EXTRACELLULAR POLYSACCHARIDES PRODUCTION BY BACTERIA

AS A MECHANISM OF MERCURY TOLERANCE

By

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ABSTRACT OF THE DISSERTATION

Extracellular Polysaccharides Production by Bacteria

as a Mechanism of Mercury Tolerance

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Dissertation Director:

Tamar Barkay

Bacteria have adapted mechanisms of mercury (Hg) tolerance to survive in environments containing elevated Hg concentrations. The potential of extracellular polysaccharides (EPS) production by bacteria as a mechanism of Hg tolerance has not been previously investigated. The objective of my dissertation was to determine if EPS produced by bacteria sorb Hg as a tolerance mechanism. Purified EPS with different chemical compositions isolated from bacterial pure cultures from microbial mats in French Polynesian atolls were assessed for Hg sorption by filtering Hg and EPS solutions after equilibration via shaking and measuring free vs. bound Hg. The data showed that EPS sorbed up to 82% of Hg from solution, that this sorption was dependent on EPS composition, and the sorption was a saturable mechanism. Hg uptake capacities ranged from 1.0 to 91.2 mg Hg/g for the different EPS. To determine if EPS production could alter Hg tolerance in bacteria during growth, an *E. coli* K-12 strain and its EPS defective mutant were obtained. A disc inhibition assay was

performed demonstrating Hg inhibited growth in a dose-dependent manner. The wild-type was more tolerant to Hg with zones of inhibition ranging from 1.4 to 6.1 mm compared to 2.5 to 7.15 mm for the mutant. Assessment of EPS production between the 2 strains by the phenol- sulfuric acid assay for sugar determination normalized to protein content of bacterial pellet showed the wild-type produced 2 times more EPS than the mutant. Finally, EPS production and Hg tolerance were examined in bacteria isolated from an environment with naturally elevated Hg concentrations. Eight obligate and facultative chemolithoautrophic bacteria tolerant to 10 µM $HgCl_2$ were isolated from the East Pacific Rise at 9ºN. Two of the isolates were selected for further characterization. Purge and trap experiments revealed that neither isolate reduced Hg^{2+} to Hg^{0} suggesting that the *mer* operon is not used for mercury tolerance. The phenol-sulfuric acid assay for sugar determination normalized to protein content of bacterial pellets of 3 facultative chemolithoautotrophs revealed that all 3 isolates produced EPS ranging from 0.5 to 38 µg EPS/mg protein. For 2 of strains, there was 8% and 32% increase in EPS production during growth in the presence of Hg, suggesting that EPS may be involved in tolerance. Together these data suggest EPS production is a potential mechanism of mercury tolerance in bacteria.

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Dedication

This dissertation is dedicated to my family. To my Mom and Dad for all their love and support throughout my life and fostering my love of science. To my brother Tim for his compassion and always being someone I can rely on. To my brother Jon for being the best lab partner and never minding having his little sister in all his classes from high school to graduate school. I have loved every minute of it!! Finally to my fiancé PJ for all his love, companionship, and unwavering belief in me.

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Chapter 1 – Introduction

Mercury

Mercury is one of the most toxic metals in the environment with no known biological function and is a human and wildlife health hazard (1-5). Humans have known about mercury since ancient times using it to make amalgams with other metals as early as 500 BC. It is still used today in the extraction of gold, dental amalgams, vaccines, certain drugs, and in technological devices (2, 6). Elemental mercury is the only metal liquid at room temperature and is also a vapor. Mercury exists in a mercurous (Hg^{1+}) and mercuric form (Hg^{2+}) bound to other elements, including one or more carbon atoms forming stable organometallic compounds. Organic mercury, e.g. methyl mercury, is the most toxic form and is of ecological importance (6, 7).

Humans are exposed to methyl mercury through dietary consumption of food, especially fish (2, 4, 8-10). Although inorganic forms are also found in fish, their levels are usually well below the toxic range. Methyl mercury is a potent neurotoxicant and is able to transverse the blood brain barrier (2, 9, 10). Some biochemical effects are non-specific cell injury by binding to sulfhydryl groups of proteins, interrupting microtubule formation, protein and DNA synthesis, inhibiting enzymes, causing oxidative stress and neuronal cell death (2). This leads to localized damage in the central nervous system (sensory and motor coordination functions) due to the inability of certain neuronal cell populations to repair the

damage. The resulting toxicities include paresthesia, ataxia, dysarthia, diminution of vision, and loss of hearing with severe poisoning resulting in blindness, coma, and death. The effects of methyl mercury toxicity can take weeks to months to manifest after exposure (2, 5, 11, 12).

One of the first major documented cases of methyl mercury poisoning was in Minamata Bay, Japan, when methyl mercury containing wastewater from the Chisso chemical plant went into the bay. People were admitted to the hospital for ataxia, sensory disturbances, dysarthia, tremors, hearing loss, and vision problems. In 1956, the disease was officially described as Minamata disease, and methyl mercury determined as the causative agent. At the time, there were 200,000 residents surrounding the bay whose livelihood mostly depended on fishing. Fetuses exposed to methyl mercury from their mothers' consumption of fish during pregnancy had serious brain pathologies. Since it was first discovered, over 2,200 patients have been diagnosed with Minamata disease (12-14).

Another major incident of methyl mercury poisoning occurred in Iraq during 1972 when farmers and their families used wheat seeds treated with a methyl mercurial fungicide imported from Mexico. The farmers and their families were exposed to methyl mercury through consumption of homemade bread made from the seeds, livestock or game birds that ate wheat seeds, and in infants through mothers' milk. There also were cases of dermal and inhalation exposures from handling and grinding of wheat. Over 6,000 people were

admitted to the hospital for mercury poisoning with over 400 associated deaths (11, 12).

Unlike the two aforementioned incidents where mercury was released in its organic form, in most cases mercury enters the environment through anthropogenic and environmental sources as inorganic mercury and as vapor (Figure 1.1) (9, 15).
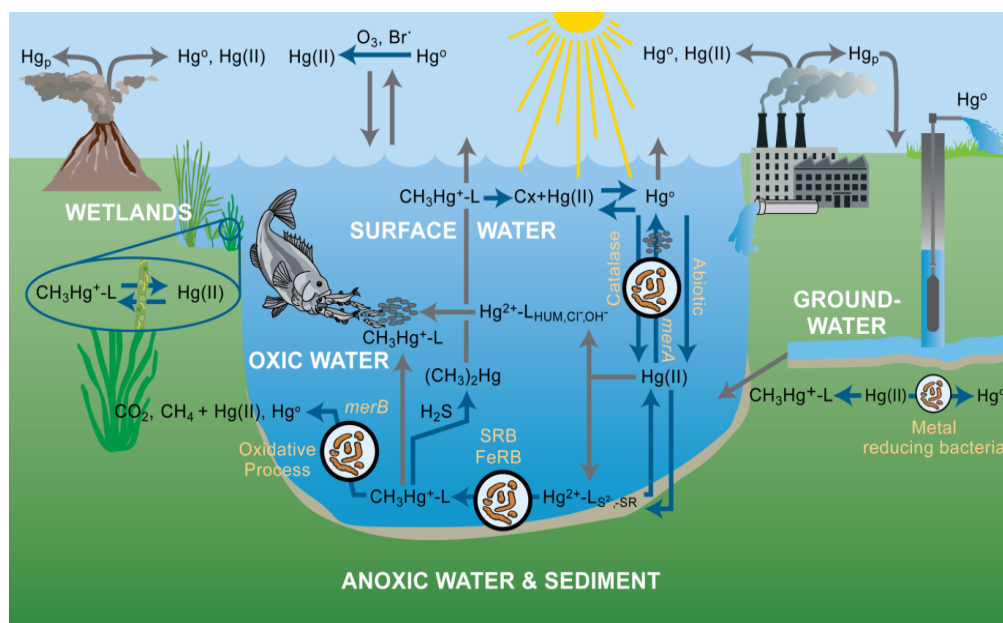
Figure 1.1 Global biogeochemical cycling of mercury in the environment (15)

Once in the atmosphere, mercury enters a complex cycle of transformations and transport through the environment (Figure 1.1). Elemental mercury vapor is stable and can stay in the atmosphere for a year or more leading to global distribution until it becomes oxidized. Once oxidized it is more water-soluble and deposited with rainwater entering natural bodies of water. Oxidized mercury can be reduced back to a vapor or converted to an organic form (2, 9). The organic form methyl mercury is of great human and wildlife health concerns because it is

readily absorbed through the gastrointestinal tract where it has been found that 95% of the consumed methyl mercury was absorbed in fish. This is in contrast to mercuric mercury of which only 7% of the consumed mercury is absorbed in the GI tract. Once methyl mercury is absorbed, it is rapidly transported throughout the body and incorporated into the tissue of animals, specifically through interactions with thiol complexes of cysteine, glutathione, homocysteine, and proteins (4, 9, 10, 16). The half-life of methyl mercury in human whole blood is 44 days with 90% of excretion through feces. There is extensive enterohepatic recycling, where the methyl mercury is excreted into the intestinal tract via bile and then reabsorbed leading to an accumulation of mercury in tissue (8-10). When animals that have accumulated mercury in their tissues are preyed upon, the predator will assimilate the mercury into their tissue resulting in a bioaccumulation and biomagnification of mercury (4, 8-10, 14, 17, 18). The amount of mercury that is assimilated by the predator depends on the subcellular location of the mercury. Mercury associated with proteins are the most bioavailable and are readily assimilated by predators (4, 18).

Mercury concentrations in non-polluted soil are around 5 to 10 nmol/l and in ocean water range from 1 to 2 pmol/l (3, 19). Transformations between redox states and between inorganic and organic forms of mercury are due in part to activities of microorganisms (Figure 1.1). Bacteria have been shown to methylate, demethylate, reduce, and oxidize mercury. These chemical alterations affect the mobility and bioavailability of mercury in the environment. Since mercury is an element it cannot be degraded and removed from the environment.

It can only be transformed to reduce bioavailability. Therefore understanding

mechanisms by which bacteria handle mercury can provide a way to modulate

the bioavailability of mercury in the environment by inhibiting mercury

methylation, enhancing demethylation, or promoting internal sequestration.

Mechanisms of mercury tolerance that involve intracellular or extracellular

sequestration could be exploited for the bioremediation of contaminated sites to

remove mercury from the environment. The recovered mercury could then be

recycled and used by industry (2, 3, 9, 15).

The main mechanism of mercury resistance among heterotrophic, aerobic

bacteria is encoded by the *mer* operon. It is a well-characterized set of genes

encoding for proteins involved in the reduction of inorganic and organic mercury

(Figure 1.2). The most important function of the operon is carried out by the

cytosolic mercuric reductase (MerA protein), a flavin disulfide oxidoreductase

using the cofactor NAD(P)H. Mercuric reductase carries out the conversion of

ionic mercury (Hg^{2+}) to elemental mercury (Hg^0). The elemental mercury

produced is a vapor, relatively inert, and readily leaves the cell (3, 20). The

second enzyme encoded by the *mer* operon is an organomercurial lyase, MerB

which releases ionic mercury from organomercuricac compounds for reduction

by MerA. Additionally, the *mer* operon encodes for proteins involved in the

transport of mercury into the cytosol and gene regulation. Periplasmic MerP and

transmembrane bound MerC and MerT are involved in the transport of mercury

from the periplasm into the cytosol for reduction. The operon is regulated by

MerR, a metal-responsive transcriptional activator inducing transcription when mercury is present, and its antagonist MerD (20).
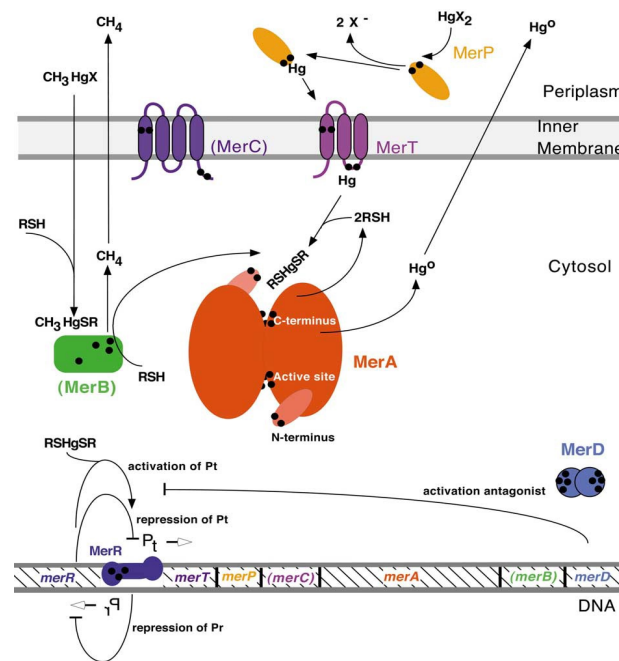
Figure 1.2 The *mer* operon of a typical Gram negative bacterium. Black dots indicate cysteine residues and X indicates a nucleophile. RSH represents a cystolic thiol and parentheses indicate the gene is not present in every operon (20).

Deep-sea hydrothermal vents and their microorganisms

Deep-sea hydrothermal vents are an environmental source of mercury emissions resulting in a natural elevation of mercury at these sites. The diverse organisms living at deep-sea hydrothermal vents have had to evolve ways to handle mercury stress. Deep-sea hydrothermal vents and their biological communities were discovered over forty years ago along mid- ocean ridges. At these ridges, there is sea-floor spreading due to tectonic plates being pulled apart and hot molten rock from deep in the Earth filling the gap forming new Earth's crust. Deep-sea hydrothermal vents account for greater than 75% of the

Earth's volcanic activity (3, 21-25). These areas are comprised of several different focused and diffuse flow vents ranging in size from a hundred to millions of square meters and are found across the globe; however the majority of research on vent systems has focused on areas of the eastern Pacific and north-central Atlantic Ocean (23).

Vent fluid is formed when seawater seeps into the Earth's crust (Figure 1.3) becoming heated to ~ 400°C due to magma chambers in the Earth's crust. At this temperature, solubilities of metals and sulfur found in basalt are greatly increased causing them to leach into seawater and the seawater becomes anoxic. The heated vent fluid is then forced back out of the Earth's crust with minimal pre-emission mixing with seawater to form focused hot flows or with significant pre-emission mixing with seawater to form low- temperature diffuse flows (23-25).
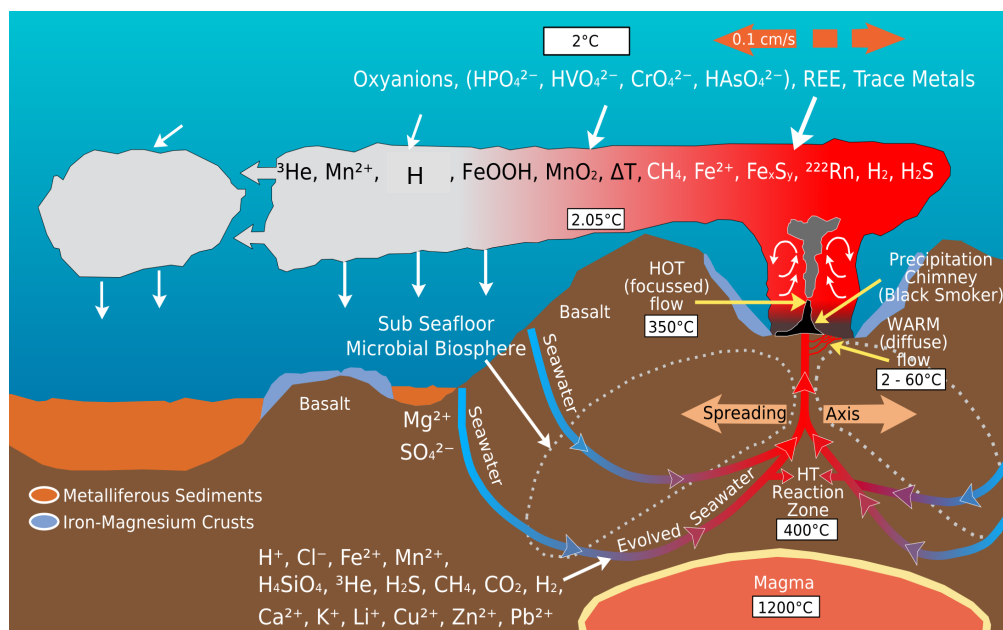
Figure 1.3 Diagram of hydrothermal vent fluids at mid-ocean ridge spreading centers. (Image adapted from the National Oceanic and Atmospheric Administration <<http://oceanexplorer.noaa.gov/explorations/02fire/background/hirez/chemistry-hires.jpg>>)

Focused hot flows are divided into black and white smokers. Black smokers evolve rapidly after a volcanic eruption and emit fluids reaching temperatures of 400°C. Only months after an eruption, mature sulfide chimneys will form exceeding 5 m in height. The fluid emitted is super heated and acidic. It mixes with cold, alkaline seawater causing metal sulfides present in the fluid to precipitate, resulting in the black color observed. White smokers are similar to black smokers except that the fluid emitted from white smokers tends to be cooler than black smokers, between 100°C to 300°C. The decrease in temperature results in a decrease in carrying capacity of metal sulfides, leading to a decrease in precipitation when the fluid mixes with seawater. Also, mixing of vent fluid with seawater may occur before it is emitted from the Earth's crust, so precipitation of metals will occur in the ocean crust leading to a white precipitate. The mixing of seawater with vent fluid prior to release from the Earth's crust can also give rise to low- temperature diffuse flows. Temperatures at diffuse flow vents can be as high as 60°C but are usually below 40°C. They are ideal to support microbial and invertebrate communities. Diffuse flow vents can be found in the porous surfaces of black or white smokers or from cracks in basalt lavas (23, 25).

Chemolithoautotrophic microorganisms form the base of the food web in hydrothermal vent ecosystems, providing organic carbon for zooplankton and benthic organisms. Microbes that use chemicals as an energy source, fix CO_2, and use inorganic electron donors are known as chemolithoautotrophs. Carbon dioxide fixation provides the vent ecosystems with a carbon source. The principle

energy sources in the vent ecosystem are oxidation of elemental sulfur and sulfide, but iron (II) oxidation, hydrogen oxidation, and methanogenesis also occur (23-27).

It has been found that some vent microorganisms are not obligate chemolithoautotrophs but are chemomixotroph because they can use a mixture of inorganic and organic electron donors and carbon sources. This is advantageous because vent fluid chemistry will change over time and the oxidative state of water changes in relation to distance from the flow source. The ability to change metabolism allows an organism to optimize its survival capabilities when conditions are altered (3, 23, 25).

Besides nutrients in the vent fluid, there are a lot of heavy metals present including lead, cadmium, and as mentioned previously mercury (19, 24, 26, 28, 29). Levels of mercury at vent sites will vary depending on vent fluid composition but are elevated compared to seawater. Seawater has an average mercury concentration of 1 to 2 pmol/ L, whereas mercury concentrations at vent sites have been found to be between 13.9 and 445 pmol/L with upper levels comparable to those of contaminated surface waters (19, 30). Mercury precipitates out of solution with sulfur. Bacteria growing on the surfaces of the precipitate will oxidize the sulfur, potentially mobilizing the metals. Bioleaching of metal sulfides by bacteria is a biotechnological approach for the recovery of heavy metals, such as copper, cobalt, and zinc. Although this technique has not been used for the recovery of mercury, it has been shown that mercury resistant *Acidithiobacillus ferrooxidans* strains are capable of mobilizing mercury from

cinnabar (19, 31, 32). Therefore in the microenvironment of the microbe, mercury levels will be elevated. To handle this stress, microorganisms at vent sites must have developed mechanisms of detoxification. Consistent with this hypothesis, It has been shown that certain heterotrophic bacteria isolated from deep-sea hydrothermal vents contained the *mer* operon (33).

Mercury detoxification mechanisms of chemolithoautotrophic bacteria are not well characterized. Studies suggest these mechanisms, in conjunction with metabolites produced by vent microbes, could have significant biotechnological applications (22, 26, 28, 29). For instance, EPS produced by *V. diabolicus* contains equal amounts of uronic acids and hexosamines (hyaluronic acid like) and has been shown to enhance bone healing in rats (24, 34). High pressure and high temperature tolerant enzymes have been isolated from vent organisms, including proteases and DNA polymerases that could be used for high pressure organic synthesis and PCR reactions respectively (22). When a flourscent strain of *P .aerugonisa* isolated from deep-sea hydrothermal vents grow under aerobic conditions, it produces sulfide which can percipitate cadimum onto the cell wall. Percipitation of cadmium to the cell wall decreases the bioavailability of cadmium in the environment. This capablility could be exploited to remediate cadimum contaminated sites (35). Elucidating the mechanisms of mercury tolerance in deep-sea hydrothermal vent chemolithoautotrophic bacteria could lead to a similar discovery resulting in a new method for the bioremidation of mercury contaminated sites. It will further our knowledge of the role of bacteria in the mercury cycle and possible ways the environment could be manipulated to

stimulate the growth of or introduce bacteria capable of decreasing mercury's bioavailability. If the mercury is sequestered in or on the cell wall like in the case of cadmium for *P. aerugonisa*, the mercury could be recovered from the environement and used.

Exocellular polysaccharides

Exocellular polysaccharides (EPS) are high molecular weight sugars excreted outside of the cell. They can either be completely disassociated from the cell and released into the surrounding environment or remain associated with the cell surface (24, 27, 36, 37). EPS does not play a role in the structure of an individual bacterial cell but can play an important role in forming complex structures with other bacterial cells known as biofilms. They also form a protective barrier to changes in environmental conditions. The composition of EPS varies greatly between microorganisms and even between different strains of the same species. Although they can be homopolysaccharides, most contain two or more monosaccharides in repeating units of 10 or less monosaccharides. It is mostly composed of carbohydrates but can contain inorganic and organic substitutes including acetate, pyruvate, amino acids, acyl groups, sulphates, and phosphates. Most EPS are polyanionic in nature (24, 36, 38-40).

EPS production is a complex process and can be altered due to changes in pH or temperature, presence of metals, phase of growth, starvation, and dehydration (36, 39, 40). The EPS production was shown to be enhanced in the presence of chromium and/or copper for *Pseudomonas* sp., *Micrococcus* sp.,

and *Ochrobactrum* sp. This is believed to be due to activation of gene transcription caused by free radicals and oxidative stress when metal cations enter the cell (39, 40).

It is known that both deep- sea hydrothermal vent bacteria associated with macrofauna and those free-living produce EPS. Some of these EPS have shown to possess unique characteristics with the potential for biotechnological applications (22, 27, 28, 41-43). As mentioned previously, EPS produced by *V. diabolicus* has the potential application of aiding in bone healing in humans (34). EPS from the aerobic, heterotrophic *A. macleodii* subsp. *fijensis* has chemical similarities to xanthan and could potentially be used as a thickening agent in food. This EPS is also capable of binding lead and could potentially by used in water treatment plants (24). Additional hydrothermal vent bacteria, including symbiots with vent macrofauna, have been found to produce an EPS capable of binding heavy metals, including copper, lead, and iron (28, 29, 42). It is hypothesized to be due in part to the high uronic acid content of marine EPS of up to 50% (24). None to date have been tested for their ability to bind mercury.

It is widely known that EPS produced by bacteria, fungi, and algae bind heavy metals preventing the metal from entering the cell and causing damage (39, 40, 43-45). The application of EPS for the bioremediation of heavy metal contaminated sites has received considerable attention in the past couple of decades as an eco-friendly and cost effective method for metal removal (29, 39, 40, 44, 46). The ability and degree to which an EPS will bind a metal cation depends on chemical composition of the polymer and properties of the metal.

Carboxylic groups (mainly from uronic acids) are the major functional groups involved in the interaction of metals ion to polysaccharides but hydroxyl and other functional groups also play a minor role in metal binding (40, 46, 47). Very few EPS have been studied for their ability to bind Hg. Only a couple examples of mercury binding to microbial EPS existing (44, 48).

Objective and Hypotheses

The overall objective of my dissertation was to determine if the production of exocellular polysaccharides produced by bacteria is a mechanism of tolerance in bacteria. From this objective, I generated two hypotheses. The first hypothesis was EPS produced by bacteria sorb mercury. The second hypothesis was EPS prevents mercury from entering the cell causing damage thereby increasing the bacteria's mercury tolerance.

Specific Objectives

To test these hypotheses, I addressed three specific objectives:

1. To determine if EPS purified from pure bacterial cultures isolated from French Polynesian atolls are able to sorb mercury.

2. To determine if model strains of bacteria with differing capabilities to produce EPS during growth also differ in their mercury tolerance.

3. To determine if chemolithoautotrophic bacteria isolated from deep-sea hydrothermal vents contain the *merA* gene in their genome, are capable of

reducing mercury (II) to elemental mercury (0), and produce an EPS that differs in production in the presence of mercury.

Chapter 2 – Mercury sorption to purified extracellular polysaccharides from pure bacterial cultures isolated from French Polynesian atolls microbial mats

Introduction

Biosorption is the non-active metal uptake by biomass (14). Functional residues on biological surfaces can interact with dissolved metal ions and play a role in metal bioavailability and toxicity (3, 14, 48). Mercury has been shown to bind to polysaccharide capsules surrounding filamentous cyanobacteria (48) either bound to the cell wall or released into the medium (49).

Metal sorption to extracellular polysaccharides (EPS) is due to the attraction of positively charged metal ions to negatively charged functional groups present in the EPS (14). Carboxylic acid residues are the main functional group for binding of metals to EPS due to their lone-pair electrons available for interaction with metal ions. For neutral EPS, oxyanions and hydroxyl residues can interact weakly with metal ions but affinity decreases for metal ions with large ionic radii (14, 50).

In this Chapter, I describe the ability of purified EPS, products of bacteria isolated from microbial mats located in French Polynesian atolls, to sorb mercuric chloride. For the purpose of this dissertation, binding of a metal cation to EPS refers to strong ionic bonds between functional groups present in the EPS with metal cations. Sorption refers to weak ionic bonds and/or van der Waals forces between functional groups present in the EPS and metal cations.

Materials and Methods

Exocellular Polysaccharides:

Four previously purified EPS from pure bacterial cultures isolated from microbial mats in French Polynesian atolls were provided by Dr. Guézennec for mercury binding studies (47). Each EPS had different chemical compositions (See Table 2.1 for details) as reported by Dr. Guézennec.

EPS	Strains	Neutral Sugars	Uronic acids	Hexos-amines	Sulph-ates	Protein	Sub-stituents	Metals Binding	Hg Sorption
RA 19[1]	*Paracoccus* sp	48	8	-	29	3	Acetate	Cu^{2+}, Fe^{2+}	Yes
Mo 203[1]	*Altermonas* sp	46	20	-	-	4	-	Cu^{2+}, Fe^{2+}, Ag^+	Yes
Mo 245[1]	*Vibrio* sp	11	27	30	-	2	Acetate	n/d	Yes
GG[2]	Nd	100	-	-	-	-	-	n/d	Yes

1. Guézennec J., Moppert X., Raguénes G., Richert L., Costa B., and Simon-Colin C. (2011). Microbial mats in French Polynesia and their biotechnological applications. *Process Biochem*. 46: 16-22.
2. Personnel communication with Dr. Guézennec, July 2, 2013
Nd No information
n/d Ongoing investigations into binding capabilities with metals

Table 2.1 Chemical compositions of the bacterial EPS (% in w/w) that were used in this study.

Mercury binding experiments:

Mercury binding experiments were performed following the protocol described in Loaëc et al. (1997). EPS was dissolved in ultrapure water to a concentration of 1% weight per volume. Mercuric chloride concentrations ranged from 0.1 to 1,000 milligrams per liter EPS solution. Flasks containing dissolved EPS and mercury were shaken at 200 RPM for three hours at room temperature. After three hours 1 mL samples were collected and passed through a 3 kDa Millipore Ultracell low binding regenerated cellulose membrane (Amicon Ultra-0.5, EMD Millipore Billerica, MA) via centrifugation for twenty minutes at 14,000 x g (28). A pre-treatment of passing ultrapure water through the filters was

performed before the addition of sample. Controls included EPS solution with 0

mg/l mercury and mercury solution containing no EPS. Samples of the EPS-

mercury solution were taken before and after filtration. In order to assess the

nature of mercury sorption to EPS, there was an additional step of moving the

filter to a new microcentrifuge tube and passing washing solutions with various

pH's through the filter. Samples of the wash after filtration were also collected.

The pH's of the washing solutions were 4.0 (buffered by potassium acid

phthalate), 6.92 (ultra pure water, unbuffered), and 9.0 (buffered by 0.5 M N-

cyclohexyl-2-aminoethanesulfonic acid [CHES]).

    All samples were digested with approximately 0.2 N bromine

monochloride overnight at room temperature to prepare them for analysis. Filters

for preliminary experiments were digested overnight in 0.5 ml of 0.2 N bromine

chloride before analysis. Filters for all remaining experiments were frozen at -

20ºC for storage without digestion by bromine monochloride. Mercury

concentrations were determined by a Hydra AA Automated Mercury Analysis

cold vapor atomic adsorption spectrophotometer (CVAAS) as recommend by the

manufacturer (Leeman Labs, Inc., Hudson, NH). Experiments were performed in

triplicate.

Mercury binding analysis:

    The mercury uptake capacity (U, mg metal/ g EPS) for each EPS for the

equilibrium isotherms graph was determined using the equation (14, 51, 52):

$$U = ((Hg_i - Hg_f) \times V) \div m$$

where Hg_i was the initial mercury concentration and Hg_f was the concentration of mercury in the filtrate or free/ unbound mercury. V was the volume of the filtered solution and m was the mass of the EPS.   .

In addition, mercury binding was compared by percent mercury removed from solution. Statistical analysis was performed using a 2-way ANOVA with GraphPad Prism 5.04 for Windows software with a Bonferroni post hoc test. Overall alpha was set to 0.05.

Percent removal of mercury mass from EPS by washing (R) was determined by using the equation:

$$R = (Hg_w \div Hg_E) \times 100$$

where Hg_w is the mercury mass found in the washing filtrate and Hg_E is the mercury mass sorbed to EPS.

Results

Experimental Design

No mercury could be detected in the filtrate or filter when EPS solutions with 0 mg/l mercury were tested for all experiments, indicating the filtration system did not contain mercury (Figure 2.1). Only 8.0%, 3.8%, and 3.6% of the total mercury (initial mercury concentration of 25 mg/L [Figure 2.1a], 50 mg/L [Figure 2.1b], and 100 mg/L [Figure 2.1c] respectively) added to the system could be detected in association with filters when EPS was not added to solution indicating mercury did not readily sorb to the filter. Only when EPS was present was a majority, 64%, 50%, and 69%, of the total mercury mass (initial mercury

concentration of 25 mg/L, 50 mg/L, and 100 mg/L respectively) associated with the filter. This indicates EPS is associating with mercury and preventing it from passing through the filter. When initial stock solution, filters, and filtrate were measured mass balance was always achieved (ranging from 100% to 119% recovery of mercury) and therefore after these preliminary experiments filters were no longer analyzed but stored at -20ºC
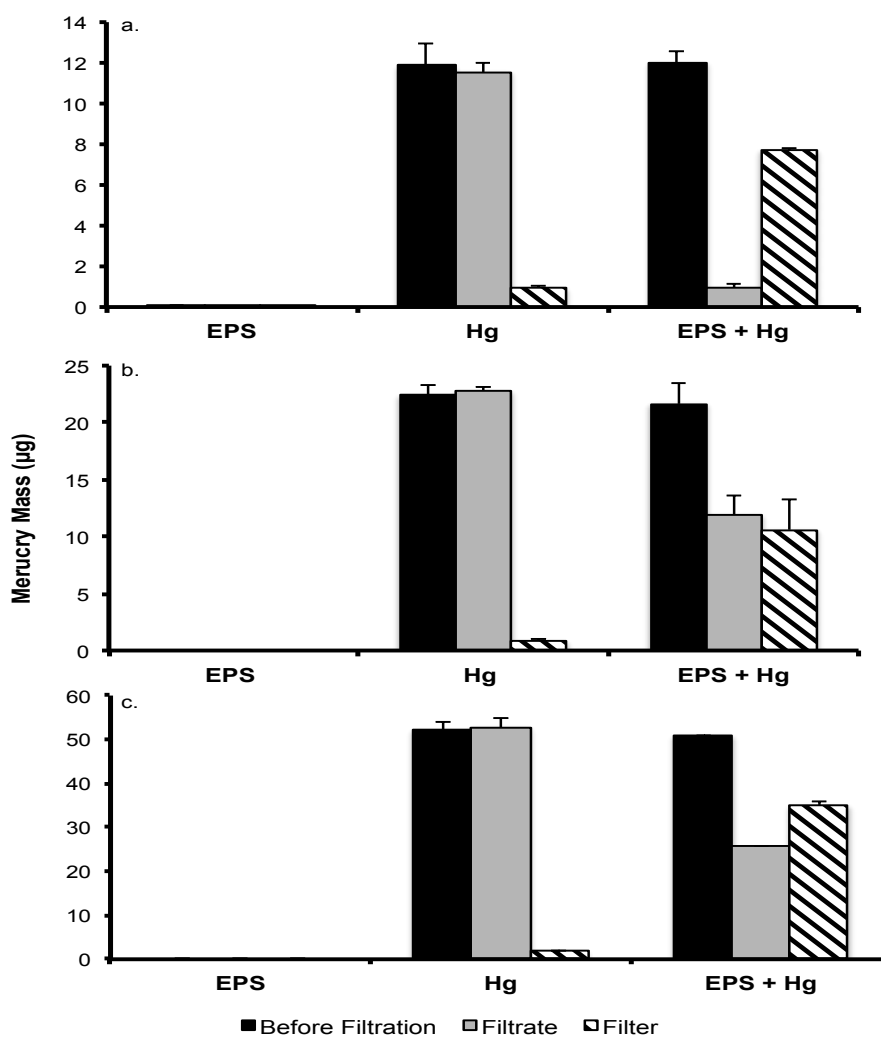
Figure 2.1 Testing of the experimental filtration system. Initial mercury concentration of a. 25 mg/L (total mercury mass filtered 12.5 µg) b. 50 mg/L (total mercury mass filtered 25 µg) c.100 mg/L (total mercury mass filtered 50 µg) with mercury concentration before filtering (solid black), after filtration (solid gray), and associated with filter (stripes) measured.

Removal of mercury from solution by EPS

To assess the efficiency of mercury sorption to each EPS, percent removal of mercury from solution was determined (28). All four EPS sorbed mercury to varying degrees indicated by lower concentrations in filtrates compared to starting solutions. For RA 19, Mo 203, and Mo 245, there was a decrease in percent mercury removed with increasing mercury concentrations, with a steep change in percent mercury removed at lower concentrations of mercury 10 mg/l to 150 mg/l. At higher mercury concentrations, the decline in mercury removal begins to taper off indicated by flattening of the curve (Figure 2.2).
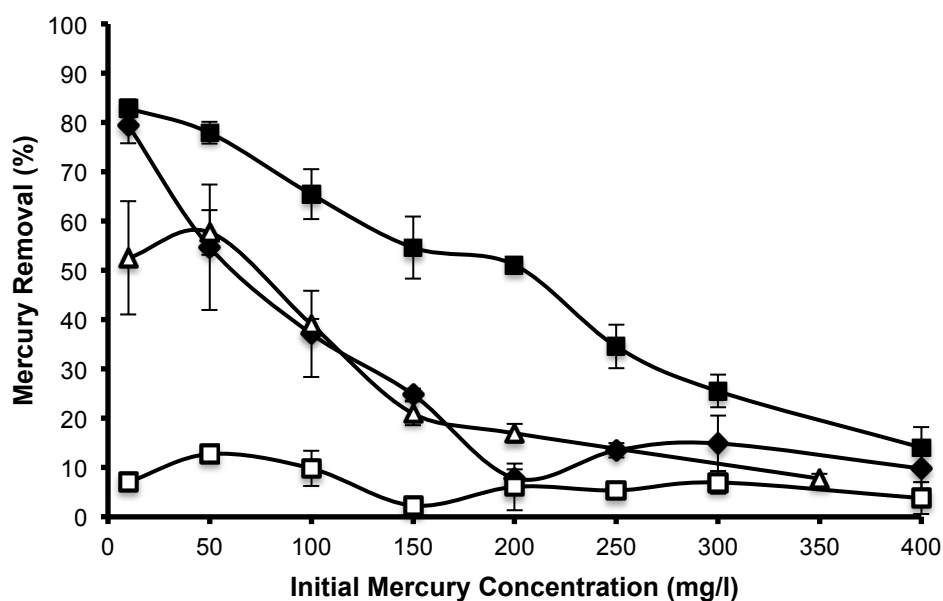
Figure 2.2 Percent mercury removed of added Hg by RA 19 (◆), Mo 245 (■), Mo 203(Δ), and GG ( ). Error bars depict standard deviation with certain error bars smaller than markers.

The capacity of different EPS to sorb mercury, expressed as percent sorbed mercury, (Figure 2.2) was statistically significant ($p \leq 0.0001$). With the

exception of GG, there was a statistically significant decrease in percent mercury removed with increasing mercury concentration ($p \leq 0.0001$). RA 19 and Mo 203 removed similar amounts of mercury. At the lowest concentration of mercury, 10 mg/L, RA 19 removed 79.4% of mercury compared to 52.5% for Mo 203; this was shown to be statistically significant ($p \leq 0.0001$). However at higher mercury concentrations, percent mercury removal were not statistically different ranging from 54.7% at 50 mg/l to 9.8% at 400 mg/l for RA 19 and 57.7% at 50 mg/L to 7.8% at 350 mg/l for Mo 203. Mo 245 had the highest percentage of mercury removal for each mercury concentration tested with statistically significant differences between 50 mg/L to 200 mg/L ($p \leq 0.0001$). Mercury removal ranged from 82.9% at 10 mg/L to 14.1% at 400 mg/L. In contrast to the other three EPS, percent mercury removed by GG did not decrease with increasing mercury concentration, but remained consistently between a minimum of 2.2% and a maximum of 12.2%. This was statistically different between 10 mg/L and 150 mg/L ($p \leq 0.0001$). GG is an entirely neutral EPS, removing the lowest percentage of mercury at each concentration tested. Mercury removal ranged from 7.1% at 10 mg added Hg/L to 3.8% at 400 mg added Hg/L. These findings indicate that all EPS are able to sorb mercury with Mo 245 the most efficient at binding mercury and GG the least efficient at binding mercury.

Equilibrium sorption isotherms

Equilibrium sorption isotherms show distribution of mercury in solution and bound to EPS versus mercury concentration at equilibrium and can indicate if

binding to EPS is saturable. As mercury concentration increases so will sorption

as long as binding sites aren't saturated. Once saturated, sorption will no longer

increase (28, 46). Equilibrium isotherms indicate for RA 19, Mo 203, and Mo 245,

sorption of mercury to the biopolymer was saturable depicted by a plateau of

metal uptake above a certain mercury concentration (Figure 2.3b). At this point,

all the binding sites are saturated and EPS is no longer able to sorb additional

mercury atoms. Metal uptake by Mo 245 was the highest compared to the other

three EPS (Figure 2.3b). Metal uptake values for Mo 245 ranged from 4.7 mg/g

at initial mercury concentration of 10 mg/l to 91.2 mg/g at initial mercury

concentration of 200 mg/l. RA 19 and Mo 203 had similar metal uptake curves

with values ranging from 4.8 mg/g to 39.6 mg/g and 4.1 mg/g to 39.9 mg/g,

respectively (Figure 2.3b). GG had the lowest metal uptake values ranging from

1.0 mg/g to 36.6 mg/g with the shallowest curve (Figure 2.3a). The mercury

concentrations for GG were lowered to assess if low metal uptake values were

due to saturation of EPS already occurring at the 10 mg/l (the lowest mercury

concentration tested. Mercury sorption was tested for GG (Figure 2.3b) at

mercury concentrations ranging from 0.1 mg/l to 8 mg/l. Mercury uptake values

ranged from 0.05 mg/g to 0.90 mg/g and were consist with the mercury uptake

values at higher concentrations for GG.
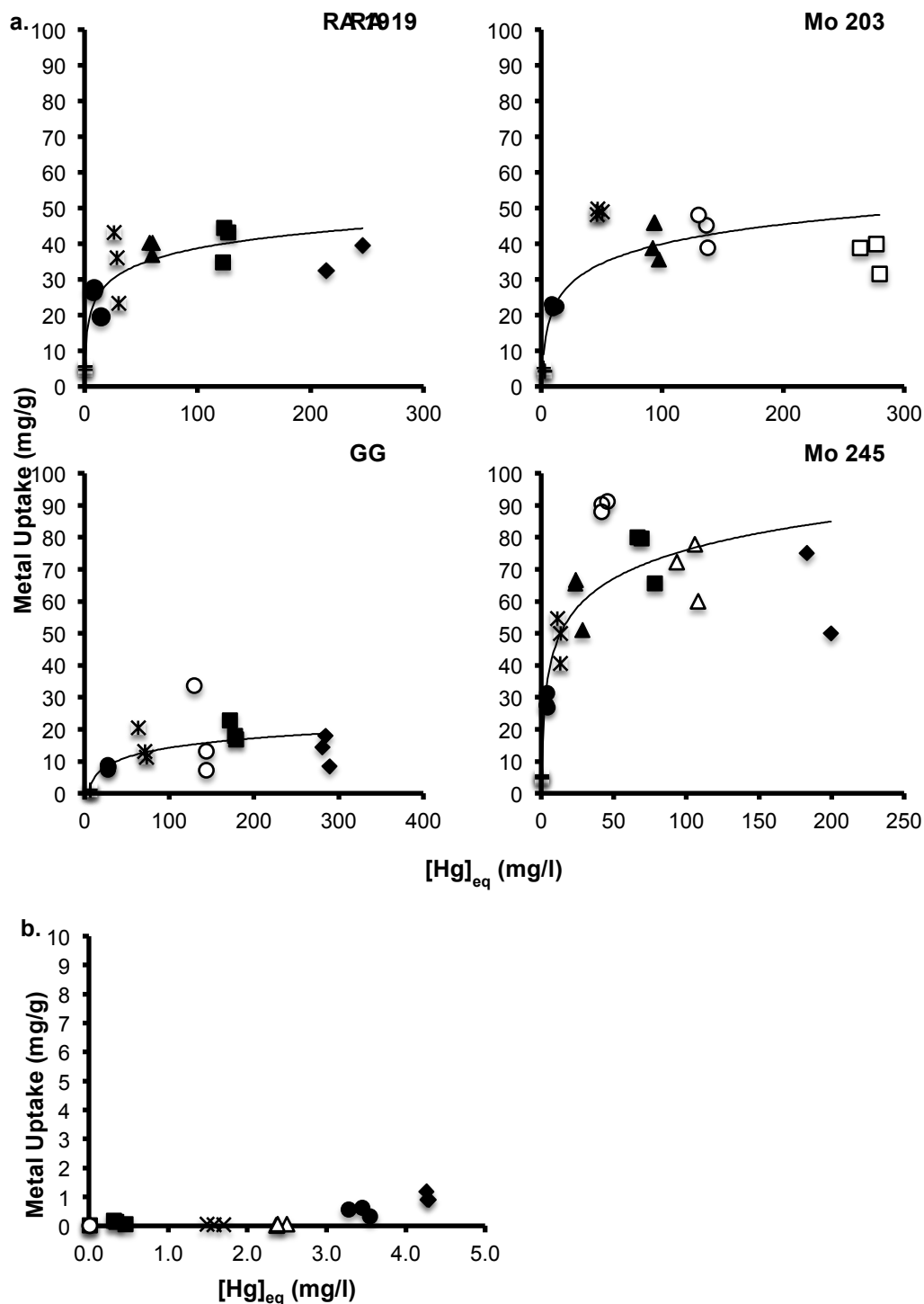
Figure 2.3 Equilibrium sorption isotherms for mercury by EPS from pure bacterial cultures isolated from microbial mats. $[Hg]_{eq}$ is measured mercury concentration at equilibrium prior to filtration. Metal uptake was determined using the equation: $U = ((Hg_i - Hg_f) \times V) \div m$. Triplicates for each mercury concentration studied are individually represented on graph with corresponding marker shapes. **a.** All four EPS with initial mercury concentrations ranging from 10 to 400 mg/l. **b.** GG only with initial mercury concentrations ranging from 0.5 to 8 mg/l.

<u>Nature of Mercury Sorption</u>

Mo 245 was selected to determine if mercury could be removed from the EPS by washing the filter-bound EPS-mercury complex with solutions of various pH's. Two mercury concentrations were selected, 50 mg/l and 200 mg/l, because they represented two unique points on the equilibrium isotherms curve. 50 mg/l was at the beginning of the curve and was well before saturation of the EPS, and 200 mg/l was further along the curve and was right before saturation of the EPS was reached. Concentrations above 200 mg/L were not selected for this experiment due to increased variability in replicates above this concentration.

For both mercury concentration tested, a similar pattern was observed (Figure 2.4). The EPS-mercury complex was initially washed with ultrapure water (pH=6.92) to determine if mercury was weakly associated with the EPS and could be readily removed. For the lower concentration of mercury (Figure 2.4a), 7.89 µg of mercury was associated with the EPS and 0.43 µg of mercury was removed by water (5.4% of total mercury bound). For the higher concentration of mercury (Figure 2.4b), 9.06 µg of mercury was associated with the EPS and 4.42 µg of mercury was removed by water (48.8% of total mercury bound to EPS). At the higher concentration of mercury tested, mercury was easier to remove by washing with water. This increase in weakly associated mercury with increasing mercury concentrations could be due reduction in availability of strong binding sites for mercury cations. Mercury could be in association with EPS via van der Waals forces. The EPS-mercury complex was then washed with an acidic solution (pH= 4). The mass of weakly associated mercury (removed by water)

was subtracted from the total mass of mercury removed by the acidic solution to determine how much mercury was removed by the acidic pH. At the lower concentration of mercury, 7.934 µg of mercury was bond to EPS and 1.75 µg of mercury was removed by the acidic solution (22.0% of total mercury bond). A similar amount of mercury was removed, 1.332 µg, by the acidic solution for the higher concentration of mercury tested; however mass of mercury bound to EPS was higher, 10.18 µg, so the percent mercury removed was 13.0%. For both mercury concentrations, a small percentage of mercury was removed by washing suggesting mercury is still tightly associated with functional groups present on the EPS. Finally, the EPS-Hg complex was washed with a basic solution (pH=9). The mass of weakly associated mercury (removed by water) was subtracted from the total mass of mercury removed by the basic solution to determine how much mercury was removed by the basic pH. The greatest amount of mercury was removed for both concentrations of mercury tested for the basic solution. At the lower concentration of mercury, 8.152 µg of mercury was associated with EPS and 4.512 µg was removed by washing (55.2% of total mercury bond). At the higher concentration of mercury, 8.52 µg of mercury was associated with EPS and 3.582 µg was removed by washing (42.0% of total mercury bond). The results suggest that at a high pH protonation of functional groups present in the EPS changes resulting in the disassociation of mercury. The functional groups being altered are responsible for mercury sorption. Mo 245 contains hexosamines, which have pK_a values of 7.87 and 8.49. At a pH of 9 the protonation these functional groups would be altered. resulting in the release of

mercury from the EPS, therefore hexosamine are likely involved in the sorption of
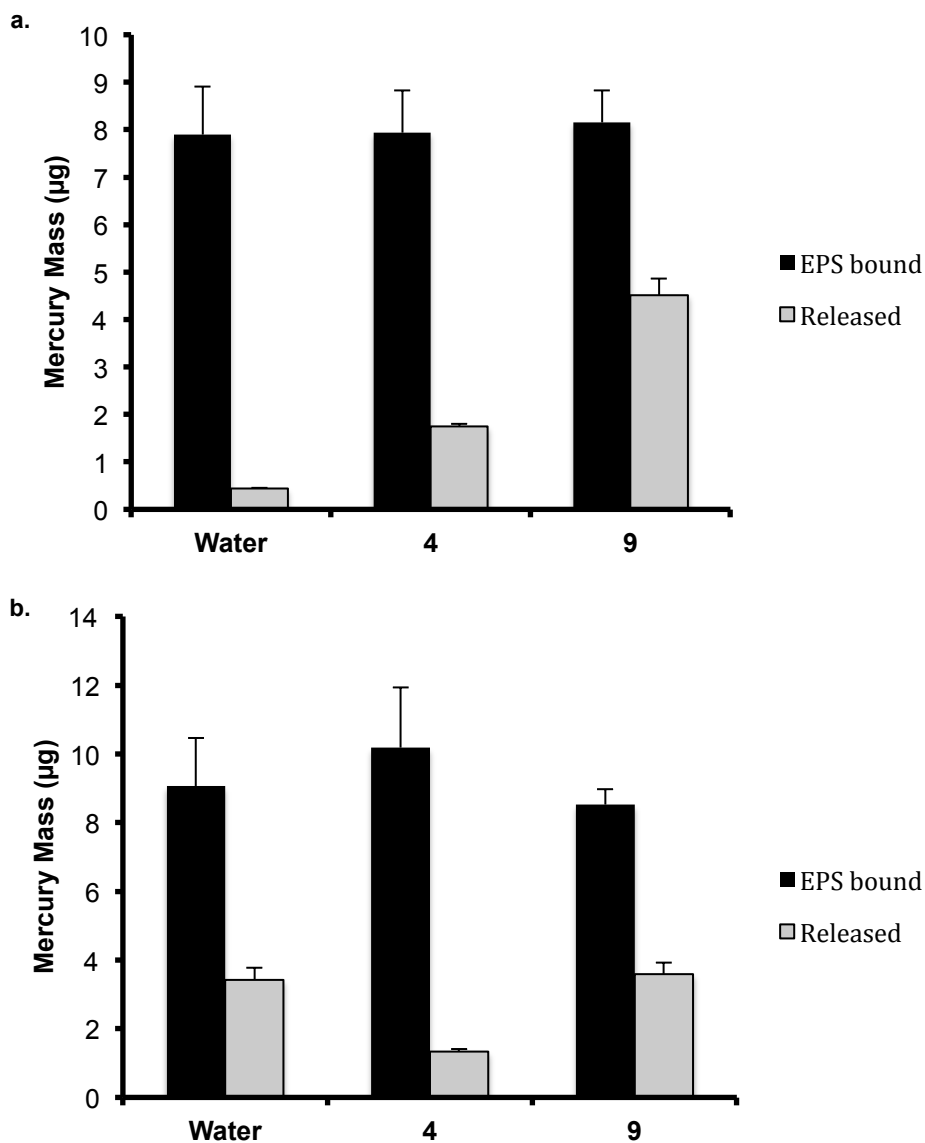
mercury.

Figure 2.4 EPS bound mercury (black bars) released from EPS (gray bars) by washing with solutions of various pH values. Initial mercury concentrations used for experiments 50 mg/L (a) and 200 mg/L (b). Mercury released from water was subtracted from mercury mass released by pH 4 and pH 9 solutions.

Discussion

In this Chatper, I report mercury sorption to purified isolated EPS. All EPS tested were able to bind mercury to varying degrees. As the sorption isotherm graphs depict, all four EPS reached saturation; however, the metal uptake capacity varied greatly between the EPS (Figure 2.3). The uptake of metal species sorbed to the EPS will increase with increasing metal concentration until saturation where it is believed there are no more binding sites for the metal to interact with (14). Another important feature of sorption isotherms is the shape of the curve. High affinity for mercury is indicated by a steep curve from the origin, where mercury concentrations are low (14). Mo 245 had the highest metal uptake capacity with values reaching almost 90 mg/g (0.45 mmol/g). The sorption isotherm is the steepest for Mo 245 suggesting it has the highest affinity to mercury. RA 19 and Mo 203 had very similar metal uptake capacities with values at saturation, averaging around 41 mg/g (0.20 mmol/g) and 44 mg/g (0.22 mmol/g), respectively. GG had the lowest metal uptake capacity with almost all values below 20 mg/g (0.17 mmol/g) with a very shallow sorption isotherm at the origin suggesting a low affinity for mercury. Moppert et al. (2009) followed the same protocol as reported here for RA 19 for cupric copper and ferrous iron. They calculated metal uptake capacities of 9.84 mmol/g and 6.9 mmol/g, respectively, much higher than the metal uptake capacity for mercury (46).

These differences in metal sorption may be due to differences in properties of the metals. Mercury is considered a soft acid, meaning it is a large atom with low positive charge containing unshared paired electrons. Both cupric

copper and ferrous iron are considered borderline metals containing properties of

both hard and soft acids resulting in differences in ligand affinity between

borderline acids and soft acids (53). It has been found that other metals, such as

cupric copper and ferrous iron, are more competitive than mercury for binding to

various biopoylmers suggesting a higher affinity to the EPS. However,

polyaminated chitosan (deacetylated polymer of chitin) was highly selective for

mercury and was more competitive than other metals for binding sites (48, 54-

57). This is due to the higher specificity of mercury for amino groups (57). Since

the structures of the Polynesian EPS are unknown it makes it difficult to compare

it to a pure polysaccharide preparation, however it could elucidate some

knowledge of how mercury is interacting with functional groups present. RA 19

contains no hexosamines, but only neutral sugars and uronic acids (Table 2.1)

and would therefore not be expected to have specificity for mercury (14, 50). This

might account for some of the differences seen between RA 19 metal uptake

capacities of these metals. The sorption of silver and copper to Mo 245 is

currently under investigation at Brest University (personal communication Dr.

Guézennec, March 12, 2014). It will be interesting to see how silver and copper

compare to mercury since it is the only EPS in my study to contain hexosamines

(Table 2.1), which would preferentially sorb mercury (57, 58).

Differences in uptake capacities were reflected in percent mercury

removed from solution (Figure 2.2). Differences among the EPS in uptake

capacities and percent mercury removed from solution are expected because

each EPS has a unique composition containing variable percentages of key functional groups involved in heavy metal sorption (46, 47).

It is not surprising that Mo 203 and RA 19 have similar percent mercury removal (Figure 2.2) since they both contain large amounts of neutral sugars (predominately rhamnose, fucose, galactose, and glucose (47)), 46% and 48% respectively, and no hexosamines (Table 2.1). Mo 203 and RA 19 differ in their uronic acid composition, 20% to 8%, and sulphate composition, 0% to 29% respectively. Since Mo 203 and RA 19 have similar efficiency for removing mercury from solution, despite having very different sulphate compositions (Table 2.1), it suggests that sulphate does not play a major role in sorption of mercury. If sulphate did sorb mercury strongly, I would expect RA 19 to be more efficient at binding mercury than Mo 203. Similarly, uronic acids do not seem to play a major role in mercury sorption. Like sulphate, Mo 203 and RA 19 have very different uronic acid composition (Table 2.1). If uronic acid sorbed mercury strongly then Mo 203 should be more efficient at binding mercury than RA 19.

Mo 245 had the highest percentage of mercury removed at every concentration tested and the highest metal uptake capacity; therefore it was the most efficient at removing mercury from solution. It is the only EPS tested to contain hexosamines (N-acetyl glucosamine and N-acetyl galactosamine, (47)), 30%, and the highest content of uronic acids, 27% (Table 2.1). Since removal of mercury was similar for RA 19 and Mo 203, it appears that sulfates and uronic acids play similar roles in mercury sorption. Therefore it is most likely the

presence of hexosamines that lead to the increase in mercury removal by Mo 245 (47).

It is known that functional groups containing amino groups have an affinity for mercury ions (58), and the addition of amino groups to chitosan beads increased mercury sorption by the beads (56). Although acetylation of amino groups does reduce binding of mercury, there is still a specificity of mercury for these groups (58).

Mo 245 was further assessed for how readily sorbed mercury could be removed by washing. Two mercury concentrations were selected due to their location on the equilibrium isotherm curve (Figure 2.3). One, 50 mg/l, is located at the beginning of the curve, where the mercury uptake capacity is increasing with increasing mercury concentrations. It is believed at this point not all binding sites are associated with mercury atoms, so some sites are still available to bind mercury. Mercury is mostly likely associated to the sites that have the highest affinity for mercury. The second mercury concentration, 200 mg/l, is located right before the curve has plateaued. Here it is believed that the majority of binding sites are no longer available for mercury sorption. There could be an increase in weak association of mercury to the EPS since the mercury concentration is high. If mercury is only weakly associated with the EPS and not truly sorbed, the mercury should be easily removed by washing with water. At 50 mg/l, only 5.4% percent of the mercury was removed from the EPS confirming that mercury is sorbing to the EPS (Figure 2.4). At the higher mercury concentration, 200 mg/l, 48.8% of the mercury was removed from the EPS. This increase in percent

mercury removed from EPS is most likely due to the fact that at this mercury concentration the availability of binding sites have decreased. In addition to mercury interacting with strong binding sites, it might also be weakly associated with other functional groups or trapped at the surface due to the high concentration of mercury added. This mercury would be readily removed by washing since it is not forming strong interactions with the EPS.

The washing solution pH was adjusted to be either acidic (pH=4) or basic (pH=9) to gain insight into interactions occurring between Mo 245 and mercury. Each functional group has a different acid dissociation constant, pK_a, the pH value where half of the hydrogen atoms are dissociated from the molecule (59). Selecting a pH value above or below this value will alter the functional groups ionization state and determine whether or not mercury will be able to associate with it. If a higher percentage of mercury were released by a basic or acid solution, it would suggest alterations in the ionization of particular functional groups are the cause of the release. When the EPS was washed with a basic solution, a high percentage of mercury was removed, 42% and 55.2% respectively, for both the high and low concentration of mercury tested.

The equilibrium sorption isotherms suggested that hexosamines are the main functional group involved in mercury sorption. Hexosamines have pK_a value are between 7.87 and 8.49, therefore at a pH of 9 the ionization state of the functional groups will have shifted (60). This shift in ionization would alter the ability of mercury to associate with the functional group causing it to be released into the filtrate. Therefore having a high percentage of mercury removed during

washing supports the hypothesis that hexosamines are a major component in mercury sorption. The release of mercury from EPS when washing in an acidic solution could correlate to carboxyl groups present. Carboxyl groups are traditionally thought of as the main functional group responsible for metal cation sorption (14). The pH of Carboxyl groups' pK_a is 4.76, so a pH of 4 would alter the ionization state of the functional group and the ability of mercury to sorb (60). A smaller percentage of mercury was released by the acidic solution (22% and 13%) compared to the basic solution suggesting that carboxyl groups play a smaller role in mercury sorption. In summary, Mo 245 was the only EPS to contain hexosamines, which may account for its enhanced ability to bind mercury compared to the other EPS.

GG was expected to bind the least amount of mercury since it contains only neutral sugars (Table 2.1). Without anionic functional groups, the metal ion will interact weakly with oxygen molecules in hydroxyl residues acting as weak donors of electrons, which would explain why GG has such a low binding capacity and the lowest percentage mercury removed (Figure 2.1) (14, 46, 50).

The mercury concentrations studied here were substantially higher than those from environments with naturally sources of mercury emissions, i.e. deep-sea hydrothermal vents have mercury concentrations ranging between 13.9 – 445 pmol/L (19, 61). At the lowest concentration of mercury tested, 10 mg/L (approximately 50 µmol/L), Mo 245 and RA 19 were able to remove 80% of the mercury from solution and Mo 203 about 50%. Therefore, in the environment,

where the mercury concentrations are much lower, the majority of mercury could be sorbed to the bacterial-produced EPS mitigating mercury toxicity.

In this Chapter, I have shown that EPS isolated from pure bacterial cultures from microbial mats in the Polynesian French Atolls are capable of sorbing mercury. This is the first report of mercury sorption to purified EPS. The binding studies revealed a complex binding stoichiometry that is not 1:1 for metal ions to binding sites. Non-cooperative binding, conformational changes, and non-specific binding may all play a role in the complex binding stoichiometry. Infrared spectroscopy and scanning electron microscopy have been used in the past to elucidate functional group- mercury interactions and to observe conformational changes (46). These tools could be used in the future to gain further understanding of mercury- EPS binding.

In this study, for one of the purified EPS, RA 19, metal uptake capacity values were 35 to 50 times less for mercury compared to the previously reported by Moppert el al. (2009) metal uptake capacity values for copper or iron; however at the lowest concentration of mercury tested, mercury was almost completely sorbed by two of the EPS. Since known concentrations of mercury in the environment are significantly lower than even the lowest mercury concentrated tested, it is plausible that in the environment, EPS may sorb mercury surrounding the cell mitigating mercury toxicity. In Chapter 3, differences in mercury tolerance in bacteria during growth of model organisms were studied.

Caveats in Experimental Design

The EPS used in this study were only partially characterized with percent content of functional groups and no structure. This inhibited modeling the sorption of mercury to EPS. Without the structure of the EPS, it was difficult to infer a lot of information from the data. It could be that the flattening of curve observed in the equilibrium isotherms graph (Figure 2.3) is not due to saturation of EPS with mercury but rather a high concentration of mercury at the surface preventing further penetration of mercury into the EPS. Another experiment could be performed where the EPS concentration is lowered. This could allow for a looser confirmation increasing the EPS' surface area alleviating this situation.

The experiment examining the nature of mercury sorption to EPS using buffered pH solutions (Figure 2.4), it was assumed that the chemicals used to buffer the solution would not interact with mercury. This assumption was based on previous experiments performed in the laboratory. Based on this assumption, the release of mercury from EPS was solely due to changes in protonation of functional groups altering interactions with mercury. This might not be the case; some of the mercury released when washed could be due to interactions between the buffer and mercury. To determine if this is the case, the experiment could be repeated using the same pH solution but buffered by different chemicals. The same amount of mercury should be released by the EPS if the buffer is not interacting with mercury.

Chapter 3 – A relationship between extracellular polysaccharides production and mercury tolerance in an *E. coli* model system

Introduction

In Chapter 2, I demonstrated that purified exocellular polysaccharides (EPS) capable of sorbing mercury. EPS are known to increase adsorption binding capacity of metal ions to a cell by interactions between negatively charged acidic functional groups in EPS with positively charged metal ions (62, 63). The amount of EPS produced by an organism can be altered by the medium composition, incubation conditions, and the ability to attach to a solid surface (64, 65). Environmental stresses, including starvation, dehydration, and heavy metals can stimulate EPS production as a protective barrier (39, 40). It has been hypothesized that hyper production of EPS may be induced by the production of free radicals from heavy metal ions inside of the cell resulting in oxidative stress activating gene transcription of EPS and possibly other detoxification mechanisms (40).

I selected *E. coli* K-12 strains to study differences in growth, EPS production, and mercury tolerance between strains to gain insight into the role of EPS in mercury tolerance of growing bacteria.

Materials and Methods

<u>Bacterial strains and growth conditions</u>

Model strain selection was based on availability of a mutant strain with an altered ability to produce EPS (produces less and takes longer for production to commence) and ease of cultivation. *Escherichia coli* strain ZK2686 (wild-type) and *E. coli* strain ZK2687 (mutant) were provided by Dr. Kolter (Danese, Pratt, & Kolter, 2000).

The mutant has an insertion of a mini-Tn*10cam* transposon in the 107th codon of the *wcaF* gene of strain ZK2686 resulting in a disruption of the open reading frame (38). The *wcaF* gene belongs to a cluster of genes responsible for the production of colanic acid. It encodes for a protein that is closely related to the NodL protein, which belongs to a large family of acetyltransferases and *O*-acetylates the *Rhizobium* Nod factors. Therefore, it is believed that the WcaF protein is involved in the *O*-acetylation of colanic acid. There is a second putative *O*- acetylate gene present in the operon (66). This results in a defective, rather than a null mutant for colonic acid production. Thus, it is able to produce a biofilm (although deformed), a consequence of EPS production, after approximately 17 hours of growth in liquid culture. Biofilm formation is less compared to the wild-type at 17 hours but continues to form and is comparable in amount to the wild-type at approximately 48 hours (38).

Frozen stocks were created in 20% glycerol and stored at -80ºC. The mutant *E. coli* strain was grown in media supplemented with chloramphenicol at a final concentration of 25 µg/ml unless otherwise indicated. Media used to grow

the *E. coli* strains were Luria- Bertani agar (LB) plates composed of (L^{-1}): tryptone (10g), NaCl (10g), yeast extract (5g), and agar (20g) and a modified M9 media (MM9) composed of (L^{-1}): 200 mL of 5x Salts (NaCl (2.5g), NH_4Cl (5g), and K_2HPO_4 (11.5g)), 10 mg/mL thiamine (34mL), 20% casamino acids (20mL), 1M $MgSO_4 \cdot 7 H_2O$ (2mL), 1M $CaCl_2 \cdot 2 H_2O$ (0.1mL), 0.335 g/mL 3-morpholinopropane-1-sulfonic acid (25mL), 500 mM L-arginine (2mL), and 100% glycerol (4mL). All reagents were added to sterilized ultra pure water post autoclaving. Both wild-type and mutant strains were incubated at 37ºC for all experiments. When grown in liquid media the cultures were shaken at 200 RPM (length of incubation is discussed below).

A standard nucleotide BLAST search of the National Center for Biotechnology Information (NCBI) nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) was performed on the genome of *E. coli K-12* to show that it does not contain a *mer* system, which would be a confounding factor. No *merA* gene was located.

Disc inhibition test for mercury tolerance in *E. coli* K-12 strains

Disc inhibition tests were performed using a modified protocol described by Barkay et al. (1990). *E. coli* strains were grown from frozen stocks on LB agar plates. Plates were incubated at 37ºC overnight. The day two a colony was transferred to MM9. The cultures were shaken overnight. The day three the cultures were diluted 1:20 into fresh MM9 media and incubated for 4 hours at

37ºC with shaking at 200 RPM. After 4 hours, cultures were centrifuged for 10 minutes at 10,000 x g and supernatant discarded. The pellets were re-suspended in cold 0.85% NaCl solution and 1 mL spread- plated on MM9 plates (2% agar). After inoculation, the plates were dried for 10 minutes in a laminar flow hood before a sterile filter disc was placed on the center of each plate. Filter discs were impregnated with 10 µL of mercury stock with final amount mercury ranging from 0 to 1,000 nmoles (performed in triplicate). Plates were incubated for 24 hours following which zones of inhibition were measured. Zones of inhibition were measured by placing plates under a dissecting microscope and measuring the distance from the center of the filter disc to the edge of growth. Each zone of inhibition was measured four times. The experiment was performed in triplicate. A 2-way ANOVA was used for statistical analysis using GraphPad Prism 5.04 for Windows software with an overall alpha set to 0.05.

EPS production by *E. coli* K-12 strains when grown on sugar free minimal solid medium plates

E. coli strains were grown from frozen stock in liquid MM9 media overnight. The next morning a 1:100 dilution into fresh liquid MM9 was performed and cultures incubated for 4 hours. After 4 hours, the cultures were centrifuged for 10 minutes at 10,000 x g. The supernatants were discarded and pellets suspended in 0.85% sterile saline solution. MM9 plates were dried for 10 minutes in a laminar flow hood before 0.1 ml of culture were spread onto the surface of each. The plates were then incubated for 16 hours before biomass was

harvested from the plates' surfaces. The biomasses were harvested with 0.85% sterile saline solution, then centrifuged for 10 minutes at 10,000 x g. The supernatant was collected and frozen at -20ºC until EPS production analysis could be performed. The pellets were washed with sterile saline solution two times. The first washes were collected and frozen at -20ºC until the phenol-sulfuric acid assay for sugar determination could be performed (assay described in detail below). The pellets were frozen at -20ºC until protein content could be determined. A student's t-test was performed for statistical analysis with an alpha set to 0.05 and n = 3 for all samples.

EPS production was determined using a modified phenol sulfuric acid assay for sugar determination (67). After the addition of 80% phenol (0.025 ml/ ml sample or 2.5% v/v) and concentrated sulfuric acid (2.5 ml/ ml sample), the samples were cooled for 10 minutes at room temperature before optical density was measured with a spectrophotometer at 490 nanometers. The water bath step of shaking for 10 - 20 minutes at 25ºC - 30ºC as described by Dubois et al. (1956) was omitted from the procedure since it did not affect optical dentistry values. D-glucose was used to generate a calibration curve and treated in the same manner as the samples. The calibration curve ranged from 0 to 100 μg/mL of D-glucose. EPS production was normalized to protein content of the pellet.

Protein content of the pellet was analyzed using the BioRad microassay for protein determination following the protocol outlined by the manufacturer. Optical density values were read at 595 nanometers on a Sunrise remote by Tecan plate reader (Männedorf, Switzerland). The pellets were prepared for the

microassay by re-suspending in buffer composed of (mL^{-1}): 1 M Na$_2$HPO$_4$ (16.2 μL), 1 M NaH$_2$PO$_4$ (3.8 μL), 0.5 mM EDTA (10 μL), and 0.1% β – mercaptoethanol (1 μL). The samples were boiled for 20 minutes and allowed to completely cool before the BioRad microsassay was performed. Bovine serum albumin (BSA) was used to create the calibration curve (final concentrations ranging from 0 to 80 μg/mL) and prepared for spectrophotometry in the identical manner as the samples.

EPS production by *E. coli* K-12 strains when grown in sugar free minimal liquid medium in the presence of varying mercury concentrations

Comparison of EPS production in the presence of varying concentrations of Hg for the *E. coli* strains was performed by growing the wild-type and mutant strain from frozen stock on LA plates incubating overnight at 37ºC. A colony was selected, transferred to liquid MM9, and incubated overnight at 37ºC with shaking at 200 RPM. The next morning a 1:100 dilution into fresh liquid MM9 was made and shaken at 200 RPM and 37ºC until the optical density (OD) of the cultures at 600 nanometers (A$_{600}$) was 0.1. Once an OD value of 0.1 was obtained, a 1:100 dilution into fresh MM9 media with Hg concentrations ranging form 0 to 2 μM HgCl$_2$ (performed in triplicate) was performed and shaken for 16 hours at 200 RPM, 37ºC. After 16 hours, the cultures were centrifuged at 10,000 x g for 10 minutes. The supernatants were collected and frozen at -20ºC until EPS analysis could be performed. EPS production was determined using the phenol-sulfuric acid assay for sugar determination (described above), The pellets were washed twice with sterile 0.85% NaCl solution before freezing (-20ºC) for protein

determination using the BioRad protein assay (described above). All experiments were performed in triplicate. A 2 way ANOVA was performed for statistical analysis using GraphPad Prism 5.04 for Windows software with an alpha set to 0.05.

Results

Disc inhibition assay for Hg tolerance in *E. coli* K-12 strains

In order to compare the mercury tolerance of the wild-type and mutant *E. coli* strains, the disc inhibition test was performed. For this test, varying concentrations of mercury were impregnated onto a sterile filter paper disc placed on a solid medium plate inoculated with a lawn of bacteria. The mercury diffuses into the medium from the filter paper with the mercury concentration highest close to the filter paper disc and decreasing as the distance from the disc increases. The bacteria are only able to grow to the region where the concentration of mercury is tolerable (Figure 3.1). Therefore, a larger zone of inhibition indicates that the bacterial strain is more sensitive to mercury (68).

Zones of inhibition around mercury-impregnated discs increased in a dose- dependent manner for both the wild-type and mutant strain. There was no inhibition of growth for either strain when sterile ultra pure water was placed on the disc as indicated by zones of inhibition equal to zero millimeters (mm) (data not shown). The mercury mass ranged from 100 to 1,000 nanomoles and with the exception of the increase from 100 to 200 nanomoles, mercury increased by intervals of 200 nanomoles between treatments. The wild-type had zones of

inhibition ranging from 1.4 mm to 6.1 mm. The zones of inhibition increased

approximately 0.96 mm with each increasing mercury mass. For the mutant type,

the zones of inhibition ranged from 2.5 mm to 7.15 mm with more variability in

the increase in zone with increasing mercury mass (ranging from 0.6 mm to 1.3

mm). Increase in size of zone of inhibition with increasing mercury amount was

statistically significant at a p- value ≤ 0.05. At each mass of mercury tested, the

mutant strain had a zone of inhibition approximately 1 mm larger (standard

deviation of 0.1) than the wild-type for the corresponding mercury mass. This

difference was statistically significant at p- value ≤ 0.05 (Figure 3.1).

a.
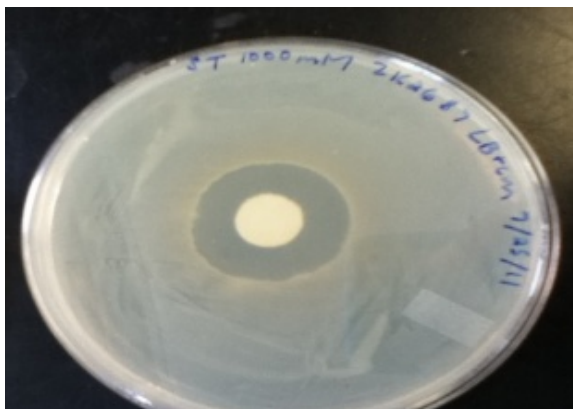
b.
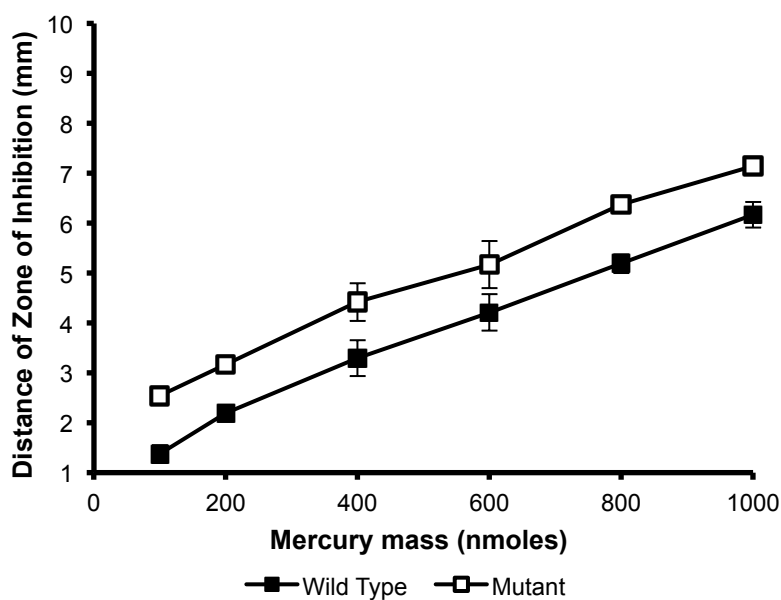
Figure 3.1 Mercury disc inhibition test. **a.** Example plate of mutant with filter disc impregnated with 1000 nmoles of mercury showing zone of inhibition. **b.** Graph depicting zones of inhibition for wild-type (filled) and mutant (open). 2- way ANOVA used for statistical analysis. All points statistically significant at p-value ≤ 0.05.

The disc inhibition test showed that mercury inhibits growth in a dose-dependent manner. The wild-type was more tolerant to mercury than the mutant strain at each mercury mass tested. Since the only difference between the two strains is the efficiency of EPS, i.e., colanic acid, production, it suggests that EPS increases mercury tolerance in *E. coli* K-12 strains growing on solid media.

Difference in EPS production between *E. coli* K-12 strains growing on solid media

EPS production by the bacterial strains was normalized to protein content of the pellet. The use of protein content to normalize bacterial polysaccharide production is widely used method (65, 69). The wild-type produced more than twice as much EPS per mg of protein than the mutant strain with 6.4 µg EPS per mg protein compared to 2.7 µg EPS per mg protein, respectively (Figure 3.2). These values were statisticaly significant with a $p \leq 0.05$. There appeared to be no EPS loosely bound to cells as indicated by the lack of EPS detected in the wash (data not shown). Thus, the higher Hg tolerance of the wild-type (Figure 3.1) might be related to the lower production of EPS by the mutant strains.
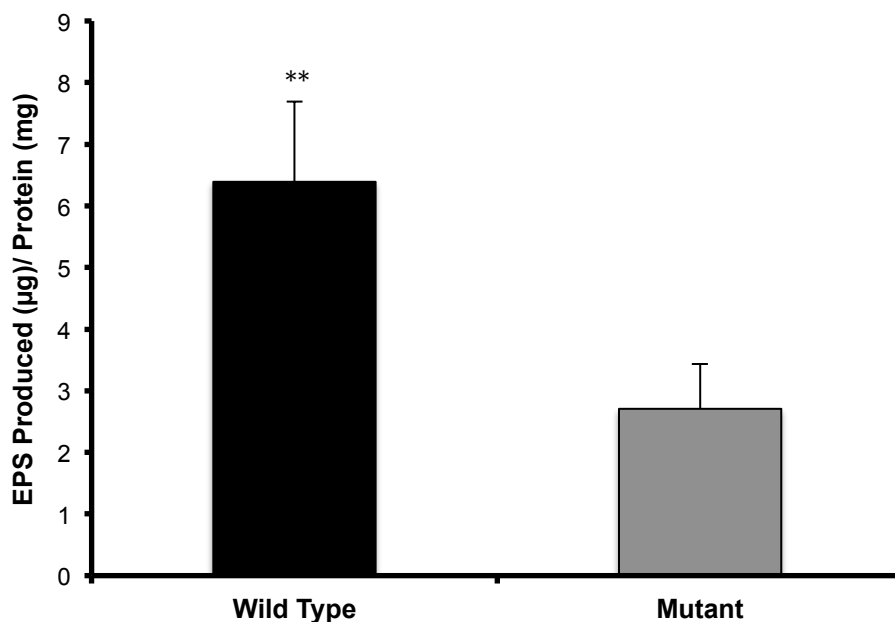
Figure 3.2 Comparison of EPS produced (µg) normalized to protein content of cell pellets (mg) between wild-type (black bar) and mutant (gray bar) *E. coli* K-12 strains. Double asterisk indicates p- value ≤ 0.05.

Effect of mercury on EPS production in *E. coli* K-12 strains

An advantage to studying EPS production of bacterial cultures in liquid media is the ability to normalize EPS production easily with protein content of the bacterial pellet. However, a caveat of growing cultures in liquid media is the variability of mercury tolerance depending on the experimental design, i.e. growth in glass test tubes verses growth in microtiter plates. In order to determine the mercury concentration that is high enough to cause toxicity but low enough to allow for growth, EPS production was determined with varying concentrations of mercury. Another objective of the study was to determine if EPS production was induced in the presence of mercury. It is known that EPS production can be induced in the presence of heavy metals (39, 40).

There was no increase in EPS production in the presence of mercury for either the wild-type or mutant strain (Figure 3.3). The wild-type produced on average 11.1 µg of EPS per mg of protein (standard deviation of 1) at each mercury concentration tested. The mutant strain produced on average 9.8 µg of EPS per mg of protein (standard deviation of 0.9) at each concentration of mercury tested. The wild-type produced on average 1.3 µg more EPS per mg of protein than the mutant strain, but this difference was not found to be statistically significant.
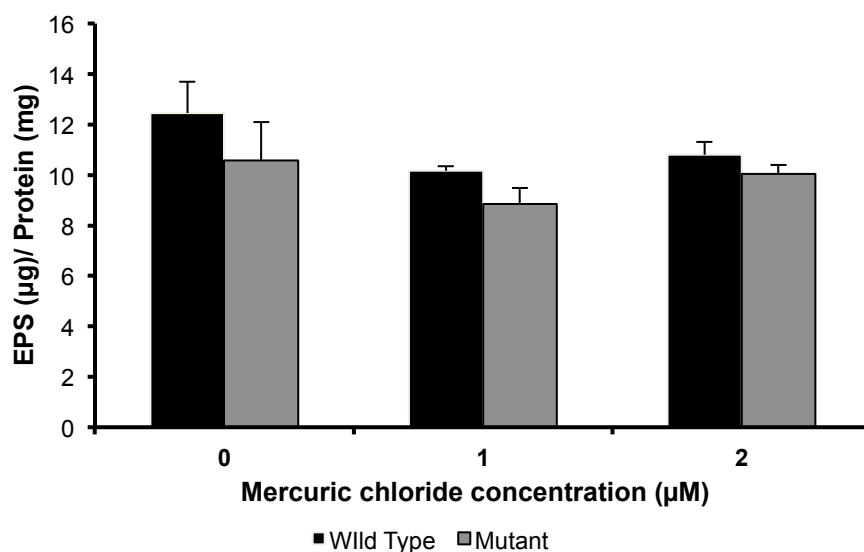
Figure 3.3 Comparison of EPS production (µg per mg protein) in the presence of varying Hg concentrations during growth in liquid media for wild-type (black bars) and mutant (gray bars). EPS production was determined using phenol- sulfuric acid assay for sugar determination and protein content determined using BioRad assay for protein determination.

There was no statistically significant difference in EPS production in the presence of mercury as compared to its absence. EPS production was determined after the cells had already reached stationary phase (approximately 16 hours). Although Danese at al. (2000) showed observable differences in

biofilm formation at 17 hours; it might be that differences in EPS production at 17

hours might be not as great explaining why no difference could be observed

between the wild-type and mutant strains. To determine if this is truly the case,

this experiment should be repeated with time points selected to reflect different

levels of EPS production during the course of the growth cycle.

Another aspect of EPS production examined during the course of the

study was the binding of EPS to the cell surface compared to its secretion into

the spent medium. When EPS is secreted from the cell it can either stay attached

to the surface of the cell or be released to the medium and found in the spent

medium. The composition and properties of the EPS can vary between the

bound and free forms (36). In order to determine the presence of the bound EPS,

cells were grown to stationary phase and then the pellet was washed with sterile

saline solution to remove EPS bound to the cells' surface (44). EPS measured in

the spent medium was compared to EPS present in the wash. The effect of

growth with mercury on the partition of EPS between the bound and excreted

forms was also assessed.

[Hg] (µM)	Wild-Type EPS (µg/OD unit)				Mutant Strain EPS (µg/OD unit)			
	Free		*Bound*		*Free*		*Bound*	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
0	41.1	0.29	23.3	2.1	45.0	1.6	21.5	3.7
0.5	49.0	1.4	36.3	2.5	22.5	0.50	51.1	3.1
1	40.1	1.3	0.0	0.0	21.2	2.9	23.4	4.1
2	39.3	2.8	2.2	2.3	45.5	0.44	7.3	2.9
3	n/d	-	47.4	0.57	n/d	-	n/d	-
4	63.9	4.8	n/d	-	n/d	-	n/d	-

Table 3.1 Comparison of free EPS vs. bound EPS between the wild-type and mutant strains. n/d indicates no sugar could be detected and SD is an abbreviation for standard deviation.

For the wild-type at 0, 0.5, 1, and 2 µM mercury, most of the EPS (64%, 57%, 100%, and 95% respectively) was free and not bound to the surface. Growth was substantially inhibited at 3 µM (only bound EPS was detected) and almost completely inhibited at 4 µM (only free EPS could be detected). Detection of EPS in the wash at 4 µM (indicated as free EPS in Table 3.1) could be due to experimental error. When the wash was collected some bacterial pellet might have also been collected. If this were the case, cellular sugars in addition to EPS sugars would have been measured resulting in false positive for sugars. Growth of cultures were determined by comparing OD at 595 nanometers values of the no mercury control (OD = 0.94) versus cultures grown in the presence of 3 µM mercury (OD = 0.4) and 4 µM mercury (OD = 0.1). For the mutant strain, no EPS could be detected in mercury concentrations of 3 or 4 µM, even though cultures grew to OD of 0.618 and 0.614 respectively compared to 0.854 for the no mercury control. For the remaining concentrations of mercury, 0 and 2 µM mercury had more EPS free compared to bound (68% and 86% respectively) and 0.5 and 1 µM had less EPS free compared to bound (31% and 48% respectively). There was variability in total EPS production in the presence and absence of mercury for both strains. Total EPS production ranged from 40 µg to 95 µg per OD unit for the wild-type and 45 to 75 µg per OD unity for the mutant strain (Table 3.1).

A statistically significant difference in mercury tolerance and EPS production between the wild-type and mutant strains of *E. coli* K-12 was only observed when cultures were grown on solid media. The difference in EPS

production between the strains was small, but this correlates with the small difference in mercury tolerance between the strains.

Discussion

The model organism for the role of EPS production on tolerance to mercury was *E. coli* K-12 and its colanic acid defective mutant. The wild-type strain is positive for production of the EPS colanic acid producing mucoid colonies (Danese, Pratt, & Kolter, 2000). Colanic acid is produced across a wide array of *E. coli* strains and is a high molecular weight sugar polymer. It is thought to play an important role in survival of *E. coli* outside of its animal host and prevent desiccation (66). Assesment of EPS production between the two strains revealed reduction of EPS production by the mutant strain was observed only when the strains were grown on solid medium plates (Figure 3.2).

Mercury inhibited growth in a dose-dependent manner for both the wild-type and mutant *E. coli* strains indicated by increasing zones of inhibition with increasing mercury concentrations. The wild-type strain was more tolerant to mercury than the mutant at each mass of mercury tested (Figure 3.1). This small difference in mercury tolerance might be due to the fact that colanic acid contains uronic acids and neutral sugars with no hexosamines (70). In Chapter 2, neutral sugars were shown to sorb mercury poorly and uronic acids, although capable of sorbing mercury, are not as efficent as hexosamines. Therefore, the small difference in mercury tolerance might be due to poor sorption of mercury to the EPS allowing some mercury to enter cell causing toxicity. Bacterial strains

known to produce an EPS containing hexosamines may have a greater difference in mercury tolerance. *E. coli* K-12 strains have been engineered to contain multicopy plasmid clones of of the *pga*ABCD locus. I was unaware of the availability of these strains when the experiments were performed. These genes are involved in the production of a hexosamine rich EPS composed of almost entirely β-1,6-*N*-acetyl-*D*-glucosamine. The EPS was originally found in *Staphylcoccus epidermidis* and *S. aureus* but through horizontal gene transfer are now found in *E. coli*. The engineered strain with multicopy plasmid clones of the *pga*ABCD locus produces hexosamine rich EPS. In addition, a mutant strain defective in the production of the hexosamine rich EPS has also been created. Both strains no longer produce colanic acid due to knockout mutations (71). An interesting study would be to examine the difference in mercury tolerance between these two strains. If hexosamine rich EPS are more efficient in removing mercury from solution, I would expect the strain able to produce EPS to be more tolerant to mercury than the mutant defective in its production. I would also expect there to be a greater difference in tolerance between the two strains than the differences I observed in my studies.

Although the difference in tolerance is small it was statistically significant, and this difference may be important in certain environments where competition is high. It is known that adding heavy metals to a community will alter the community structure leading to an increase in metal- tolerant strains (72, 73). Areas like deep-sea hydrothermal vents contain low concentrations of mercury (13.9 to 445 pmol/L) (19) compared to the concentrations used in my

experiments. Therefore, a low level of protection by the EPS could enhance the competitiveness of producing strains.

It is well documented that deep-sea hydrothermal vent bacteria produce EPS. Purified EPS from these bacteria are capable of binding heavy metals, including cadmium, copper, and zinc, when dissolved in solution. This was demonstrated by dissolving purified EPS in water with varying concentrations of metal ions. The solutions were allowed to equilibrate then passed through a filter with a small enough molecular weight cut off to prevent EPS from passing through. The difference in metal concentration before and after filtration was measured. Decrease in metal concentration after filtration was considered metal bound to EPS. However the ability of EPS to bind mercury was not explored in these studies (28, 29). Very little is known of mercury binding to EPS. A recent study has shown that environmental bacteria from soils, sediments, and mine effluents collected from environments containing elevated concentrations of mercury produced an EPS capable of binding Hg (44). To examine this possibility, I explored mercury tolerance and EPS production of chemolithoautotrophic bacteria isolated from deep-sea hydrothermal vents in Chapter 4.

Caveats in Experimental Designs

For the disc inhibition assay, the mercury concentrations at the edge of zones of inhibition were not measured. It could be that mercury concentrations at the edge of zones of inhibition were the same regardless of initial mercury mass impregnated on the disc. If the same exact point were compared for a plate with

a low mercury mass disc and a plate with a high mercury mass disc, the mercury concentration at that point would be different because for the high mercury mass disc more mercury would be diffusing into the media. Points closer to the disc would have a higher concentration of mercury than their counterpart on the lower mercury mass disc. To get the same mercury concentration on the high mercury mass disc plate as found on the low mercury mass disc plate, a point further away from the disc would need to be selected. This could explain the parallel lines I observed for the disc inhibition test. The *E.coli* strains are inhibited at a certain mercury concentration, they stopped growing at this concentration on plate. The location of this concentration was different for each disc explaining the parallel lines.

For the experiment determining the free versus bound concentrations of EPS in the presence and absence of mercury, it could be that not all of the bound EPS was removed by washing the pellet with saline. It is known that for a hexosamine rich EPS produced by certain strains of *E.coli* a more vigorous method of EPS removal is required (71). However since the strains of *E.coli* used in these studies only produce colanic acid, which is loosely associated with the cell wall, this should not have been the case (74).

For most the experiments, EPS production was measured after 16 hours of growth. This is around the same time frame that Danese et al. (2000) determined the mutant would start producing visible biofilm (17 hours). Since EPS is needed for biofilm formation, EPS production is also occurring at this time. To truly examine the difference in mercury tolerance and EPS production

for these strains when growing in liquid culture an earlier time point should have been selected.

Differences in mercury tolerance of genetically engineered *E.coli* strains producing a hexosamine rich EPS was not studied. This was due to the unavailability of strains during the course of experiment. Time constraints prevented the generation of these strains myself. This data could have strongly supported my findings in Chapter 2 in regards to sorption of mercury to hexosamine functional groups. This experiment should be performed in the future.

Chapter 4 - Mercury tolerance in chemolithoautotrophic deep-sea hydrothermal vent bacteria isolated from East Pacific Rise 9 ºN

Introduction

Mercury is one of the most toxic metals, has no known biological function, and is a human and wildlife health hazard (1, 2, 5). Organisms use various adaptive strategies to survive mercury exposure. Eukaryotes, including plants and animals produce phytochelators or metallothioneins, heavy metal complexing compounds which sequester mercury, and prevents it from interacting with cellular components (75). Some microorganisms, however, are highly tolerant to mercury via the mercury resistance (*mer)* operon, a detoxification mechanism whereby an intracellular mercuric reductase (MerA) catalyzes the reduction of inorganic mercury to the highly volatile elemental mercury. Elemental mercury readily diffuses out of the cell allowing for cellular growth (20, 75).

Deep-sea hydrothermal vent emissions can contain high concentrations of mercury resulting in naturally elevated levels of mercury at these sites. Mercury concentrations at vents range between 13.9 and 445 pmol/L (19, 76) with upper levels comparable to contaminated surface waters (30, 77). These concentrations are 2 to 3 orders of magnitude higher than bottom seawater (1 to 2 pmol/L) (19), and suggest that the diverse organisms living at these sites must

have mechanisms enabling their survival in the presence of toxic concentrations of mercury.

Heterotrophic bacteria from the East Pacific Rise (EPR) at 9 °N were previously isolated on artificial seawater media under aerobic conditions. They were shown to be adapted to mercury, reduce ionic to elemental mercury, and carry *merA* genes (33).

Chemolithoautotrophic microorganisms form the base of the food web in hydrothermal vent ecosystems, providing fixed carbon and energy to the abundant and diverse vent communities (21-23). Adaptation to mercury among deep-sea chemolithoautotrophs is therefore critical to the sustenance of life in these ecosystems. Indeed, most probable number estimates showed that up to 2.4×10^7 cell/mL of chemolithoautotrophic thiosulfate-oxidizing bacteria from mercury enriched vent fluids grew in the presence of 10 µM mercury, suggesting that these microbes were highly resistant to mercury (19). *mer* operons and mercury reduction have been documented among chemolithoautotrophs, mostly acidophiles belonging to the *Proteobacteria* that are common in acid mine sites (78, 79). Deep-sea hydrothermal vent bacteria produce exocellular polysaccharides (EPS). This EPS is capable of binding heavy metals, including cadmium, copper, and zinc (28, 29, 41). Very little is known of mercury binding to EPS but a recent study has shown that bacteria isolated from the metal rich soils of the Vík í Mydral black sand beach of Southern Iceland, the sediments of the Petit Saut reservoir in French Guiana, and the mining effluents of the Tinto and Odiel Rivers in Spain produced EPS capable of binding mercury (44). My

research has shown that EPS sorb mercury (Chapter 2) and that EPS production increases the tolerance of *E. coli* to mercury (Chapter 3).

The mechanism of mercury- tolerance among deep-sea vent chemolithoautotrophs has never been investigated. Here I report the isolation of pure cultures of mercury tolerant obligate and facultative chemolithoautotrophs that lacked *merA* gene homologs and did not reduce mercury during autotrophic growth with 10 µM mercury. All three facultative chemolithoautotrophis produce EPS in the presence and absence of 0.2 µM mercury with 2 isolates producing more EPS in the presence of mercury.

Materials and methods

Isolation of pure cultures and culture conditions

Chemolithoautotrophic bacteria were isolated from hydrothermal fluid samples from the East Pacific Rise (EPR 9° 50'N, 104° 17'W) during oceanic expeditions during April 2004, May 2005, and January 2007 on board R/V Atlantis. Isolations were carried out in defined autotrophic 142 medium prepared as described by Crespo- Medina et al. (2009), containing thiosulfate as an energy source and carbon dioxide as a carbon source. Isolates were obtained from either serial dilution of a sample or by direct enrichment. Cultures were transferred to a plate then a single colony was selected and transferred to a new plate. This was performed three times after which the culture was considered pure. To achieve high growth yields, the strains were grown in medium 142 supplemented with 10 mM acetate (142 + A). The isolates were tested for

mercury tolerance by growth in medium 142 that was supplemented with 10 µM

HgCl$_2$. Genomic DNA was extracted and 16S rRNA genes PCR amplified and

sequenced as previously described (19). Isolate identification, isolation details,

and growth characteristics are summarized in Table 4.1.

Two strains were selected for further characterization of mercury tolerance

based on ease of cultivation and ability to grow to high biomass resulting in

turbidity that could be measured with a spectrophotometer. The two strains were

Thiomicrospira sp. EPR85 (strain EPR85) and *Halothiobacillus sp.* EPR155

(strain EPR155)*.* For all experiments, cultures were grown statically in Medium

142 + A at 28°C.

Isolate Number	Most closely related to (16S rRNA sequence similarity)	Class/Order	Sites/Sample	In situ temperature (°C)
EPR74	*Thioclava pacifica* TL2T (97%)	*Alphaproteobacteria/ Rhodobacterales*	East Wall 04/04 E Vent	11
EPR75	*Thiomicrospira crunogena* XCL-2 (98%)	*Gammaproteobacteria/ Thiotrichales*	Tica Vent	2.5
EPR85	*Thiomicrospira thermophila* 178[T] (99%)	*Gammaproteobacteria/ Thiotrichales*	East Wall 04/04 E Vent	50-70
EPR96	*Thiomicrospira thermophila* 178[T] (99%)	*Gammaproteobacteria/ Thiotrichales*	Mk 89	8
EPR115	*Hydrogenvibrio marinus* MH-110 (99%)	*Gammaproteobacteria/ Thiotrichales*	Tam Town	12
EPR125	*Pelagibaca bermudensis* HTCC2601[T] (99%)	*Alphaproteobacteria/ Rhodobacterales*	Tam Town	12
EPR129	*Halothiobacillus hydrothermalis* R3 (99%)	*Gammaproteobacteria/ Chromatiales*	Crab Spa MkF	28
EPR155	*Halothiobacillus hydrothermalis* R3 (99%)	*Gammaproteobacteria/ Chromatiales*	Mk 19	NA

[1]All strains grew in presence of 10 µM HgCl$_2$
[2]NA – not available

Table 4.1 Mercury tolerant chemolithoautotrophic bacteria isolated from EPR 9°N[1]

Phylogenetic analysis

A phylogenetic tree was generated from 16S rRNA gene sequences of the isolated chemolithoautotrophic bacteria and of marine bacteria known to carry the *mer*A gene (33) using ClustalX (version 2.0, (80)for alignment and SeaView (version 4.2.8, (81) for tree generation. The Jukes-Cantor model was used for genetic distance and the neighbor-joining method for tree building. Bootstrap values greater than 50, obtained by 500 replications, were indicated at branching points (Figure 4.1).

Mercury speciation modeling

Modeling of mercury speciation in growth media was performed using the chemical equilibrium model, MINEQL+ (version 4.5).(82). The National Institute of Standards and Technology database and MINEQL+ were used to obtain input parameters (19). A 10 µM mercuric chloride concentration was used to model mercury speciation. This was selected since it was the concentration used for isolation of the chemolithoautotrophic strains.

Determination of mercury tolerance levels

Tolerance to mercury was quantified by generating growth curves in medium 142 and medium 142 + A in the presence or absence of 10 µM mercury. Growth was determined by measuring the optical density of the cultures at 660 nanometers (A_{660}). Cultures were initiated from frozen stocks and grown for 48 hrs before being transferred to fresh medium containing 5 µM mercuric chloride.

These cultures were grown for 48 hrs and then diluted 20 fold into fresh medium either in the presence or absence of 10 μM mercury and A_{660} was measured periodically until cultures reached stationary phase. The experiments were performed in triplicate.

Detection of *merA* in mercury tolerant chemolithoautotrophic vent bacteria

The presence of the *merA* gene, encoding for the mercuric reductase enzyme, was determined by PCR using primer sets that were specifically designed for the detection of this gene among *Gammaproteobacteria* (primer set A1s.F/ A5-nRv (33) and primer set 2 from Wang et al. (83) and *Alphaproteobacteria* (primers Al-fw/Al-Rv (84)). PCR conditions are presented in Table 4.2.

Primer set	PCR conditions
A1s.F/A5-nRv[1]	35 cycles: 30 sec at 94ºC, 30 sec at 54ºC, 30sec
Primer set 2[2]	30 cycles: 1min at 95ºC, 30 sec at 61ºC, 90 sec at 72ºC
Al-fw/Al-Rv[3]	30 cycles: 30sec at 95ºC, 30 sec at 63ºC, 1min at 72ºC

[1]Schaefer JK, Yagy J, Reinfelder JR, Cardona T, Ellickson K, Tel-Or S, Barkay T. 2004. The role of the bacterial organomercury lyase (MerB) in controlling methylmercury accumulation in mercury contaminated natural waters. *Environ. Sci. Technol.* 38:4304-4311.
[2]Wang Y, Boyd E, Crane S, Lu- Irving P, Krabbenhoft D, King S, Dighton J, Geesey G, Barkay T. 2011. Environmental conditions constrain the distribution and diversity of archaeal *merA* in Yellowstone National Park, Wyoming. *Microb. Ecol.* 62:739- 752.
[3]Oregaard G, Sørersen S. 2007. High diversity of bacterial mercuric reductase genes from surface and sub-suface floodplain soil (Oakridge, USA). *ISME J.* 1:453- 467.

Table 4.2 *merA* primer sets and amplification conditions used in PCR reactions in this study.

Plasmid pHG103, containing cloned *merA* from *Serratia marcescens* plasmid

pDU1358 (22), was used as a positive control for both gammaproteobacterial

primer sets. Since mercury resistance has not yet been described among the

Alphaproteobacteria, the specificity of the alphaproteobacterial primer set was

demonstrated using 10 resistant soil isolates that were shown by 16S rRNA gene

sequencing to belong to this order (AD Chatziefthimiou, PhD dissertation,

Rutgers University, 2012). Ten percent of each PCR reaction was loaded on a

1% agarose gel stained with ethidium bromide to assess if bands of the expected

sizes (a 309 and a 1247 base pair fragment for the gammaproteobacterial primer

sets A1s.F/A5-nRV (33) and 2 (83), respectively, and 780 base pairs fragment for

primer set Al-fw/Al-Rv (84)) were produced. *In-silico* PCR amplification of *merA*

genes from the genomes of related bacteria species obtained from the Integrated

Microbial Genomes and Metagenomes database (https://img.jgi.doe.gov) were

performed using Thermo Scientfic online multiple primer analyzer

(http://www.thermoscientificbio.com/webtools/multipleprimer/).

Reduction and volatilization of mercury

Cultures were grown from frozen stocks in medium 142 + A at 28°C until

turbidity was apparent. The cultures were then diluted 20 fold into fresh medium

and grown for 48 hrs before a further 20 fold dilution in fresh medium containing

10 µM mercuric chloride for an additional 48 hrs of incubation. A 20 fold dilution

of these cultures were used to inoculate 10 ml of fresh medium in acid- washed

50 ml sealed serum bottles (denoted as reactors) to which mercury was added to a final concentration of 5 µM. All incubations were carried out at 28°C.

A heterotrophic vent isolate, *Acinetobacter* sp. EPR148 (strain EPR148), was used as a positive control for mercury reduction. This strain volatilizes mercury as determined by the x-ray assay (data not shown) and carries the *mer*A gene in its genome (33). EPR148 was grown in medium 142 + A.

Heat killed controls were obtained by placing cultures in an 80°C water bath for one hour before the addition of mercury. Uninoculated media containing mercury were also included as controls. Time zero samples, inoculated media that was spiked with 5 µM mercury followed immediately by the addition of 50 µl of concentrated trace metal grade hydrochloric acid to stabilize the mercury, were sealed and stored at 4°C until analysis.

The reactors' headspace was purged with sterile air for 10 minutes every 8 hours to ensure a sufficient supply of oxygen for growth. During each purge the reactors were connected to vials with mercury trapping solution consisting of 0.5 ml of concentrated H_2SO_4, 0.5 ml of concentrated HNO_3, 1.5 ml of 5% w/v $KMnO_4$, and 0.8 ml of 5% w/v $K_2S_2O_8$. After 48 hours a final purge of 45 minutes with sterile air of both the headspace and liquid was performed. The same trapping solutions were used with each reactor throughout the experiment thus collecting all volatile mercury that was formed during the incubation. At the end of the experiment, 50 µl of concentrated trace metal grade hydrochloric acid was added to both the reactor and trapping vials and samples were stored at 4 ºC until analysis.

Mercury analysis

Total mercury concentrations in the reactors and trapping vials were
determined using EPA protocol 245.1 (85). The samples were then analyzed
with a Hydra AA Automated Mercury Analysis cold vapor atomic absorption
spectrophotometer (CVAAS) as recommended by the manufacturer (Leeman
Labs, Inc., Hudson, NH). Statistical analysis of mercury concentrations was
performed using one-way ANOVA on GraphPad Prism 5.04 for Windows
software with alpha set to 0.05.

EPS production by facultative heterotrophic deep-sea hydrothermal vent bacteria
during growth in presence of varying mercury concentrations

Three facultative chemolithoautotropic marine bacteria (see Table 4.3)
that could grow heterotrophically were chosen for EPS analysis: *Thiomicrospira
sp.* strain EPR74 (EPR74), *Pelgibaca sp.* strain EPR125 (EPR125), and
Halothiobacillus sp. strain EPR155 (EPR155). Marine isolates were grown from
frozen stock on medium 142 - A plates with 10 µM $HgCl_2$ (the concentration used
for isolation) and incubated at 30ºC until visible growth was observed. A colony
was selected and inoculated into a heterotrophic, modified minimal artificial
seawater (MM-ASW) medium composed of (L^{-1}): NaCl (23.6g), KCl (0.64g),
$MgCl_2$ • 6 H_2O (4.53g), $MgSO_4$ • 7 H_2O (5.94g), NH_4Cl (0.65g), $CaCl_2$ • 2 H_2O
(1.3mg), Na_2HPO_4 • 7 H_2O (43 mg), $NaHCO_3$ (46mg) and adjusted to pH of 6.5.
After autoclaving, the medium was supplemented with 20 mL of 10% casamino

acids, 10 mL of 1 M Na acetate, 1 mL of vitamin solution, 1 mL of trace element solution SL-10, and 1 mL of 1 µmol L^{-1} vitamin B-12 solution.

The cultures were shaken overnight at 30ºC. One ml of inoculum was used to start fresh cultures of 35 mL of MM-ASW and 35 mL of MM-ASW containing 0.2 µM HgCl$_2$ (a concentration that allowed for reliable growth and sufficient biomass to perform assays) and were shaken at 30ºC overnight (performed in triplicate). The next day, cultures were centrifuged for 10 minutes at 10,000 x g. The supernatant was collected and frozen at -20ºC until EPS analysis could be performed. The pellet was washed twice with sterile 0.85% NaCl solution before freezing (-20ºC) for protein determination. EPS production was determined using the phenol- sulfuric acid assay and protein content determined with the BioRad assay for protein determination following the procedure outlined in Chapter 3. Student's t-test using VasarStats: Website for statistical computation (vasarstats.net) was used for statistical analysis with alpha set to 0.05.

Results

<u>Mercury speciation in growth media</u>

MineQL+ modeling of the speciation of mercury in medium 142 with and without 10 mM acetate showed that in both media all of the mercury was only bound in negatively charged complexes of either mercury di- (60%) or trithiosulfate (40%), and acetate had no effect on mercury speciation in solution.

<u>Selection and identification of mercury tolerant chemolithoautotrophic bacteria</u>

<u>from EPR 9º N</u>

Eight chemolithoautotrophic bacterial isolates were enriched from water samples that were collected on the EPR at 9 ºN where total mercury concentrations ranged from 13.9 to 445 pmol/l and 0.2 to 24.6% of the total MPN counts were tolerant to 10 µM mercury (19). 16S rRNA gene sequences were used to classify the chemolithoautotrophic deep-sea vent bacteria and to place them within a phylogenetic framework in a neighbor-joining tree (Figure 4.1).
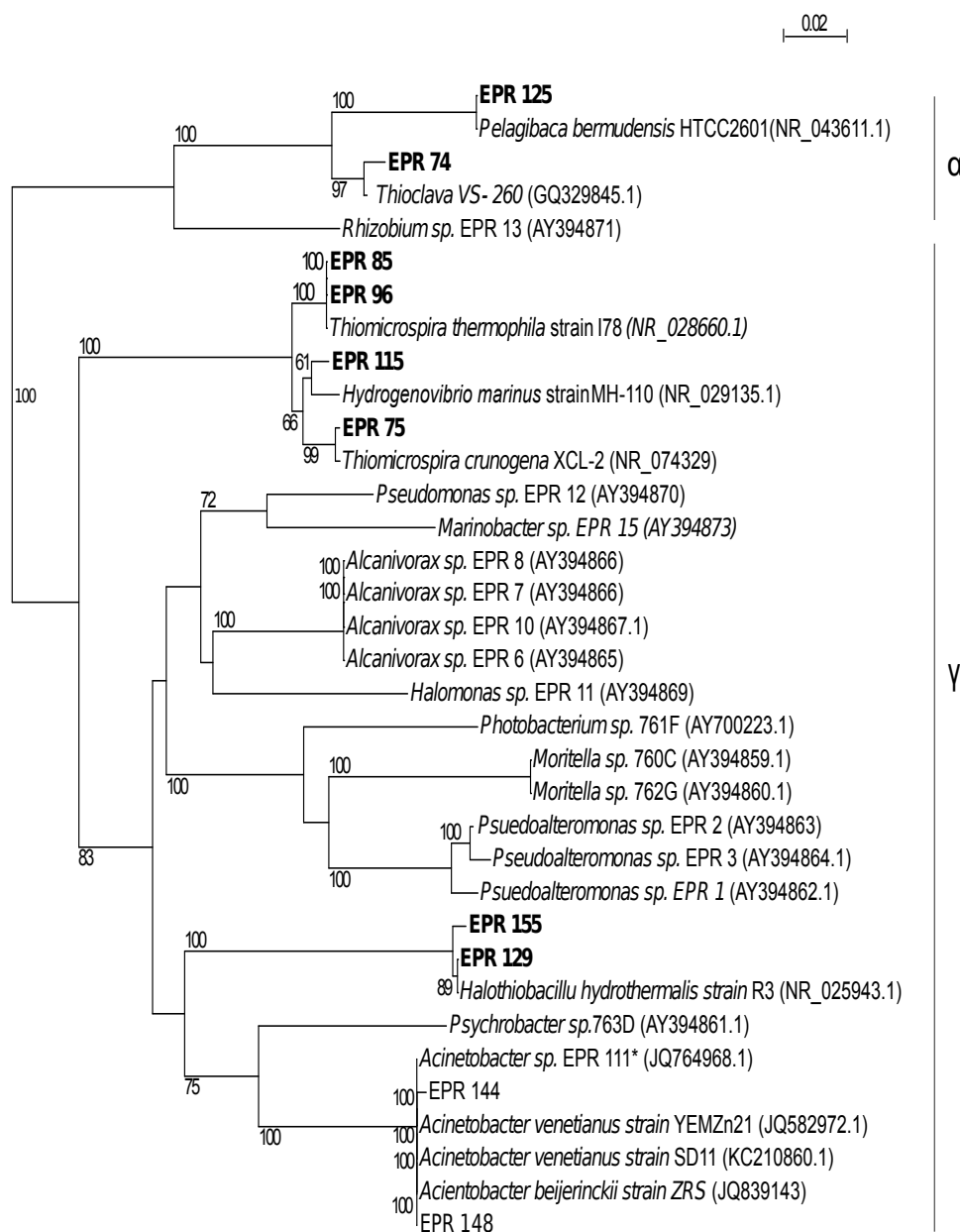
Figure 4.1 A neighbor joining phylogeny of deep-sea hydrothermal vent isolates and of marine bacteria known to contain the *mer*A gene. 16S rRNA gene sequences were used for tree construction. Organisms' designation in bold identify chemolithoautotrophic isolates that do not contain the *mer*A gene but are tolerant to mercury. Bootstrap values resulting from 500 iteration of the tree are provided at branching points. The bar indicates distance corresponding to 2% nucleotide substitution rate. Asterisk next to EPR111 indicates that this organism does not contain *mer*A and is sensitive to mercury.

All eight strains belong to the phylum *Proteobacteria* with the majority (six) most similar to *Gammaproteobacteria* and two isolates belonging to the *Alphaproteobacteria* order *Rhodobacterales* in which strains possessing *mer*A homologs occur. Most strikingly, *Pelagibaca bermundensis* HTCC2601, the most similar organism to *Pelagibaca* sp. EPR125 possesd a *mer*A homolog (86); *mer*A has not been reported among *Thioclava* spp. nor are complete genome sequences of this genus available in public databases.

The six *Gammaproteobacterial* chemolithoautotrophs belong to the order *Thiotrichales* (*Thiomicrospira* spp. EPR75, EPR85, EPR96 and *Hydrogenovibrio* sp. EPR115) and *Chromatiales* (*Halothiobacillus* spp. EPR129 and EPR155). Indeed, these cluster in two separate clades with reference strains belonging to the same orders (Figure 4.1). BlastP searches of complete genome sequences (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenesBlast&page=geneSearchBlast) showed *merA* homologs among representatives of both orders including in *Halothiobacillus neapolitanus* c2 (*Chromatiales*) and *Thiomicrospira arctica* DSM 13458 (*Thiotriachales*). These findings suggest that marine bacteria related to the new mercury tolerant deep-sea vent chemolithoautotrophs have the genetic potential to detoxify mercury via *mer*-operon mediated mercury reduction.

Effect of mercury on growth

Comparison of deep-sea chemolithoautotroph growth in the presence and absence of mercury was assessed by a commonly used approach (68). Three

parameters of growth may express tolerance; growth rate, growth yield, and the length of the lag period. These were delineated in medium 142 in the presence or absence of 10 µM mercury for *Thiomicrospira* sp. EPR85 and *Halothiobacillus* sp. EPR155, representing the two orders of tolerant *Gammaproteobacteria* in my collection (Figure 4.2). Under all conditions tested, there was an increase in lag phase in the presence of mercury for both strains. When either strain was grown chemoheterotrophically, the length of lag phase when grown in the presence of mercury was increased compared to growing chemoautotrophically. For strain EPR85 grown chemolithoautotrophically, i.e., in medium 142, the length of the lag phase and growth yield best expressed toxicity with the lag phase lasting for 7 hrs at 0 µM and 13 hrs at 10 µM mercury. The growth yield was also diminished in the presence of mercury with OD at 600 nm of 0.34 at 0 µM and 0.23 at 10 µM mercury (Figure 4.2a). When grown chemoheterotrophically in medium 142+A, the lag phase was elongated from 7 hrs at 0 to 16 hrs at 10 µM. However, the growth yield was not affected by the presence of mercury (Figure 4.2c). The doubling time of strain EPR85 in medium with acetate was 1.37 hrs at 0 µM and 5.63 hrs at 10 µM mercury. Mercury was less toxic to strain EPR155 than to EPR85. Nevertheless, the length of the lag phase in medium 142 increased from 7 hrs in presence of 0 µM mercury to 13 hrs in presence 10 µM mercury (Figure 4.2b). The length of the lag phase increased in the presence of mercury and acetate (Figure 4.2d) and it was slightly shorter for the no mercury cultures, 13 vs. 19 hrs. It should be noted that in spite of the increase in lag

phase in the presence of acetate, strain EPR155 achieved similar cell densities,

i.e., growth yield, in the absence and presence of mercury.
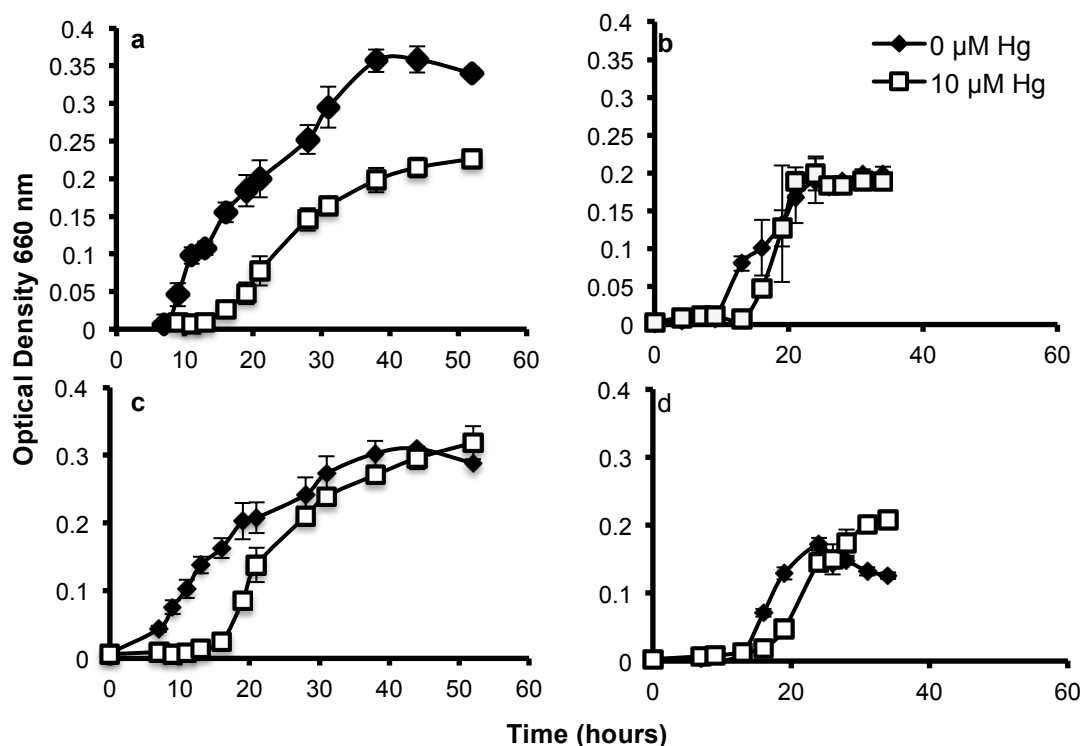
Figure 4.2 Effect of mercury on growth of chemolithoautotrophic deep-sea hydrothermal vent bacteria. *Thiomicrospira* sp. EPR85 (a and c) and *Halothiobacillus* sp. EPR155 (b and d) in medium 142 in the absence (a and b) or presence (c and d) of 10 mM acetate.

Presence of *merA* in the genomes of chemolithoautotrophic vent bacteria

To try and detect *merA* homologs in the genomes of the mercury tolerant

chemolithoautotrophs PCR with primer sets specifically designed for the

detection of this gene between the *Alpha-* and *Gammaproteobacteria* were used.

While appropriate positive controls resulted in PCR products of expected sizes

as observed in ethidium bromide stained agarose gels, these were not observed

when DNA templates from the chemolithoautotrophic mercury tolerant strains

were used. These results suggested that either *merA* genes were not present in

these strains or that these genes were divergent and could not be detected with

the employed PCR primers used in the experiment reported here. At the time of

experimental design, no representative genome sequences were available for

bacteria related to these isolates. Such genomes have become available since

then for six of the eight isolates and the complete genome sequences of

Halothiobacillus neapolitanus c2, *Thiomicrospira arctica*, and *Pelagibaca*

bermudensis HTCC2601 contain *merA* homologs. *In silico* PCR amplification of

mercuric reductase genes from these representatives with the primer sets used

showed that none of my primer sets would have amplified these *merA* homolgs.

These findings suggest the possibility of undetected merA homologs at least in

EPR75, EPR85, EPR96, EPR125, and EPR155 that belonged to the genra

Thiomicrospira, *Pelagibaca*, and *Halothiobacillus*.

Reduction of mercury (II) to elemental mercury during growth of

chemolithoautotrophic vent bacteria

Because *mer*-independent reductive detoxification of mercury was

reported in acidophilic chemolithoautotrophic bacteria (87) and to further test the

possible involvement of *mer* in mercury resistance, I followed the production of

mercury (0) by two deep-sea vent chemolithoautotrophic strains when grown in

presence of mercury (II). When grown in presence of 5 µM mercury (II) for 36

hrs, strains EPR85 and EPR155 did not produce volatile mercury (as indicated

by less than 1% mercury present in trapping vials of reactors) even though cell

growth occurred as suggested by an increased turbidity of the cultures in the

reactors and/or by media acidification. (Medium 142 contains phenol red, which

turns yellow when thiosulfate, provided as an energy source, is converted to

sulfuric acid (88)). Volatile mercury was not produced by the heat-killed cultures

(Figure 4.3). In comparison, the positive control, *Acinetobacter* sp. EPR148,

which contains *merA*, converted 25.8% of the added mercury (II) to volatile

mercury to produce statistically significant ($p \leq 0.05$) more mercury in the

trapping solution as compared to traps that were connected to reactors with the

marine chemolithoautotrophs with less than one percent of added mercury (II)

converted to volatile mercury (Figure 4.3). Mercury reduction activity by strain

EPR148 was lost in heat-killed cultures showing that only metabolically active

cells could reduce mercury (II). Recovery rates of mercury either remaining in

reactors or found in traps ranged from 84 to 111% of the added 5 µM mercury

(II). These results indicated that for two strains of deep-sea

chemolithoautotrophs isolated from EPR 9ºN mercury (II) is not reduced to

elemental mercury and mercury volatilization is not the resistance mechanism.
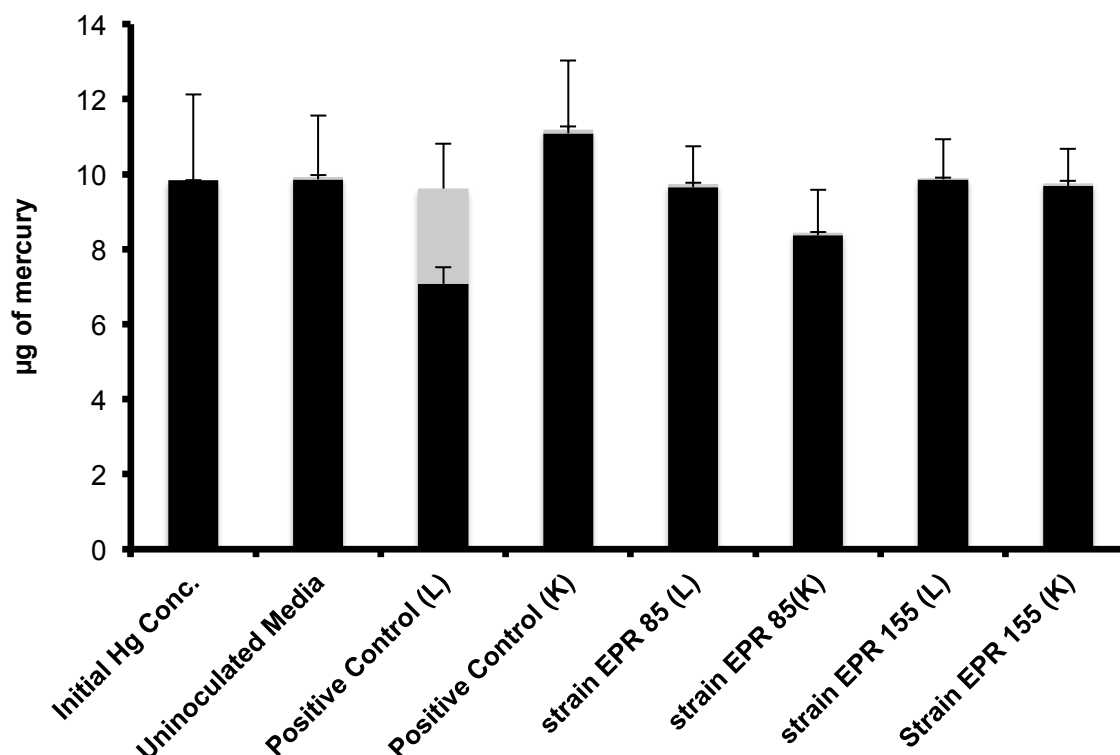
Figure 4.3 Production of volatile mercury during growth of chemolithoautotrophic deep-sea hydrothermal vent bacteria. Black bars represent the amount of mercury that remained in incubation reactors, and gray bars the amount of mercury collected in vials containing trapping solution. L - reactors containing live cultures; K - reactors containing heat killed controls. Positive control was *Acinetobacter* sp. EPR148, a bacterium known to contain the merA gene and reduce mercury. The amount of mercury added to the reactors at the beginning of the experiment was 10 µg.

Selection of chemolithoautotrophic deep-sea hydrothermal vent bacteria for EPS

studies

Out of the eight EPR 9ºN chemolithoautotrophic isolates that were tolerant

to 10 µM Hg, five were obligate autotrophs and three, facultative heterotrophs

(Table 4.3). The three facultative heterotrophs were selected for analysis of EPS

because of their ability to grow in medium MM-ASW. The choice of this medium

was dictated by the impossibility of using spent medium 142, the

chemolithoautotrophic medium, for the phenol-sulfuric acid assay for sugar

determination. It was determined that thiosulfate added to the medium as an

energy source interacted with the assays resulting in a green, chalky precipitate.

Accurate optical density values could not be obtained for making a standard

curve with this precipitate present. Minimal ASW does not contain thiosulfate and

is a heterotrophic medium containing casamino acids and acetate to support

growth. Nitrate was removed from the medium so as to not conflict with the

assay. The removal of nitrate from the media did not alter growth of the strains as

ammonium replaced nitrate as a nitrogen source in the modified medium.

Isolate Number	Species ID	Class	Metabolic Strategy
EPR74	***Thioclava sp.***	**α- *Proteobacteria***	**Facultative heterotroph**
EPR75	*Thiomicrospira sp.*	γ- *Proteobacteria*	Obligate autotroph
EPR85	*Thiomicrospira sp.*	γ- *Proteobacteria*	Obligate autotroph
EPR96	*Thiomicrospira sp.*	γ- *Proteobacteria*	Obligate autotroph
EPR115	*Hydrogenvibrio sp.*	γ- *Proteobacteria*	Obligate autotroph
EPR125	***Pelagibaca sp.***	**α- *Proteobacteria***	**Facultative heterotroph**
EPR129	*Halothiobacillus sp.*	γ- *Proteobacteria*	Obligate autotroph
EPR155	***Halothiobacillus sp.***	**γ- *Proteobacteria***	**Facultative heterotroph**

Table 4.3 Chemolithoautotrophic strains from EPR 9ºN able to grow in the presence of 10 µM Hg. Marine isolates selected for EPS studies are in bold.

<u>Effect of mercury on EPS production in facultative heterotrophic deep-sea hydrothermal vent bacteria</u>

The three facultative heterotrophic bacteria tolerant to at least 10 µM

mercury were tested for the production of EPS and also the potential induction of

EPS during growth in the presence of mercury in order to examine if EPS plays a

role in mercury tolerance for these vent organisms.

In the absence of mercury, all three isolates produced EPS ranging from 0.6 µg EPS/mg protein for EPR74 to 38 µg EPS/mg protein for EPR125 (Figure 4.4). EPR125 produced substantially more EPS than the *E.coli* strains tested in Chapter 3 (wild-type produced 6.4 µg EPS/mg protein and mutant produced 2.7 µg EPS/mg protein). EPR155 produced similar amounts of EPS compared to the *E.coli* strains and EPR74 produced substantially less than the *E.coli* strains. There was an 32% increase in EPS production in the presence of mercury for EPR155. Although there was an 8% and18% increase, respectively, in EPS production in the presence of mercury for EPR74 and EPR125, this was not statistically significant (Figure 4.4). It should be noted that at the concentration of mercury tested, there was no inhibition of growth for EPR74 and EPR155 as indicated by protein content of cell pellet in the absence and presence of mercury (69.1 mg versus 69.8 mg and 95.4 mg versus 89.9 mg respectively, student's t-test, p-value ≤0.05). There was a small decrease in protein content for EPR125 in the presence of mercury that was statistically significant (student's t-test, p-value ≤ 0.05), 32.7 mg versus 25.5 mg. Attempts to isolate and purify EPS from all 3 isolates were unsuccessful due to the inability to generate enough EPS from bacterial cultures (data not shown).
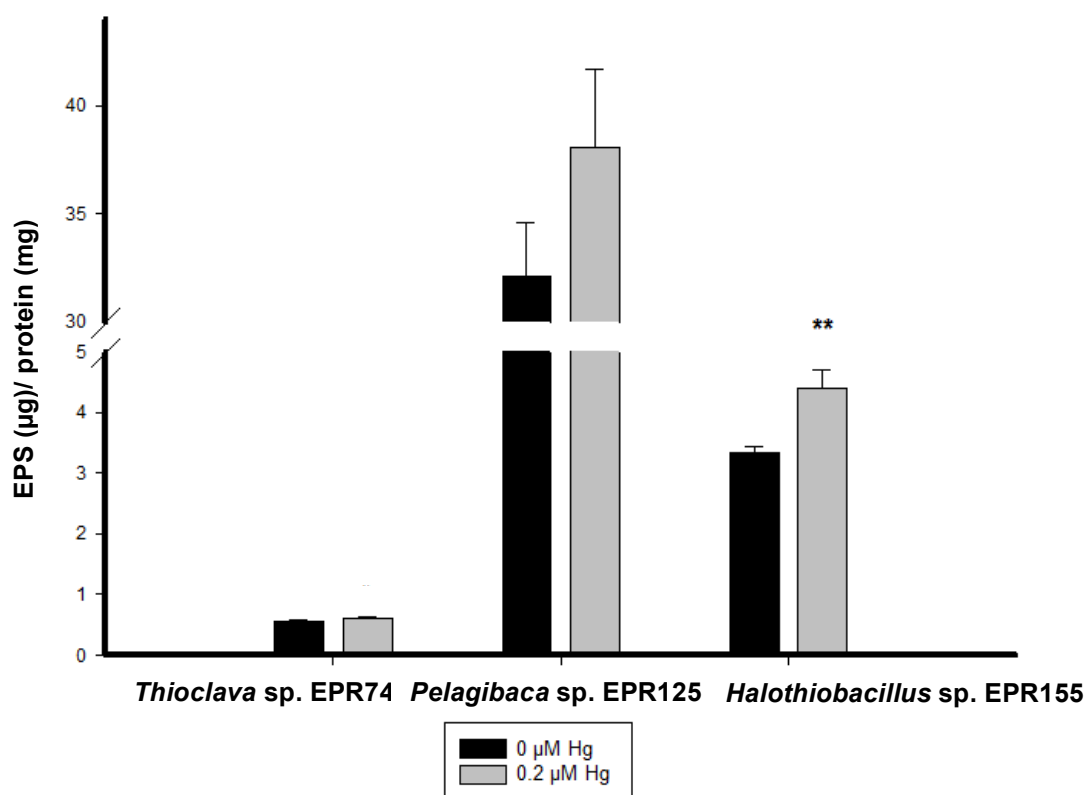
Figure 4.4 EPS production in absence (black bar) and presence (gray bar) of 0.2 $HgCl_2$ during growth. EPS production measured using phenol-sulfuric acid assay for sugar determination and normalized to protein using BioRad assay for protein determination. Student's t- test used for statistical analysis. Double asterisk indicates p- value ≤ 0.05.

Discussion

In this Chapter, eight deep-sea mercury tolerant chemolithoautotrophic bacteria may not possess the most common and well-understood prokaryotic mercury resistance system, *mer*. This finding is surprising and implies that if mercury detoxification is occurring by primary producers in the vent environment, it may not proceed by reduction of this toxic metal. Three of the isolates that had been isolated as chemolithoautotrophs from EPR 9°N were later found to be

facultative heterotrophs and the remaining were obligate autotrophs (Table 4.3).

This finding is not surprising since it is not uncommon for deep-sea hydrothermal

vent organisms to utilize numerous different carbon and electron sources and

this versatility in lifestyles has resulted in scientists referring to them as

mixotrophs (23). The three facultative heterotrophs (EPR74, EPR125, &

EPR155) were selected for further analysis because of their ability to grow in

medium MM-ASW. This is important because the thiosulfate used an energy

source for the autotrophic medium used to grow the isolates, medium 142,

reacted adversely with the phenol-sulfuric acid assay for sugar determination and

could not be used to measure EPS content. MM-ASW does not interact

negatively with the assay.

16S rRNA gene sequencing identified most of the isolates as

Gammaproteobacteria with two strains representing the *Alphaproteobacteria;*

merA genes and *mer* operons were previously described in representatives of

these classes (33, 84, 89). Prior findings with culture- independent methods

showed that the majority of 16S rRNA gene sequences from deep-sea vents

belonged to the *Epsilonproteobacteria* and to a lesser extent to the *Gamma*- and

Alphaproteobacteria (90-92). Pure epsilonproteobacterial cultures of thiosulfate

oxidizing mesophilic aerobic chemolithoautotrophs were previously described in

deep-sea hydrothermal vents (Reviewed in (93)). *Epsilonproteobacteria,*

however, prefer anaerobic and/or microaerophilic conditions (94) and the use of

fully oxygenated media in my enrichments might have limited their growth.

The deep-sea chemolithoautotrophs were enriched in media containing 10 µM mercury, a concentration documented to be highly toxic to most microbes (20). The growth response to mercury varied between the strains and growth conditions consistent with various modes by which mercury exerts its toxicity. Thus, the length of the lag phase is a function of how rapidly cells produce new enzymes and cell constituents (95) and the increased length of this period for *Thiomicrospira* sp. EPR85 and *Halothiobacillus* sp. EPR155 suggests that mercury stress necessitates biosynthesis of new molecules. Another possible cause for the elongated lag phase could be generalized suppression of metabolism and reproduction induced by mercury toxicity. The length of the lag phase was elongated further when either strain was growing chemoheterotrophically (utilizing an inorganic carbon source). In addition to increase in lag phase, a decrease in growth yield was observed for strain EPR85 growing chemoautotrophically. For these strains, mercury is entering the cell indicated by alterations in growth patterns implying toxicity, and the presence of tolerance mechanism(s) in deep-sea chemolithoautotrophic bacteria.

This is the first report of mercury tolerant chemolithoautotrophic bacteria, isolated from hydrothermal vents, which may lack a *mer* system but are able to grow at 10 µM mercury (II) without its reduction to mercury (0) (Figure 4.3). While the majority of known *mer*-carrying bacteria are aerobic heterotrophs (86, 96), *mer* systems have been described among acidophilic chemolithoautotrophs, mostly sulfur and iron oxidizing bacteria, such as *Thiobacillus* spp. and *Acidithiobacillus* spp., where reductase activities and *merC*-type mercury

transporters were documented (78, 97). Furthermore, the majority of neutrophilic

thiosulfate oxidizing moderately thermophilic bacteria from mercury-rich

terrestrial springs reduced mercury and possessed *merA* gene homologs (88).

The difference between this group and the deep-sea chemolithoautotrophic

bacteria may rest in the growth temperature and/or the taxonomic affiliation of the

enriched isolates, which in the terrestrial study belonged to the Gram-positive

order *Bacillales*. *mer* systems are common among marine heterotrophic bacteria

(96) including deep-sea vent isolates (33). Thus, the absence of *mer* systems

among the deep-sea chemolithoautotrophs reported here is not related to their

ecological or taxonomical affiliations. These observations raise the question of

how these microbes can tolerate micromolar concentrations of mercury.

Resistance to mercury that is not associated with reduction to the volatile

elemental form is known. The production of extracellular polysaccharides (EPS)

and binding of mercury to this matrix was reported for soil bacteria that tolerated

30 to 100 µM mercury (44). It is known that some deep-sea sediment and

hydrothermal vent bacteria are strong producers of EPS (98), with a potential to

bind heavy metals such as Cu, Cd, Pb, and Zinc (28, 29, 99). Therefore, I tested

the possiblity that chemolithoautotropic isolates from the EPR may tolerate to

mercury by producing EPS that sequester the metal outside the cell.

EPR155 demonstrated an increased EPS production when grown in

presence of 0.2 µM Hg compared to the no mercury control (Figure 3.5). This

may suggest an induction of EPS production by exposure to mercury. EPS

production, a complicated process involving many different genes, varies greatly

among microbial species. Some species will produce different EPS that are regulated differently. For instance, *Erwinia amylovara* secretes two different EPS, one that is constitutively expressed and the other is encoded by a separate operon, which is inducible. Levan is a neutral homopolymer synthesized by levansucrase, which is constitutively expressed. Amylovoran is a heteropolymer consisting of galactose and glucuronic acid residues, whose synthesis is controlled by the family of positive regulators RcsB. Overexpression of RcsB enhances the production of amylovoran while reducing the production of levan (66, 100). The amount of EPS produced by an organism can be effected by the medium composition, incubation conditions, and the ability to attach to a solid surface (64, 65). Environmental stresses, including starvation, dehydration, and toxic heavy metals can stimulate EPS production as a protective barrier (39, 40). It has been hypothesized that toxic metal ions result in the production of free radicals which in turn lead to oxidative stress which then induce the expression of EPS production genes and possibly other detoxification mechanisms (40).

Copper has been shown to induce EPS production as much as 4 fold in a *Pseudomonas aeruginosa* strain resistant to this metal; however copper had no effect on EPS production in a *Pseudomonas aeruginosa* strain sensitive to copper (40). Similarly, EPS production was increased in the presence of chromium (VI) for *P. aeruginosa, Micrococcus* sp., and *Orchrobactrum* sp. In these organisms, EPS production was directly linked to chromium (VI) removal capacity with the organism producing the most EPS also having the greatest chromium (VI) removal capacity (39). Since EPS induction varies between strains

and species, it is not surprising that when I compared EPS production by marine chemolithoautotrophic bacteria from different genera, exposure to mercury resulted in increased EPS production in two of the facultative heterotrophic strains but not by the third. However, more studies need to be performed to clearly demonstrate that EPS is induced by the presence of mercury.

The results reported here together with prior observations suggest that mercury tolerance may be mediated by different mechanisms among primary and secondary producers in deep-sea hydrothermal vent communities. Both are adapted to the presence of mercury as indicated by the relationship between the concentration of mercury in vent fluids and level of tolerance (19, 33). Many of the resistant heterotrophs, i.e., secondary producers, possess *merA* and reduce mercury while autotrophs isolated from this study do not. This conclusion is also supported by observations that genome sequence of several vent *Epsilonproteobacteria*, i.e. primary producers, lack *mer* genes as do all epsilonproteobacterial genomes (86, 96). Sorption of mercury to EPS produced by autotrophs is potentially involved in mercury tolerance in autotrophic bacteria. My results suggest heterotrophic guilds provide mercury detoxification via mercury reduction and autotrophic guilds may further enhance mercury tolerance in this ecosystem through the production of EPS. These interactions between the two may be critical for sustenance of life in deep-sea hydrothermal vents.

Caveats in Experimental Design

The mercuric reductase genes present in representative chemolithoautotrophic isolates could not amplified via *in silico* PCR amplification using the primers from these experiments. These findings suggest that the negative *mer*A results obtained in this study might be false negatives. These primers are not available and would need to be generated. Primers should be generated for the mercuric reductase genes for the representative isolates to determine if the *mer*A gene is truly not present in EPR 9°N isolates.

When assessing the effect of mercury on growth for the two chemolithoautotrophic isolates, only the mercury concentration the organisms were isolated on was tested. At this concentration, mercury was only slightly toxic to the isolates (Figure 4.2). Growing the isolates with increasing mercury concentrations until no growth could be observed would be beneficial to determine these organisms' levels of mercury resistance. This could then be compared to resistance levels of bacteria known to contain mercuric reductase.

A major disadvantage of chemolithoautotrophic deep-sea hydrothermal vent organisms is the gap in knowledge of the optimum nutrients demands of these organisms. Although there are media that these organisms can be cultured in (such as 142 used in the above experiments), they do no grow to high yields nor is their growth consistent. This makes reproducibility of experiments difficult. The mercury concentrations had to be dropped over the course of the dissertation in order to deal with these issues. Developing a media that closer

matches the metabolic demands of the organisms might result in increased

growth yield and more reliable growth.

Chapter 5 – Conclusion

Abiotic and biotic processes mediate mercury's complex geochemical cycle. Microorganisms play a critical role in the biotic processes involved in the movement of mercury throughout the cycle. The oxidation, reduction, methylation, demethylation, and binding of mercury by microorganisms alters bioavailability and causes a redistribution of mercury in the environment (2, 3, 9, 15).

The main objective of this dissertation was to determine the role of exocellular polysaccharides (EPS) produced by bacteria in the development of tolerance to mercury.

Purified EPS from pure bacterial cultures isolated from microbial mats in French Polynesian atolls were obtained to assess if EPS are capable of sorbing mercury. Until this study, no work has been done to assess mercury sorption to purified EPS.

Four purified EPS with different chemical compositions were tested for their ability to sorb mercury. All four EPS were capable of sorbing mercury by a saturable system. The composition of the EPS affected the extent of mercury binding (14, 43). The EPS consisting mostly of neutral sugar removed the least amount of mercury from solution (Figure 2.2) with sorption most likely due to interactions with oxygen atoms in hydroxyl groups acting as weak electron donors (29). Not surprisingly, the only EPS containing hexosamines was the most effective in removing mercury from solution (Figure 2.2). A washing

experiment (Figure 2.4) was used with the majority of mercury released from the

EPS when washed the EPS-mercury complex was washed with a basic solution

(pH=9). This supports the hypothesis that hexosamines play a role in mercury

sorption This EPS could be potentially used as a chelator for acute mercury

poisoning by ingestion in patients. The use of bacterial EPS for clinical treatment

of disease has already gained attention over the years when it was discovered

that the deep-sea hydrothermal vent bacterium *V.diabolias* produced an EPS

capable of enhancing bone healing. Interestingly, this EPS contains equal

amounts of hexosamines and uronic acids, which is very similar to the chemical

composition of Mo 245 (24, 34). Currently activated charcoal is administered in

patients with acute mercury poisoning; however it's efficiency as a treatment is

debated. Other treatment options include the chelators such as penicilliamine,

dimercapol, and unithiol; but these medicines have strong side effects including

nephrotoxicity, hypersensitivity, GI disorders, convulsions, and headache (101).

Bacterial EPS might be a safer way to effectively ingested mercury. This EPS

could also potentially be used as a filter in the environment to prevent mercury

from a contaminated site from leaching into water systems or in water treatment

plants to remove mercury from water.

In order to assess if EPS production alters mercury tolerance in bacterial

cultures, model *E. coli* strain ZK2686 (wild-type) and *E. coli* strain ZK2687

(mutant) were selected. The wild-type produces colanic acid as its sole EPS

constituent with the mutant having diminished colanic acid production (38). When

the wild-type and mutant strains were grown on solid media inhibited growth for

both strains in a dose-dependent manner (Figure 3.1). However, the wild-type was more tolerant to mercury than the mutant at all mercury masses tested (Figure 3.1) (68). I determined that the wild-type produced twice the amount EPS per mg of protein as the mutant when growing on solid media (Figure 3.2). Since genetically, the only difference between the two strains is the ability to produce EPS, these findings suggest EPS may alter mercury tolerance. The difference in mercury tolerance was small but statistically significant between the wild-type and mutant. A greater difference in mercury tolerance might have been observed if I used bacteria strains capable of producing an EPS with a different chemical composition. I showed that uronic acid, the major functional group present in colanic acid, was not the prominent functional group involved in mercury sorption by the marine EPS. Instead hexosamines appeared to be the major group involved in mercury sorption. In the future, the disc inhibition assay should be performed on *E. coli* strains engineered to produce a hexosamine rich EPS and their defective mutants (71) since hexoasmines were involved in the sorption of mercury by marine EPS. I would expect there to be a greater modulation in mercury tolerance in these organisms than I observed in my strains.

Finally, mercury tolerance and EPS production were assessed in bacterial isolates from an ecosystem with naturally elevated mercury concentrations, where they would have had millions of years to evolve an EPS capable of sorbing mercury. Eight facultative and obligate chemolithoautotrophic deep-sea hydrothermal vent bacteria were isolated from EPR 9ºN (Table 4.1), which has

naturally elevated mercury concentration ranging from 13.9 to 445 pmol/l (19). As primary producers chemolithoautotrophic microorganisms are critical in deep-sea hydrothermal vent ecosystems forming the base of the food (23-27). These eight isolates were found to be resistant to 10 µM mercuric chloride, a concentration known to be toxic to microorganisms indicating the presence of tolerance mechanism (20).

To taxonomically and phylogenetically identify the mercury resistant isolates, we sequenced their 16S rRNA and created a neighbor joining tree (Figure 4.1). Six of the isolates belong to the class *Gammaproteobacteria* with the remaining belonging to the class *Alphaproteobacteria.* For isolates most similar to *Thioclava* spp., *merA* homologs have not been reported in this genus. nor a complete genome sequence available in a public database to perform a blast search to search for a *merA* homolog. For other isolates, strains in the same genus or order have been shown to possess *merA* homologs suggesting marine bacteria related to our isolates may be capable of detoxifying mercury via the *mer* operon in addition to sorption to EPS.

Attempts to amplify *merA* genes in the eight chemolithautotrophic bacteria were unsuccessful, suggesting these bacteria do not contain a *merA* gene and use an alternative mechanism of tolerance to mercury. However, at the time of the experimental design, no representative strain genome sequences for any isolates were available. Since then, three representative strain genomes have been sequenced, each containing a putative *merA* gene. *In silico* PCR amplification of *merA* genes of these strains showed no amplification with the

primers that were employed by this study, suggesting the possibility of false negative results. With this new information, primers should be designed for the *merA* genes of the representative strains and the experiment repeated with the newly designed primers.

To gain insight into the effect of mercury on growth of the isolates and determine if they are capable of volatizing mercury regardless of PCR amplification of *merA*, two isolates, *Thiomicrospira* sp. EPR85 (EPR85) and *Halothiobacillus* sp. EPR155 (EPR155), were further characterized.

Growth curves for EPR155 (Figure 4.2) indicated a slight decrease in growth yield and increase in lag phase when growing chemoheterotrophically, utilizing organic carbon sources, versus chemoautotrophically, utilizing inorganic carbon sources in the absence of mercury. When grown in the presence of mercury, there was an increase in the lag phase with no difference in growth yield compared to the same mode of growth (heterotrophic or autotrophic) in the absence of mercury. Chemoheterotrophic and chemoautotrophic growth was the same for EPR85 in the absence of mercury (Figure 4.2). EPR85 was more sensitive to mercury than EPR155, as indicated by an almost doubled lag phase when growing chemautotrophically and more than doubled lag phase when growing chemoheterotrophically in the presence of mercury. Growth yield was decreased by 24% in the presence of mercury when growing chemoautotrophically compared to in absence of mercury. These data suggest that for both strains 10 µM mercury is only slightly toxic. A commonality between the strains and conditions was the increase in lag phase in the presence of

mercury. This could be due to the need to biosynthesize new molecules in presence of mercury stress (95). As previously noted, this concentration is known to be toxic to bacterial cells (20) and suggests a mechanism of tolerance is present in order to cope with mercury toxicity. A future study with higher concentrations of mercury that greatly inhibit growth could elucidate a dose-response relationship that could provide additional information into the effect of mercury on growth.

To determine if the mechanism of tolerance is volatilization, regardless of *merA* detection*,* I performed a purge and trap experiment. None of the mercury added to the culture was reduced by either EPR85 or EPR155 (Figure 4.3), suggesting that even if these two strains contained *merA* gene homologs not detected by PCR amplification, these genes do not specify mercury reduction activities. Together these findings suggest that chemotrophic vent bacteria tolerate high concentrations of mercury (even though they do not volatilize it) by an alternative mercury tolerance mechanism.

Deep- sea hydrothermal vent bacteria are known to produce EPS, which is critical for their survival in the vent ecosystem (22, 27, 28, 41-43) by allowing cells to alter their environment and compete in a complex community and in a changing environment. EPS allows bacteria to sequester nutrients, adhere to surfaces, and form aggregates and biofilms (24). It is well established that EPS produced by marine bacteria are capable of binding a wide array of heavy metals (24, 28, 29, 102) but little research to date has examined the ability of EPS from chemolithoautotrophic marine bacteria to bind mercury.

Work on the effect of mercury on EPS production during growth of the three facultative chemolithoautotrophic strains (Table 4.3) isolated from EPR 9°N showed a statistically significant increase in EPS production in the presence of mercury for one strain (Figure 4.4). It is known that heavy metals can stimulation EPS production in metal resistant bacterial strains most likely the result of free radical production by free metal ions inside of the cell. The free radicals cause oxidative stress activating gene transcription of detoxification mechanisms, such as EPS. (39, 40). These preliminary findings suggest that mercury may induce EPS production for certain vent bacteria; however more studies need to be performed to determine if this is the case. Studies to assess sorption of mercury to EPS produced by these facultative chemoautotrophs should also be performed to determine the extent mercury sorption.

Overall, EPS produced by bacteria are able to sorb mercury to varying degrees. It is plausible that EPS production is one of several mechanisms employed by marine bacteria to handle the high mercury concentrations at deep-sea hydrothermal vents. Other mechanisms that warrant investigation include the formation of insoluble HgS complexes due to the interaction of mercury with hydrogen sulfide (H_2S) produced by bacteria (3, 26), intracellular accumulation of mercury in a sulfide complex (103, 104), and efflux of mercury from the cell preventing intracellular damage (105-107).

In summary, data from this dissertation have enhanced our knowledge of the role EPS play in the extracellular sequestration of mercury by bacteria and the ability of chemolithoautotrophic bacteria from deep-sea hydrothermal vents to

tolerate high mercury concentrations. I have shown that the EPS of marine bacteria readily sorbed mercury and EPS production increased mercury tolerance in a model system. I also showed that strains of chemolithoautotrophic deep-sea hydrothermal vent bacteria are tolerant to high concentrations of mercury and do not volatize it. Furthermore, for some of the vent strains the data from this dissertation suggest that EPS production can be modulated by the presence of mercury. Together, I have demonstrated that EPS production has the potential to increase mercury tolerance in bacteria.

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