological settings requires rich knowledge of the contributing abiotic and biotic processes. Prokaryotes are major players in the global Hg cycle. Facultative and anaerobic bacteria can form MeHg(I) with consequent intoxication of wildlife and humans. Sustainable stewardship of Hg-contaminated sites requires eliminating not only MeHg(I) but also the Hg(II) substrate for methylation. Fortunately, a variety of mercury resistant (HgR) aerobic and facultative bacteria and archaea can do both things. Prokaryotes harboring narrow or broad Hg resistance (*mer*) loci detoxify Hg(II) or RHg(I), respectively, to relatively inert, less toxic, volatile Hg(0). HgR microbes are enriched in highly contaminated sites and extensive field data show they depress levels of MeHg >500-fold in such zones[2]. So, enhancing the natural capacity of indigenous HgR microbes to remove Hg(II) and RHg(I) from soils, sediments and waterways is a logical component of a comprehensive plan for clean up and stewardship of contaminated sites.

This award was a subcontract from ORNL-Univ. Tennessee, PI Jeremy Smith, to support our contributions to work in his laboratory on the proteins and nucleic acids of the bacterial mercury resistance locus. The funds of SC0005149 were used to augment corresponding projects in my lab carried out under our own award SC0005133 as described in that Final Report and also below.

SPECIFIC AIMS OF THE SUMMERS' LAB

1. STRUCTURAL ANALYSIS OF THE MerR-MerOP COMPLEX.

We continued to collaborate with investigators at ORNL and UGA with the aim of using neutron scattering, computational chemistry, and x-ray crystallography to observe and model MerR interacting with Hg and/or with operator DNA.

OUTCOME: The ORNL collaboration for biophysical and computational studies had led to a publication revealing dynamic behavior of MerR consistent with its known DNA under-winding activity (1). We provided MerR protein and detailed protocols and other guidance to the ORNL team for their efforts to obtain neutron scattering data on the MerR-MerOP complex in solution and to make and characterize mutant MerR's in this respect. Exceptions taken to our established procedures limited their success in these efforts. We also contributed extensively to the writing of a computational analysis of the basis of Hg's preference for the soft ligand sulfur over the hard ligand oxygen (2). Multiple attempts to acquire crystals of MerR (ORNL) or Mer-MerOP complex (Cory Momany, UGA) suitable for x-ray structure determination were unsuccessful. Such a structure was acquired in 2015 by a Chinese group with the related *Bacillus* MerR using a mutant genetically optimized for crystallization.

2. MerR-MerOP-METAL INTERACTIONS.

This subproject employs biophysical, biochemical, and genetic tools to assess the kinetics and thermodynamics of the ability of the MerR-MerOP complex to distinguish inducer metals and respond effectively to them – or not. Originally meant to include interactions with RNA polymerase, the project took a novel turn when an exciting discovery about the *mer* operator-promoter was made during initial experiments.

OUTCOMES:

a. Calorimetric, X-ray absorption, and hydrodynamic characterization of MerR's and MerR-OP's interactions with all 3 Group 12 metals (Zn, Cd, and Hg) established that: (i) MerR does not offer ligands sufficient for making kinetically and thermodynamically stable complexes with the former two metals; (ii) under equilibrium conditions, complexes of Zn or Cd that form do not provoke the requisite allosteric change in MerR or MerOP needed to under-wind the DNA. A publication describing this work is in revision after review at *Metallomics* (3).

b. We made the surprising discovery that under low thiol conditions the MerOP DNA itself has a very stable binding site for Hg. We decided to pursue this novel and very exciting finding which overturned the conventional wisdom that thiols are the exclusive cellular target for Hg and set aside the originally proposed analysis of crosslinking to identify points of contact between MerR, MerOP, and RNAP. Calorimetry revealed that the Hg binding site in MerOP is between two thymidines in lower strand at the center of the dyad of the MerO operator DNA and that Hg binding drives that dyadic region to adopt a cruciform structure in vitro (Fig. 1). The formation constant of this site is 10^{22} and once formed, Hg is not removed even by 24 hr dialysis in excess cysteine, despite the formation constant of the simple bidentate Cys-Hg-Cys complex being 10^{39}. The Hg-MerOP stability likely resides in the strong entropic cost

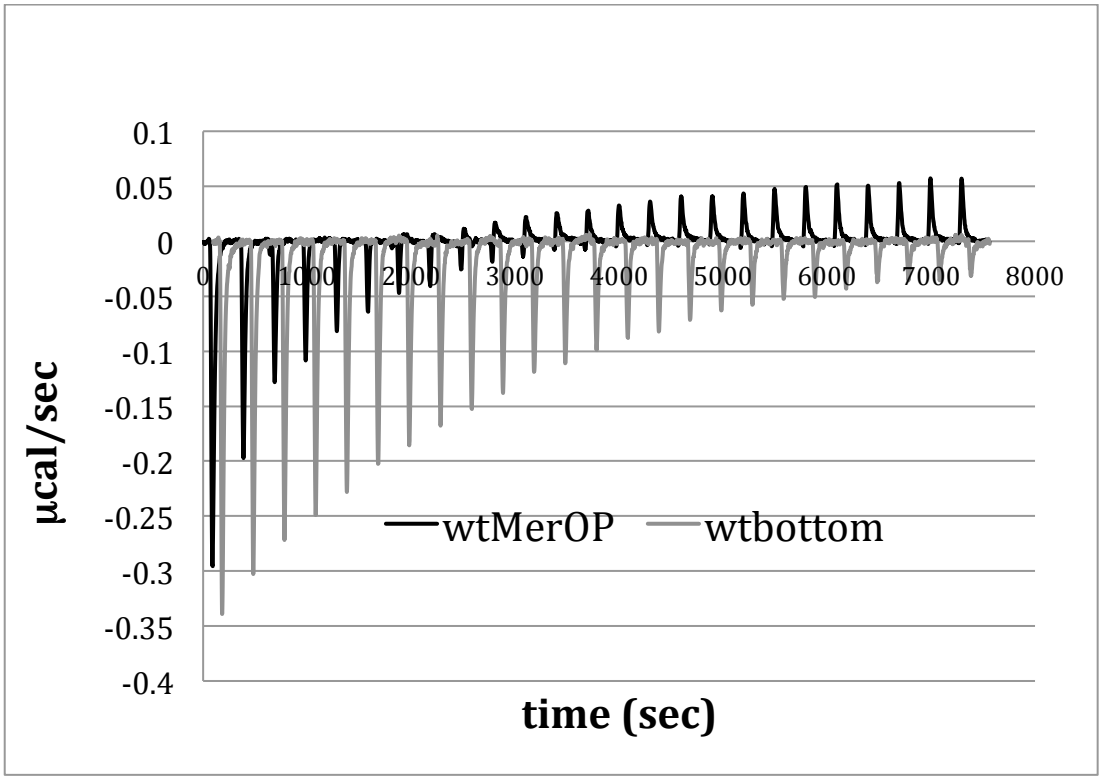

Figure 1. Titration calorimetry of Hg binding to double stranded MerOP (black) or its lower single strand (gray). MerOP has the weaker exothermic and stronger endothermic features of a strongly entropic sequential process consistent initial Hg binding provoking cruciform formation. In contrast, the lower strand is entirely exothermic consistent with simple Hg binding without any allosteric change.

inherent in chelation by the cruciform structure. The biological relevance of these in vitro results lies in the fact that exposure even to vey low levels of Hg provokes ROS formation and depletes cellular thiols, leaving unprotected other nucleophilic ligands including the N3's of thymine (Fig. 2) already well-established in synthetic nucleotides as a strong Hg-binder. In addition, our previous genetic and in vivo foot-printing work had established that one of these thymines is involved in operator function.

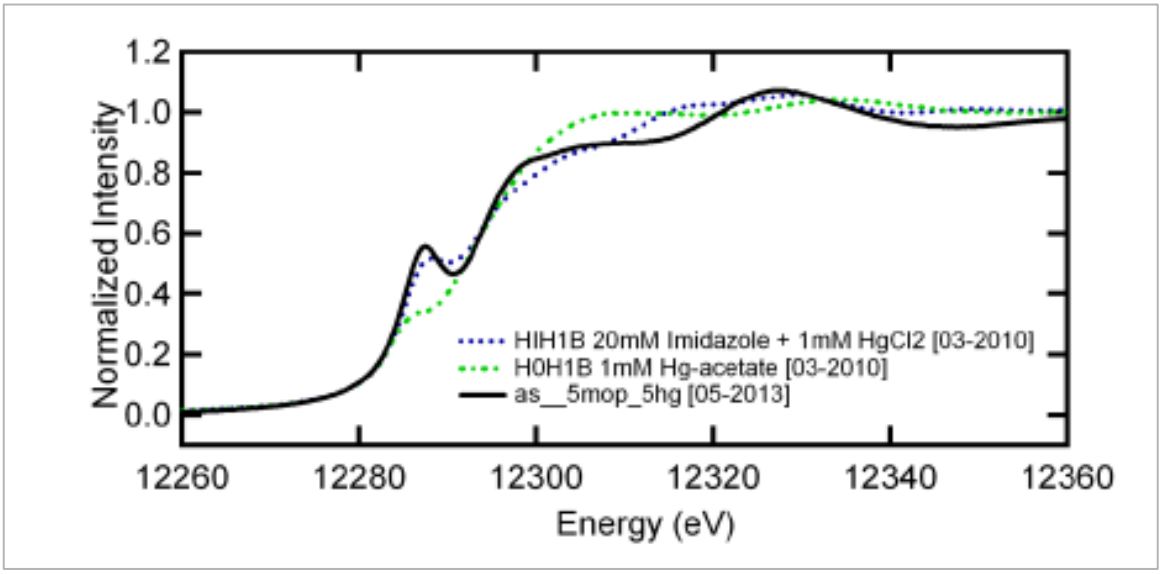

Figure 2. X-ray Absorption Near-edge Spectroscopy (XANES) of the double stranded MerOP-Hg, Hg-(imidazole)$_2$, and Hg(acetate)$_2$. Spectral features of MerOP (black) resemble those of Hg(imidazole)$_2$ (blue) more closely than those of Hg(acetate)$_2$ (green)

Loss of our long time XAS collaborator, Bob Scott who became UGA's associate vice-resident for research, slowed the collection of XAS data, but we were able to acquire sufficient data through Graham George at the Canadian Light Source and to have it analyzed by a former post-doc of Bob's. The manuscript covering calorimetry, equilibrium and competition dialysis, and XAS of wildtype and mutant operators will be submitted this summer (4).

3. HOW DOES ANTI-REGULATOR MerD WORK?

The *mer* operon has a novel regulator, MerD, to turn off the operon once Hg(II) has been eliminated from the cell by MerA. We hypothesize that MerD joins MerR at the operator-promoter region and prevents the recruitment of RNA polymerase to the promoter. An alternative hypothesis, typical of other anti-sigma regulators, would be that MerD interacts directly with sigma-70 to prevent its occupancy of the promoter. We aimed to purify MerD and examine such interactions in vitro and also to quantify alterations in *mer* operon transcription with wildtype MerD or mutant of it in vivo.

OUTCOME:
Doctoral student Taka Sasaki ($24,000/year) tried several different affinity tag constructs of MerD, but was unsuccessful in overproducing a soluble form of the protein suitable for purification. He then took a genetic approach by using a series of existing deletions of the *merD* gene to assess their affect on expression of a *mer-lac* transcriptional fusion on a separate compatible plasmid. As expected the *mer-lac* fusions expressed less LacZ with wildtype *merD* than with the *merD* deletion mutants. So Taka employed a more refined but expensive

technique using RT-qPCR to measure *mer* operon mRNA directly and again found that the mutant *merD* allowed the *mer* transcript to persist longer than the wildtype gene. Unfortunately, Taka's health deteriorated seriously after a construction-related major toxic exposure during late 2011 that affected my entire group. After a few months of recovery, despite his promising results, Taka decided to leave the lab in Spring of 2012 because the University had declined to take corrective action to prevent future exposures.

4. HOW DOES THE *Streptomyces lividans* MerR (SL-MerR) WORK?

Although most Gram positive and Gram negative bacteria have a MerR protein homologous to that of *E.coli*, the actinobacteria use a Hg(II) responsive member of the ArsR regulator family to control expression of their *mer* operons. This is about as different a family of regulators from the MerR family as one could imagine and, as noted, the actinobacteria use a very different thiol buffer, so we expected some differences in the actinobacterial MerR will partner with its host cell. Our plan was to purify the protein and characterize it biochemically and biophysically.

OUTCOME:
Using an *E.coli* strain carrying the His-tagged wildtype SL_MerR gene provided by a colleague in Germany who first described the *S. lividans mer* locus, research technician Logan Davis ($20,000/yr) used standard IMAC chromatography to purify the protein. However, yield was low and erratic. So, we had the SL_MerR gene commercially synthesized with codons optimized for *E.coli* and with the tandem His6-SUMO tag which enhances solubility of difficult proteins.

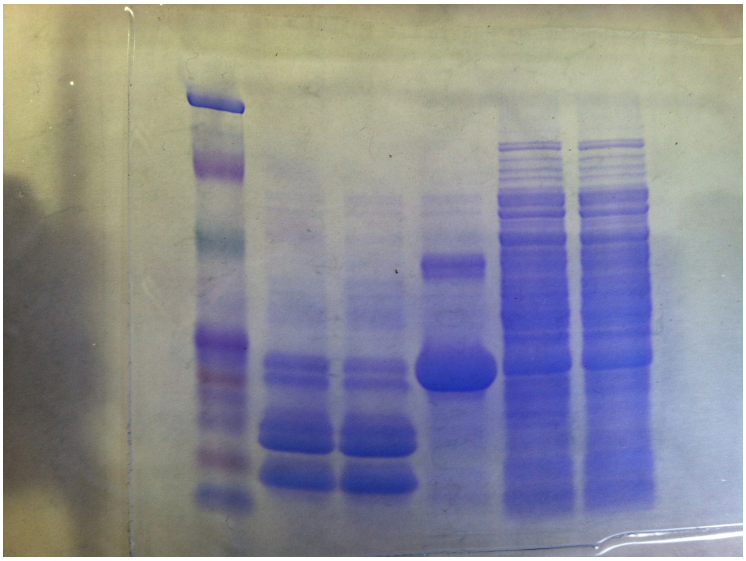

Figure 3. SUMO protease digestion of His6-SUMO-SLMerR. Lane 1, MW markers, lanes 2,3 digested eluate; lane 4, pre-digest eluate, and lanes 5,6, original lysate. The fastest (smallest) bands in lane 2 and 3 are SL-MerR released nearly completely from the fused construct (lane 4) by SUMO proteolysis. Densitometry suggests stoichiometric recovery of SLMerR consistent with its being quite soluble even when separated from the SUMO tag (the next larger band in lanes 2,3).

This tandem tag can also be removed by the highly efficient SUMO protease without leaving a scar. Logan completed production of cell pellets producing the new clone before leaving for grad school in neurobiology. Rotation student Ramya Kolli picked up the project and successfully got excellent yield and very clean cleavage of highly soluble SL-MerR (Figure 3) before moving on to her next rotation. This very promising project has since been on hold for want of funding to support a student.

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PEER-REVIEWED :

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3. Olliff, L., Cowart, D., Mahendra, U., Brick, A., Mullis, T., Scott, R.A., and Summers, A.O. (2016b). Thermodynamic, Kinetic, and Structural Aspects of the Discrimination of Group 12 Metals by the Bacterial Metalloregulator MerR. *In revision for Metallomics.*

4. Riccardi, D., Guo, H-B, Parks, JM, Hu, B, Summers, AO, Miller, SM, Liang, L, Smith, JC (2013). Why Mercury Prefers Soft Ligands. J Phys Chem Lett *4*, 2317-2322.

MEETING PRESENTATIONS:

1. Summers, et al. (2011, 2012, 2013, 2014, 2015) *Posters and Talks on Hg Biology* DOE SBR Annual PI Meetings.

2. Lavoie, SP, Polacco, BJ, Zink, EM, Purvine, SO, Wireman, J, Lipton, MS, Miller, SM, Summers, AO (2014). *Differential Proteomic and Physiological Consequences for E.coli of Organic or Inorganic Mercury Exposure.* 9th International Biometals, Duke University, Durham, NC.

3. 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.

4. 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

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

INVITED SEMINARS:

Summers, AO (2014) *Mercury: Is It Really Bad for You?* Osher Lifelong Learning Institute, University of Georgia, Athens, GA, Dec 2014.

Summers, AO (2014) *Bio-inorganic Diversity: Microbes Modulate Mercury's Mayhem*. Department of Microbiology, University of Illinois, Urbana, IL, November 2014

Summers, AO (2014) *The Hg Exposome: Discriminating Molecular Targets of Inorganic and Organic Mercurials in the Proteome and Natural Nucleic Acids* Environmental Health Sciences Dept., University of Georgia, March 2014.

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.