A Study on the Mechanisms of Mercury Tolerance in the Cyanobacterium, Synechococcus sp. IU 625

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MONTCLAIR STATE UNIVERSITY

A Study On the Mechanisms of Mercury Tolerance in the Cyanobacterium, Synechococcus sp. IU 625

by

Winder Bienvenido Perez

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Montclair, NJ

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Abstract

*Synechococcus* sp. IU 625 (*Anacystis nidulans*) is a freshwater unicellular cyanobacterium and an obligate photoautotroph that readily harbors plasmids. This organism has been used in many studies to assess the effect of heavy metal toxicity as an environmental pollutant. In one of the few studies for strain IU 625, Simon isolated 4.7-MDa and 37-MDa plasmids in 1978. The relationship between these plasmids and the 7.6-kb and 46.6-kb plasmids of strain PCC 7942 is unknown. Primers designed based on the sequence of 46.6-kb plasmid pANL of PCC 7942 exhibited positive priming with isolated plasmid DNA from *Syn. sp.* IU 625. Sequence analysis showed high homology with the respective sequences in plasmid pANL.

Cyanobacteria like *Syn. sp.* IU 625 have general metal resistance mechanisms. However, tolerance to mercury, the heavy metal with the strongest toxicity, is dependent on mercury resistance determinants (*mer*) that are commonly found in plasmids. Research into the genome of related strain PCC 7942 indicates that *mer* genes may also be located on the genome. The present study addresses the issue of plasmid versus chromosomal mediated mercury tolerance in *Syn. sp.* IU 625. Priming of pure genomic DNA from IU 625 cells with plasmid-specific and chromosomal-specific primers for mercury resistance showed positive priming with only chromosomal specific primers. Priming of isolated plasmid DNA from IU 625 with the same primers showed positive priming with only the plasmid-specific primers. Therefore, the findings suggest that *mer* determinants are located on both plasmid and genome. Preliminary findings from studying the induction of the plasmid in IU 625 in response to mercury stress suggest that the plasmid is induced early on after the introduction of the heavy metal.
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Introduction

Background on Cyanobacteria

Cyanobacteria, formerly known as blue-green algae, are oxygen producing photosynthetic bacteria (56). Evidence suggests that these microorganisms originated approximately between 2.8 and 3.7 billion years ago (26,53). Other evidence suggests that cyanobacteria may have been one of the largest groups of organisms that dominated during the Proterozoic Era of the Pre-Cambrian period (54). Thus, it has been proposed that cyanobacteria were the major contributors for the production of oxygen during that time (42, 47). The microorganisms included within this group of prokaryotes display a variety of different morphologies and cell division patterns (17, 53). The global importance of cyanobacteria today, as it has been throughout the existence of our planet, stems from the ability of cyanobacteria to undergo the process of nitrogen fixation as well as for their involvement in both the oxygen and carbon cycles (56). Cyanobacteria have the ability to undergo the process of photosynthesis in a fashion similar to plants because they are able to use water as an electron donor; they also possess chlorophyll $\alpha$, phycocyanin, and phycobiliproteins as photosynthetic pigments (53). Studies have also indicated that these organisms are able to use carbon dioxide (CO$_2$) as their sole source of carbon during the Calvin cycle (53). Due to these abilities, it is generally accepted that chloroplasts of plants and algae are derived from a common cyanobacterial ancestor (15,56).

Morphological differences among cyanobacteria have been used to divide the members of this group of microorganisms into five subsections (11,56). Cyanobacteria classified under subsections I and II (formerly known as Chroococcales and
Pleurococcusales, respectively) are unicellular coccoids. Unlike the members of subsection I that divide by means of binary fission, those of subsection II divide by multiple fission to produce cells called baeocytes (56). The members of subsections III-V (Oscillatoriales, Nostocales, Stigonematales, respectively) are all able to form filaments with varying degrees of complexity. Cyanobacteria of subsection III only have vegetative cells, and are therefore limited developmental capacity. The vegetative cells of cyanobacteria in subsections IV and V have the ability to differentiate into either heterocysts or akinetes depending on growth conditions (56). Morphologically distinct from vegetative cells, heterocysts and akinetes have the ability to perform nitrogen fixation and survive changes to the environment such as drastic fluctuations in temperature as well as droughts (59). The members of subsection V are considered to be among some of the most highly developed prokaryotes because of their ability to form characteristic branching patterns (56).

Cyanobacteria can be found throughout various types of environments, including places such as hot springs, deserts, and polar regions. They can also be found in freshwater areas such as lakes and drinking water reservoirs (35). Cyanobacteria have the ability to produce in large quantities that can lead to the development of cyanobacterial or algal blooms. These blooms are in part due to the enrichment of waters with plant and cyanobacterial nutrients from sewage, agricultural fertilizers and industrial run-off (38,40). Algal blooms have the potential to cause turbidity in enclosed bodies of water (38), produce a foul odor, cause the deaths of oxygen-requiring organism due to oxygen depletion, and give drinking water an extremely unpleasant taste (8,9).
The rise of harmful algal blooms (HABs) in freshwater areas can lead to the production of toxins that can affect both humans and animals. Toxic cyanobacteria poisonings (CTPs) are reported to be the most common cause of freshwater intoxication (7,13). Over 40 different species of cyanobacteria have been implicated in producing HABs (7). There are a number of different factors that can determine the formation and toxicity of HABs: 1) genetic composition of cyanobacterial strains, 2) factors that affect growth, 3) ratio of toxin vs. non-toxin producers in a freshwater bloom, and 4) factors that affect bioaccumulation in a marine bloom (10). Cyanobacterial toxins can be separated into three distinct groups: dermatoxins, neurotoxins, and hepatotoxins (9). Depending on the specific strains involved in the formation of HABs, these toxins can be produced individually or in combination. Hepatotoxins are the ones most commonly associated with cyanobacterial bacteria poisoning (10). Individuals who ingest an adequate of toxin-producing cyanobacteria may experience liver failure within several hours to a few days of the incident (10). Ingesting these microorganisms is not the only way that toxins can be released; cell lysis can also cause the release of toxins into the environment. Therefore, using algacidal agents to control algal blooms becomes ineffective. Using these agents may actually worsen the quality of the drinking water (16).

*Synechococcus sp. IU 625 (formerly Anacystis nidulans)*

Due to the ubiquitous nature of cyanobacteria and their adaptability to various ecological environments, they serve as excellent models for the investigation of various biological processes including membrane transport and physiological changes in
photosynthesis and respiration due to changes in environmental conditions (46,60). They also serve as good indicators of environmental pollution (12). A unicellular organism, *Anacystis nidulans*, has been used in many studies to assess the effect of heavy metal toxicity as an environmental pollutant (29-32).

*Anacystis nidulans*, is a non-motile, unicellular, rod-shaped organism, which is similar to gram-negative bacteria in cell wall structure, replication and ability to harbor plasmids (Figure 1) (32). Morphologically, it is classified under subsection I because it replicates via binary fission. *Anacystis nidulans* was first isolated by M.B. Allen in 1952 from Waller Creek, Austin Texas and further characterized by Myers and Kratz in 1955. Transmission electron microscopy analysis illustrates its cell wall as containing peptidoglycan, and the photosynthetic lamellae, which are regularly arranged in three to four concentric circles around the periphery of the cytoplasm (Figure 2). Each membrane is approximately 10nm in thickness with two electron-transparent layers surrounding an electron dense core. The area between the membranes is of even density, and is the location of the protein complexes, which contain the accessory photosynthetic pigment, phycocyanin (1,48). The central region of the cell is the nucleoplasm, which contains DNA, ribosomes and carboxysomes (polyhedral structures which contain enzymes involved in carbon fixation) (48).

As a cyanobacterium, *Synechococcus sp.* IU 625 is an obligate photoautotroph whose photosynthetic apparatus is similar to that of plants (17,22,43). It is widely accepted that ancient cyanobacteria similar to *Syn. sp.* IU 625 were responsible for today's oxygen-rich atmosphere crucial for many modern organisms (25,52) and that a
cyanobacterial ancestor was the precursor of chloroplasts in photosynthetic organisms (4,17,43).

*Synechococcus* sp. IU 625 is one of several gram-negative bacteria formerly named *Anacystis nidulans*. Other species that were formerly classified under *A. nidulans* include the nontransformable *Synechococcus elongatus* strain PCC 6301, also known as the reference strain of the genus (44), and the highly transformable strain PCC 7942, which was once designated *A. nidulans* R2 (18). Unlike PCC 6301 and PCC 7942, strain IU 625 is found rarely in literature. Better DNA techniques have reclassified all *A. nidulans* strains as members of the *Synechococcus* genus despite a rod-shaped appearance and have established a close relationship between the two most-studied strains. Genetically, *S. elongatus* strains PCC 7942 and 6301 only differ slightly in chromosome length and by a 189-kb inversion (55). However, it is unknown to what degree *Syn. sp.* IU 625 is related to either of these strains.

In one of the few studies for strain IU 625, Simon isolated 4.7-MDa and 37-MDa plasmids in 1978 (51). The relationship between these plasmids and the 7.6-kb and 46.6-kb plasmids of strain PCC 7942 is unknown. While the entire genomes of *S. elongatus* PCC 7942 and 6301 have been published (23,55, respectively), the only available genetic sequence that has been published for *Syn. sp.* IU 625 is a metallothionein gene (GenBank Accession# DQ383472).

Strain IU 625 has been used to study heavy metal resistance to toxic ions such as the light metals Ba\(^{2+}\) and Al\(^{3+}\), the non-metal Se\(^{2+}\) and the heavy metals Pb\(^{2+}\), Co\(^{2+}\), Hg\(^{2+}\) and Cd\(^{2+}\) (29-32). Other heavy metals such as Mn\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\) and Ni\(^{2+}\) are essential to
cyanobacteria (2,41), but were also found to be toxic to S. sp. IU 625 in high concentrations (32).

Figure 1. Scanning electron micrograph (SEM) of Syn. sp. IU 625 cells.

Figure 2. Transmission electron micrograph (TEM) of Syn. sp. IU 625. The photosynthetic lamellae appear on the periphery of cell.
Impact of Toxic Heavy Metals

Comprising the majority of the 90 naturally occurring elements, heavy metals are metals with densities greater than 5 g/cm³ (41). The majority of the heavy metals are transition elements whose $d$ orbitals are not completely filled. The incomplete $d$ orbitals give the heavy-metal cations the ability to form complex compounds that may have the ability to take part in reduction-oxidation reactions (41). Because of their ability to form complex compounds and their involvement in redox reactions, certain heavy-metal cations play an extremely important role as “trace elements” in biochemical reactions that govern processes such as the stabilization of protein structures and bacterial cell walls (6,41). The problem with heavy-metal ions is that as their concentrations increase in the cellular environment, they form nonspecific complex compounds within cells that can lead to toxic effects (41). Such toxic effects can occur through a number of different ways such as the displacement of the metal from its native binding site or competition of the metal with other essential molecules at oxygen sites (6).

The release of heavy metals from industrial processes into the environment has caused a number of safety concerns for human health and marine ecosystems (24). Although heavy metal pollution of marine environments naturally occurs due to the weathering processes of the Earth’s crust, industrialization and urbanization have lead to an increase in contamination of these environments. Among many other factors, this increase in contamination can be attributed mainly to industrial drainage, urban runoff, mining, soil erosion, and air pollution fall out (29-32).

Concerns continue to grow as the levels of contamination increases in aquatic environments because most of these heavy metals are being disposed of in streams, lakes
and reservoirs (37). Without a method for decomposition, these metals will continue to accumulate and the list of health issues related to them will continue to grow. Mercury (Hg) is the most common heavy metal that the Environmental Protection Agency has listed as been the most toxic at high concentrations on their target analyte list (TAL). Elevated levels of mercury integrated into the food chain can cause great harm to animals and eventually work their way up to affect the human population. The effects of various heavy metals on a number of species of cyanobacteria have been well studied (28,32,48,57). Microorganisms such as Synechococcus sp. IU 625 have been used as indicator species for the determination of heavy metal pollution in the environment. Thus, it can be said that the health of ecosystems has some dependability on a functional microbial community and any adverse effects on microorganisms may pose a serious threat (32).

Iron is biologically the most important heavy metal cation (41). It is an essential part of oxygenic photosynthetic electron transport chains as well as respiratory-transport complexes in cyanobacteria (28). Iron is also important in the production of ribosomes in both cyanobacteria and higher photosynthetic organisms (57). Despite the large amounts of iron found in aquatic environments, its low solubility at neutral pH can limit the production of phytoplankton (28). This poses a potential problem for other aquatic species as phytoplankton that are not a only natural food source for other organisms but also have the ability to fix large amounts of carbon that would otherwise be released as carbon dioxide. Iron deficiency in cyanobacteria can lead to a decrease in the levels of chlorophyll, cytochromes, and phycobilisomes. Guikema and Sherman (1984) showed that the polypeptide composition of membranes from iron-deficient A. nidulans R2
cultures were also quite different from the membranes of wild type cells (19). High concentrations of iron can also lead to neurodegenerative disorders such as Alzheimer's disease. Rottkamp et al. (2001) showed that amyloid-β toxicity, a key mediator in the pathogenesis of Alzheimer's, was greatly decreased in the presence of iron chelating agents and restored in the presence of excess iron (45).

Mercury is one of the most lethal heavy metals studied with no known biological significance (58). It is found in rocks, soil and water and is relatively insoluble in its elemental, inorganic (mercuric chloride) or organic form (methyl and dimethyl mercury) (58). Mercury is used in the production of fungicides, insecticides, wood preservatives, electrodes, and pharmaceuticals (58). Although the use of mercury has drastically decreased or stopped completely, it was also used as an amalgam in tooth fillings for decades (41). Mercury exhibits high toxicity to photosynthesis, including both light and dark reactions in phototrophic organisms (34). In humans, it may be fatal if swallowed and is harmful if inhaled or absorbed by contact with skin. Mercury is a severe irritant to the eyes, skin, and respiratory tract. It also may cause burns, damage to the kidneys and central nervous system. Mercury is also known to cause birth defects in developing fetuses. Bioaccumulation of mercury in cyanobacteria occurs in two stages: short-term physical absorption at the cell surface and long-term uptake involving intracellular accumulation (24,37). Mercury is highly toxic because of its high affinity for thiols, which play a vital role in biological functions as part of enzymes and other essential cellular proteins (6,27,39).

A number of combination studies, as the one done by Lee et al. (2002), have been done to determine the effects of the presence of two heavy metals on the growth of an
organism (32). The Lee et al. (2002) study set out to determine the effects of mercury and selenium, in combination, on the growth of *Synechococcus sp.* IU 625. This combination was used for two reasons: 1) selenium is less toxic to the growth of cyanobacteria in comparison to mercury, and 2) it was hypothesized that the lower concentrations of selenium could potentially reduce the toxicity of mercury (Lee 2002). However, the study showed that the selenium did not produce a significant beneficial effect on the toxicity of mercury in *A. nidulans* (32). Current studies exploring other combinations of heavy metals with mercury may provide some evidence to the possibility of other heavy metals providing antagonistic effects on the toxicity of mercury. For example, iron, a necessary element found in biological systems, may have an important role in reducing the toxicity of mercury. A previous study looking at the effective of ferric chloride on the growth of *Syn. sp.* IU 625 cells indicate that a concentration of 10 mg/L may enhance the growth of the cells and also help in reducing the toxicity of mercury to IU 625 cells. The results from a combination study looking at the effects of iron and mercury on IU 625 cells can be used to correlate the effects of heavy metals on cyanobacteria in their natural environments because they are more likely exposed to more than one heavy metal at a given time.

**Heavy Metal Resistance in the Microbial World**

Prokaryotic organisms have been shown to create microbial mats, which are cohesive, laminated microbial communities dominated by photoautotrophic cyanobacteria (3,14). They are also found in many extreme environments like hot springs and deep-sea hydrothermal vents (3). These microbial communities have the ability to
sequester organic compounds and heavy metals from their environments because of the complex and firm structures that they form. Similarly, organisms grown in culture may also have this ability to sequester toxic heavy metals once they have reached a particular concentration.

Bacterial cells have putative mechanisms for dealing with heavy metal stress, which may or may not be similar to mechanisms in cyanobacteria. For example, ferric-specific ligands, siderophores, are responsible for binding complex compounds of Fe(III) (41, 57). Siderophores function using a two-step process: 1) solubilization of iron in the microenvironment around the cell, which occurs by the utilization of hydrophilic siderophores, and 2) an exchange of the bound Fe(III) to a surface associated hydrophobic siderophore. Due to its low solubility, Fe$^{3+}$ is not toxic to aerobic bacteria (41), and it predominates under physiological conditions as the highly insoluble ferric hydroxide complex (28).

Other heavy metals, like zinc, cadmium and mercury have different uptake mechanisms, each depending primarily on the structure and solubility of the heavy metal. Members of a variety of different protein families can transport zinc, a component of various enzymes and DNA-binding proteins. For example, in its Zn$^{2+}$ form, it is transported quickly and without specificity by the Mg$^{2+}$ transport systems. A number of different ATPases may also be able to transport zinc by either bringing about its uptake as a byproduct of Mg$^{2+}$-uptake again, or its efflux as a means of detoxification (41).

Metal transport systems specific to magnesium (MIT), or manganese uptake systems are responsible for the uptake of cadmium (41). Also, metallothionein (Smt) proteins characterized in cyanobacteria and have been shown to provide resistance to
cells when in the presence of high concentrations of cadmium (5,20,21). Metallothioneins are low molecular weight, cysteine rich, metal-binding proteins that exists in a variety of species (12). Each domain of metallothionein binds metal ions with ligation through metal thiolates at cysteine residues, making ligation very strong (12). This characteristic suggests that metallothioneins play an important role in heavy metal detoxification by allowing the cyanobacteria to thrive in toxic conditions. Studies done by Gupta et al. have shown that increased and decreased amplification of the metallothionein locus correlates with an increase and decrease in cadmium resistance, respectively (20,21).

Mercury is considered to be the heavy metal with the strongest toxicity, and therefore has no beneficial function. Since bacteria are likely to encounter toxic Hg$^{2+}$ concentrations in their natural environments, mercury resistance determinants, mer, can be found among a number of different species of bacteria (41,49). Resistance to mercury is based on the unique characteristics of the metal. For example, its melting/boiling point (melting point -39°C, boiling point 357°C) is extraordinarily low for a metal (41). These unique characteristics are what allow living cells to reduce Hg$^{2+}$ to the metal and thus remove it by means of passive diffusion (41,50). However, once outside the cell, the Hg$^0$ may become oxidized by other bacteria and thus become volatile once again (41). Due to high affinity of Hg$^{2+}$ to thiol groups, the question has been raised that metallothioneins may also be playing a role in sequestering this highly toxic heavy metal in cyanobacteria. Due to its high toxicity and the unique combination of reducibility and the ability of the product (Hg$^0$) to become volatile make mercury an excellent candidate for bioremediation (41). Bioremediation is the process of detoxifying bodies of water or
earth from toxic heavy metals using organisms, especially ubiquitous organisms like cyanobacteria (36). Resistance mechanisms like the ones described would make resistant strains of cyanobacteria very useful in the process of bioremediation.

![Diagram of protein families involved in bacterial heavy-metal metabolism.](image)

**Figure 3.** Protein families involved in bacterial heavy-metal metabolism.

There are eight genes that can comprise the mercury resistance (*mer*) operon. It is difficult to determine what genes make up the *mer* operon in a particular strain of gram-negative bacteria because the numbers of genes that comprise the operon varies. The essential genes are *merR*, *merT*, *merP*, and *merA* and the accessory genes are *merC*, *merF*, *merB*, and *merD* (33). As previously mentioned, the mechanism of resistance to
mercury involves the reduction of Hg\(^{2+}\) to the volatile form Hg\(^0\). This transformation is mediated by the mercuric reductase, *merA*, an inducible NADPH-dependent, flavin adenine dinucleotide-containing disulfide oxidoreductase (33). The *merR* gene encodes the regulatory protein and *merT* and *merP* make up the Hg\(^{2+}\) uptake system. Even though it is considered an accessory gene, gram-negative bacteria that contain *merB*, the gene encoding organomercurial lyase, have the added benefit of being able to detoxify organomercurials, which are more toxic than Hg\(^{2+}\) (33, 41). Studies have shown that *merD* appears to act as an antagonist to *merR* (33). The remaining components, *merC* and *merF*, encode membrane proteins that appear to be involved in the transport of Hg\(^{2+}\) (33). Because the accessory genes of gram-negative bacteria encode similar proteins with similar function as the essential genes, with the exception of *merB*, it is possible that these accessory genes were acquired through evolution as the bacteria become faced with greater amounts of toxic mercury in the environment.

**Research Proposal**

The objectives of this thesis are as follows:

I. **To study the effects of iron and mercury on the growth of *Synechococcus sp.* IU 625.**

Many heavy metals (Al, Hg, Mn, Zn, Cd, Pb) have been used individually to evaluate the effects of toxic heavy metals on *Syn. sp.* IU 625. In order to determine the combined effects of heavy metals on the growth of *Syn. sp.* IU 625, cultures will be grown using a constant concentration of iron and varying the concentration of mercury. The effect of iron on the toxicity of mercury is of particular interest.
II. To isolate and study a resistant strain of *Synechococcus sp.* IU 625.

After observing the growth kinetics through three passages in response to iron and mercury, *Syn. sp.* IU 625 cells appear to become more and more resistant to mercury. Resistant cells were isolated in order to study the mechanism(s) of resistance.

III. To provide insight into the molecular mechanisms of mercury resistance in *Synechococcus sp.* IU 625.

a. Plasmid vs. chromosomal mercury resistance.

Previous studies have shown that mercury resistance appears to be mediated by plasmid DNA. However, research into the genomes of related strains *Synechococcus elongatus* PCC 6301 and PCC 7942 has shown that both strains appear to have mercury resistance genes embedded in their genomes. Therefore, one of the major goals of this study is to determine if either plasmid or chromosomal pathways or a combination of the two mediate mercury resistance in *Syn. sp.* IU 625 using a PCR-based assay.

b. To analyze DNA levels and RNA expression of mercury resistance genes.

Upon determining the concentrations that induce plasmid-mediated resistance and that the plasmid has been successfully isolated, quantitative real-time polymerase chain reaction (qRT-PCR) experiments will be carried out in order to detect the time frame of when the mercury resistant gene products have begun to be produced. Preliminary experiments will focus on analyzing the DNA levels of plasmid-specific genes, and will be followed by examining the expression levels of plasmid-specific and chromosomal specific mercury resistance genes.
Materials and Methods

I. Culture and Maintenance of *Synechococcus* sp. IU 625

*Synechococcus* sp. IU 625 stock cultures were obtained from the American Type Culture Collection, Manassas, VA (ATCC No. 27344). At approximately $1 \times 10^6$ cells/mL, *Syn. sp.* IU 625 were inoculated aseptically into 250 mL Erlenmeyer flasks. Five milliliters of cells were added to 95 mL of sterilized Mauro’s Modified Medium (3M medium), adjusted to a pH of 7.9 using 1M NaOH. The cultures were grown in an Innova™ 4340 incubator (New Brunswick Scientific, Edison, NJ) at a constant temperature of 30°C with constant fluorescent light and continuous agitation at 100 rpm. Cell growth was monitored in two ways: 1) by direct cell count using a hemocytometer, 2) a turbidity study using a Spectronic GENESYS 20 at OD$_{750nm}$.

II. Preparation of Heavy Metals Stock Solutions

A. FeCl$_3$ – A stock solution of 10% ($10^3$ mg/L) ferric chloride was prepared with triple deionized water in a sterile, opaque container and covered with aluminum foil as to not allow the entrance of light.

B. HgCl$_2$ – A stock solution of 1% ($10^4$ mg/L) mercuric chloride was prepared, as above, with triple deionized water in a sterile container.

III. Introduction of Heavy Metals into *Synechococcus* sp. IU 625 cultures

A. FeCl$_3$ and HgCl$_2$

*Syn. sp.* IU 625 cells were inoculated into 250 mL Erlenmeyer flasks containing 95 mL of sterilized 3M medium at a concentration of approximately $1 \times 10^7$ cells/mL. Each flask contained a fixed final FeCl$_3$ concentration of 10 mg/L and one of the following concentrations of HgCl$_2$: 0.1, 0.5, or 1.0 mg/L. A control culture to which the
heavy metals were not added was also used. Two repeatings were done for each inoculum. The growth of the cultures was measured by direct cell count using a hemocytometer and by turbidity study using a Spectronic GENESYS 20 at OD_{750nm} over a period of 24 days.

B. HgCl₂

*Syn. sp.* IU 625 cells were inoculated into 250 Erlenmeyer flasks containing 95 mL of sterilized 3M medium at a concentration of approximately 1x10^7 cells/mL. Each flask contained a concentration of 0.1, 0.5, or 1.0 mg/L HgCl₂. A control group to which the heavy metal was not added was also used. Two repeatings were done for each inoculum. The growth of the cultures was measured by direct cell count using a hemocytometer and by turbidity study using a Spectronic GENESYS 20 at OD_{750nm} over a period of 24 days.

IV. Culture and Maintenance of Resistant *Synechococcus sp.* IU 625 cells in HgCl₂: Second Passage

Resistant cells were isolated and grown in the presence of 0.1, 0.5, or 1.0 mg/L HgCl₂ and 95 mL of sterilized 3M medium. A control group to which the heavy metal was not added was also used. Two repeatings were done for each inoculum. The growth of the cultures was monitored as previously described for a period of 28 days.

V. Count of the Amount of Mercury Within *Synechococcus sp.* IU 625

To determine the amount of mercury within the cells with respect to the growth medium, samples were collected at 2, 4, and 24 hours for the short-term study and at 1, 3, 8, and 17 days for the long-term study. The samples were sent to Accredited Analytical Resources, LLC for analysis. The 25 mL portion of each sample was digested with
potassium permanganate and persulfate at acidic conditions in a water bath at 95°C. The mercury in the sample was reduced to the elemental state and detected by the cold vapor technique in a closed system. The analytical procedure associated with the Cold Vapor technique is derived from the EPA Method 245.1.

VI. Molecular Analysis of Heavy Metal Resistance in *Synechococcus* sp. IU 625

A. *Synechococcus* sp. IU 625 Plasmid Isolation Using the QIAGEN QIAprep® Spin Miniprep Kit (Cat. No. 27106) and a Microcentrifuge with Some Modifications

Depending on how confluent each culture was at the time samples were taken, anywhere from 500 µL to 5 mL of cells were aliquoted into sterile 1.5 mL microcentrifuge tubes or 15 mL test tubes, respectively. After making sure that the optical density of each sample was equal to 1, the samples were centrifuged for 2-3 minutes at 10,000-12,000 rpm in an eppendorf 5415 C centrifuge (Brinkmann Instruments, Westbury, NY) and Dynac™ centrifuge (Clay Adams, Parsippany, NJ), respectively. The pelleted bacterial cells were resuspended in 250 µL of Buffer P1 and transferred into sterile 1.5 mL microcentrifuge tubes. Next, 250 µL of Buffer P2 was added to the samples and mixed thoroughly by inverting gently. Please note that vortexing at this step is not recommended because it will result in the shearing of genomic DNA. This was followed by the addition of 350 µL of Buffer N3; the samples were mixed immediately and thoroughly by inverting. Samples were then centrifuged for 10 minutes at 13,000 rpm (~17,900 x g). The resulting supernatants were applied to QIAprep® spin columns by pipetting, making sure not to disturb the pelleted material.
The samples were centrifuged for 30-60 seconds at 13,000 rpm and the flow-through was discarded. Since each spin column has a maximum capacity of 800 µL, this step was repeated until all supernatant had been run through the spin column. The spin columns were washed by adding 0.75 mL (750 µL) of Buffer PE and centrifuging for 30-60 seconds at 13,000 rpm. The flow-through was discarded and the samples were centrifuged for an additional 1-minute to remove residual wash buffer. The spin columns were placed into clean 1.5 mL microcentrifuge tubes. In order to elute the desired DNA, 30-50 µL of sterile water was added to the center of each spin column, and allowed to stand for 1 minute so that the water could be absorbed into the membrane. The lower the amount of water used in the elution step, the greater the concentration of DNA will be. The samples were then centrifuged for 1 minute at 13,000 rpm. Please note that the pH of the water must be between 7.0 and 8.5 in order for it to be used. Water was chosen over buffer EB (10 mM Tris-Cl, pH 8.5), the elution solution that is provided with the kit, because buffer EB has the potential of having adverse effects during PCR amplification. After eluting the DNA, samples were stored at -20°C or -80°C for future analysis by gel electrophoresis, polymerase chain reaction (PCR), and Real-Time PCR (QPCR). Specific details for each step are described in the miniprep kit handbook (appendix 1).

B. *Synechococcus sp.* IU 625 DNA Extraction Using the InstaGene™ Matrix (Bio-Rad, Cat. No. 732-6030)

Depending on how confluent each culture was at the time samples were taken, anywhere from 500 µL to 5 mL of cells were aliquoted into sterile 1.5 mL microcentrifuge tubes or 15 mL test tubes, respectively. The samples were centrifuged for 2-3 minutes at 10,000-12,000 rpm in eppendorf 5415 C centrifuge and Dynac™
centrifuge, respectively, and the resulting supernatants were removed. The pelleted cells were resuspended by adding 200 µL of InstaGene™ Matrix to the pellets. For samples that were originally in 15 mL test tubes, the resuspended cells were transferred into sterile 1.5 mL microcentrifuge tubes prior to the addition of the InstaGene Matrix. The samples were incubated at 56°C for 30 minutes, vortexing every 10 minutes for 3-5 seconds. After the incubation period, the samples were vortexed for 10 seconds. The samples were placed in either a 100°C heat block or boiling water bath for 8 minutes. The samples were removed from the heat block or water bath and vortexed for 10 seconds and then centrifuged at 10,000-12,000 rpm for 2-3 minutes. The samples were then stored -20°C or -80°C for future analysis by gel electrophoresis, polymerase chain reaction (PCR), and Real-Time PCR (QPCR).

C. Probing of Mercury Resistance By Polymerase Chain Reaction

DNA extracts were subjected to polymerase chain reaction in order to determine the presence of mercury resistance. 12.5 µL of HotStarTaq Master Mix (QIAGEN, Cat. No. 203443) was pipetted into a sterile 0.2 mL bubble cap PCR reaction tube along with 5.5 µL of sterile H2O, 1 µL Forward Primer, 1 µL Reverse Primer, and 5 µL of DNA extract. The total volume for each reaction tube was 25 µL. The reaction tubes were placed into either a pre-heated Labnet MultiGene II thermal cycler (Labnet International, Edison, NJ) or Veriti™ 96-Well thermal cycler (Applied Biosystems, Foster City, CA). The reaction profile typically used was: initial denaturation at 95°C for 15 minutes to activate the HotStarTaq DNA Polymerase, followed by 35 cycles of denaturation at 95°C for 1 minute, primer annealing at 56-64°C for 1 minute, and extension at 72°C for 1:30 minutes. At the end of the 35 cycles, the reaction tubes were subjected to a final
extension at 72°C for 5 minutes. Once the thermal cycler cooled down to 4°C, the samples were removed and if necessary purified using the QIAquick PCR purification kit before being placed into the -20°C freezer for future analysis via agarose gel electrophoresis.

D. Primer Design for PCR-based Assay

Table 1 lists all of the primers used for PCR-based assay. Plasmid-specific primers pANL21, pANL36 and pANL53 were designed based on the published sequences of the plasmids of Synechococcus elongatus PCC 7942, pANL. The pANL21 primers were 5’-TGA GCC TAC TCC TTG GTC TA-3’ for the forward and 5’-GCA CAA AAG AAT CAC CAG AT-3’ for the reverse primer. The pANL36 primers were 5’-GTG AAT GAG ACG ATC TTG GT-3’ for the forward and 5’-ACC GTA TTT CCA CAA CTG AC-3’ for the reverse primer. The pANL53 primers were 5’-CTG TGG CAA ATC CTC TTT AC-3’ for the forward and 5’-GCA CTG AGG TCA GTC AGT TT-3’ for the reverse primer. Another set of plasmid-specific primers, merA1&A5, were designed based on the merA gene of the NR1 plasmid of Shigella flexneri. The merA1&A5 primers were 5’-ACC ATC GGC GGC ACC TGC GT-3’ for the forward and 5’-ACC ATC GTC AGG TAG GGG AAC AA-3’ for the reverse primer. Primers for chromosomal-specific mercury resistance gene CmerA were designed based on the genome sequence of Synechococcus elongatus PCC 6301. The CmerA primers were 5’-CAA ATA TTG AAG GCT TGG AG-3’ for the forward and 5’-ACT GAG AGC ACT CAT CCC TA-3’ for the reverse primer.
### Table 1: Primer Design for PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence (5' to 3')</th>
<th>Source</th>
<th>Accession no.</th>
<th>Amplicon</th>
<th>Protein product</th>
</tr>
</thead>
<tbody>
<tr>
<td>pANL21 F-</td>
<td>TGA GCC TAC TCC TTG GTC TA</td>
<td>S. elongatus PCC 7942, gene 21 of plasmid pANL</td>
<td>NC_004073</td>
<td>522 bp</td>
<td>Similar to protein from <em>Nostoc</em> sp. PCC 7120</td>
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<tr>
<td>R-</td>
<td>GCA CAA AAG AAT CAC CAG AT</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pANL36 F-</td>
<td>GTG AAT GAG ACG ATC TTG GT</td>
<td>S. elongatus PCC 7942, gene 36 of plasmid pANL</td>
<td>NC_004073</td>
<td>578 bp</td>
<td>Cysteine sulfinate desulfurase</td>
</tr>
<tr>
<td>R-</td>
<td>ACC GTA TTT CCA CAA CTG AC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pANL53 F-</td>
<td>GTG CAA ATC TCT TTT AC</td>
<td>S. elongatus PCC 7942, gene 53 of plasmid pANL</td>
<td>NC_004073</td>
<td>588 bp</td>
<td>ATPase component</td>
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<tr>
<td>R-</td>
<td>GCA CTG AGG TCA GTC AGT TT</td>
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<tr>
<td>pUH24.1 F-</td>
<td>CGT GCT TGC TGA CGA GTT G</td>
<td>S. elongatus PCC 7942, gene 1 of plasmid pUH24</td>
<td>NC_004980</td>
<td>503 bp</td>
<td>Hypothetical protein</td>
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<tr>
<td>R-</td>
<td>GAC AGC GGT GAT CGC AGT AG</td>
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<tr>
<td>merA1 &amp; merA5 F-</td>
<td>ACC ATC GGC GGC ACC TGC GT</td>
<td>Shigella flexneri, <em>merA</em> gene of NR1 plasmid</td>
<td>K03088</td>
<td>1,238 bp</td>
<td>Mercuric reductase</td>
</tr>
<tr>
<td>R-</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CmerA F-</td>
<td>CAA ATA TGG AAG GCT TGG AG</td>
<td>S. elongatus PCC 6301, chromosomal <em>merA</em> gene</td>
<td>NC_008576</td>
<td>879 bp</td>
<td>Mercuric reductase</td>
</tr>
<tr>
<td>R-</td>
<td>ACT AGC GAG ACT CAT CCC TA</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

*F. forward; R. reverse*  
*Reference: Liebert et al. 1997*

Table 1. Primers used in molecular analysis of *Synechococcus* sp. IU 625 via Polymerase Chain Reaction. Primers pANL21, 36, and 53 and pUH24.1 were designed to determine the similarities between plasmid pANL of *Syn. elongatus* PCC 7942 and IU 625. Plasmid- and chromosomal-specific *mer* primers were designed to determine the presence and location of mercury resistance genes in *Syn. sp*. IU 625.

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E. Purification of PCR Products Using the QIAGEN QIAquick PCR Purification Kit (Cat. No. 28104) with Modifications

Five volumes of Buffer PBI were added to 1 volume of the PCR sample and mixed. The color of the mixture must remain yellow (similar to that of Buffer PBI before adding the PCR sample). A QIAquick spin column was placed into a provided 2 mL collection tube. To bind the DNA, the sample mixture was applied to the column and centrifuged for 30-60 seconds at 14,000 rpm. The flow-through was discarded and the column placed back into the collection tube. The sample was washed by adding 0.75 mL (750 µL) of Buffer PE to the column and centrifuged for 30-60 seconds at 14,000 rpm. The flow-through was again discarded and the sample was centrifuged again for one minute at 14,000 rpm. The column was removed from the collection tube and placed into a clean 1.5 mL microcentrifuge tube. To elute the DNA, 30 µL of sterile water was added to the center of the column membrane, allowed to stand for 1 minute, and then...
centrifuged for 1 minute at 14,000 rpm. The column was removed and the samples now in the 1.5 mL microcentrifuge tubes were stored in the -20°C freezer for future analysis via agarose gel electrophoresis or DNA sequencing. The detailed steps can be found in the handbook (appendix 2). The handbook states that centrifugation steps should be done at 13,000 rpm (17,900 x g). The issue here is that the rotor for the centrifuge in the lab had been calculated at 14,000 rpm being equivalent to 16,000 x g. Also the final step in which the DNA is eluted, the handbook says to use 50 μL but if you want a higher concentration of DNA you should use 30 μL instead.

F. Analysis of Miniprep Samples, DNA Extraction Samples and Purified PCR Products via Agarose Gel Electrophoresis

DNA extracts and purified PCR products were visualized on 1% agarose gels. Each gel was made by weighing out 0.4-0.7g of agarose (USB Corporation, Cat. No. 32802) and combining it with 40-70 mL of 1X TAE (Tris-Acetate-EDTA) buffer in a 125 mL Erlenmeyer flask. The mixture was heated in a microwave in intervals of 30 seconds until the agarose was completely dissolved. Two Kimwipes® were placed into the opening of the flask in order to prevent the build up of condensation within the flask. Once the mixture cooled, 1 μL of Ethidium Bromide was added to the flask and mixed until it dissolved into the mixture. The mixture was poured into a gel rig and allowed to solidify. A gel comb was placed into one end of the mixture after it was poured in order to produce the wells. Once the gel had solidified, the samples were prepared for loading by mixing 1 μL of 10X loading dye with 10 μL of sample. The gel was placed in the correct orientation, 1X TAE buffer was added to the gel rig until it completely covered the gel, and the samples along with the Hi-Lo DNA marker were loaded accordingly to
the gel. The gel was run at approximately 100-115V for 30-50 minutes, depending on the lengths of the DNA fragments of interest. The gels were analyzed under UV light using a Kodak Image Station 440CF (Perkin Elmer Life Sciences, Waltham, MA).

G. High Molecular Weight Band Isolation from Agarose Gels using the QIAGEN QIAquick Gel Extraction Kit (Cat. No. 28704) with Modifications

After running the agarose gel and verifying the presence of the high molecular weight band, the DNA fragment was excised using a sterile razorblade. The gel slice was weighed in a sterile 15 mL colorless test tube. This was done by putting an empty beaker on a scale, placing a 15 mL test tube inside the beaker without the top on and zeroing the scale. Once the gel slice had been placed inside the tube and the weight of the slice calculated, 3 volumes of Buffer QG were added to 1 volume of the gel (100 mg of gel is approximately equal to 100 μL) into the test tube. For example, if a gel slice weighs 0.4 g, or 400 mg, then a total of 1200 μL of Buffer QG should be added to the tube. The samples were incubated at 50°C for 10 minutes or until the gel slice was completely dissolved. The test tube was vortexed every 2 minutes during the incubation to help dissolve the agarose. After the gel slice is completely dissolved, the color of the mixture was checked (it should remain yellow. One gel volume of isopropanol was added to the test tube and mixed by inverting the test tube 3-5 times. To bind the DNA, the sample was pipetted from the 15 mL test tube into a QIAquick column and centrifuged for 40 seconds at max speed (14,000 rpm). Depending on what the total volume is in the test tube, this step may have to be repeated several times, making sure not to add more than 700 μL of the sample at a time into the column. The flow-through must be discarded after each centrifuge. To remove any remaining agarose, 500 μL of Buffer QG was
added to the column and centrifuged for 1 minute at 14,000 rpm. The sample was washed by adding 750 µL of Buffer PE to the column and centrifuged for 1 minute at 14,000 rpm. The flow-through was discarded and the column centrifuged again for another 40 seconds at 14,000 rpm. The column was placed into a clean 1.5 mL microcentrifuge tube, 40 µL of sterilized water added to the center of the column membrane, allowed to stand for 1 minute, and then centrifuged for 1 minute at 14,000 rpm to elute the purified DNA.

**H. RNA Isolation Using the RiboPure™ – Bacteria Kit (Applied Biosystems, Part No. AM1925) with Vortex Adapter (Part No. AM10024)**

After samples for the short-term study were collected at 0, 0.5, 1, 2, 4, 6, 8, 24 and 48 hours post inoculation with HgCl₂, total RNA was isolated. Samples were centrifuged at 14,000 rpm for 30-60 seconds and the supernatant removed. For each sample, approximately 250 µL of ice-cold Zirconia Beads were placed into 0.5 mL screw cap tubes. A diagram of where the beads should come up to in the tube was provided in the kit. Next, 350 µL RNAwiz was added to the cell pellet and the cells were resuspended by vortexing vigorously for 10-15 seconds. The cells in RNAwiz were transferred to the tubes containing the Zirconia Beads. These tubes were placed horizontally on the vortex adapter with the tube caps facing the center and vortexed at maximum speed for 10 minutes to lyse the bacterial cells. The bacterial lysates, approximately 200-250 µL per sample, was transferred to RNase-free 1.5 mL microcentrifuge tubes. Next, 0.2 volumes of chloroform were added to each sample, shaken vigorously for 30 seconds, and then incubate at room temperature for 10 minutes. Samples were centrifuged at 14,000 rpm for 5 minutes at 4°C. The aqueous phase (top) of each sample, containing the partially
purified RNA, was transferred to fresh RNase-free 1.5 mL microcentrifuge tubes. Next, 0.5 volumes of 100% ethanol were added to the aqueous phase of each sample and were mixed thoroughly. Next, filter cartridges were placed into 2 mL collection tubes and samples were added to the filter cartridges. Samples were centrifuged for 1 minute at 14,000 rpm to allow binding of the RNA to the filter cartridge and the flow-through was discarded. Samples were then washed by adding 700 μL of wash solution 1 to the center of the filter cartridge and centrifuged for 1 minute at 14,000 rpm. The flow-through was discarded and the samples were washed twice by adding 500 μL of wash solution 2/3 to the center of the cartridge and centrifuging for 1 minute at 14,000 rpm and discarding the flow-through after each wash. The filter cartridges were centrifuged for an additional minute to remove excess wash solution and then transferred to fresh 2 mL collection tubes. Total RNA was eluted by applying 30 μL of elution solution, preheated to 95-100°C, to the center of the filter cartridge. The samples were centrifuged for 1 minute at 14,000 rpm and the elution step was repeated by adding another 30 μL of elution solution in order to maximize total RNA yield.

Trace amounts of genomic DNA were removed from the eluted RNA by DNase I treatment. To each eluted RNA sample with a total volume of 60 μL, 4 μL of DNase I (2U/μL) and 1/9th volume (~6.67 μL) of 10X DNase buffer were added. Samples were incubated for 30 minutes at 37°C so that the DNase I could digest the genomic DNA. After the incubation period, a volume of DNase inactivation reagent equal to 20% of the volume of RNA being treated (i.e., 12 μL for 60 μL of treated RNA), was added to each sample. Samples were vortexed immediately after the addition of the DNase inactivation reagent. The samples were incubated at room temperature for 1 minute, flicking each
sample once or twice during the incubation period to resuspend the DNase inactivation reagent. Samples were centrifuged at 14,000 rpm for 1 minute to pellet the DNase inactivation reagent and the supernatants containing the RNA were transferred into new RNase-free 1.5 mL microcentrifuge tubes. Ten microliter aliquots of each sample were used subsequently in RT-PCR for the synthesis of cDNA and the remainder was stored in a -80°C freezer.

1. **Synthesis of cDNA Using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Part No. 4368814)**

Total RNA extracts from mercury-treated and control cells were used to synthesize cDNA to study the expression levels of plasmid and mercury resistance genes. Each RT-PCR reaction sample was prepared by combining the following reagents in a 0.2 mL RNase-free PCR tube: 2 μL 10X RT Buffer, 0.8 μL 25X dNTP Mix (100mM), 2 μL 10X RT Random Primers, 1.0 μL MultiScribe™ Reverse Transcriptase, 4.2 μL Nuclease-free H₂O, and 10 μL RNA sample for a total reaction volume of 20 μL. After combining all components in each PCR tube, the samples were centrifuged briefly to spin down the contents and to eliminate any air bubbles that may have formed during the preparation. The samples were placed into the Veriti™ 96-Well thermal cycler and were subjected to the following conditions, optimized for the use with the High-Capacity cDNA Reverse Transcription kit: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds. The samples were allowed to cool down to 4°C before being removed from the thermal cycler and placed into a -20°C freezer for storage. Please note that the step at 37°C for 120 minutes was set up as two separate steps for 60 minutes each because the
thermal cycler would not permit a time greater than 99 minutes to be entered in a single step.

**J. Induction of Plasmid and Expression of plasmid and mercury resistance genes via Real-Time Quantitative PCR**

PCR products from the reverse transcription reaction and genomic DNA extracts were subjected to Real-Time PCR in order to determine the expression levels of plasmid and mercury resistance genes. The following components were added to each sample: 12.5 μL of Brilliant SYBR Green QPCR Master Mix (Stratagene, Cat. No. 600548), 9.5 μL of sterile H₂O, 1 μL forward primer, 1 μL reverse primer, and 1 μL of cDNA for a total reaction volume of 25 μL. The reaction tubes were placed into pre-warmed MX3000P QPCR thermal cycler (Stratagene, Cat. No. 401405). The reaction profile used was: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56°C for 1 minute, and extension at 72°C for 1 minute. The samples were removed from the thermal cycler and placed into the -20°C freezer for future analysis via agarose gel electrophoresis.

**K. Primer Design for Real-Time PCR**

Table 2 lists all of the primers used for Real-Time PCR. Primers needed to be redesigned so that the PCR products were between 100-200 base pairs. Plasmid-specific pANL36 primers were designed based on the published sequences of the plasmids of *Synechococcus elongatus* PCC 7942, pANL. The pANL36 primers were 5'-GGG AAC TGG CAA TTG ATG AT-3' for the forward and 5'-GTA ACC CTT CCA ACG TCA GC-3' for the reverse primer. Primers were also designed for ribosomal subunit 16S to be used a control (*rpsL*) based on genomic sequence from strain PCC 7942. The *rpsL*
primers were 5'-CCG TAT TTG GAA CGG CTT T-3' for the forward and 5'-TTG GTC ACA ACC TGC AAG AG-3' for the reverse primer. Another set of plasmid-specific primers, NR1merA, were designed based on the merA gene of the NR1 plasmid of *Shigella flexneri*. The NR1merA primers were 5’-CAG CGA GAC GAT TCC TAA GC-3’ for the forward and 5’-GTG TGT TCC CTC ACC TCG AT-3’ for the reverse primer. Primers for chromosomal-specific mercury resistance gene CmerA were designed based on the genome sequence of *Synechococcus elongatus* PCC 6301. The CmerA primers were 5’-ACG TGC AAA CAC ACT GGG TA-3’ for the forward and 5’-GCC TCA CCC ACT TGA ATT GT-3’ for the reverse primer.

### TABLE 2: Primer Design for QPCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence (5’ to 3’)*</th>
<th>Source</th>
<th>Accession no.</th>
<th>Amplicon</th>
</tr>
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<tr>
<td>pANL36</td>
<td><strong>F</strong>- GGG AAC TGG CAA TTG ATG AT <strong>R</strong>- GTA ACC CTT CCA ACG TCA GC</td>
<td><em>S. elongatus</em> PCC 7942, gene 36 of plasmid pANL</td>
<td>NC_004073</td>
<td>135 bp</td>
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<tr>
<td>rpsL</td>
<td><strong>F</strong>- CCG TAT TTG GAA CGG CTT T <strong>R</strong>- TTG GTC ACA ACC TGC AAG AG</td>
<td>Small Subunit (16S) ribosomal gene <em>rpsL</em></td>
<td>CP000100.1</td>
<td>145 bp</td>
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<tr>
<td>NR1merA</td>
<td><strong>F</strong>- CAG CGA GAC GAT TCC TAA GC <strong>R</strong>- GTG TGT TCC CTC ACC TCG AT</td>
<td><em>Shigella flexneri</em>, <em>merA</em> gene of NR1 plasmid</td>
<td>K03089</td>
<td>198 bp</td>
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<tr>
<td>CmerA</td>
<td><strong>F</strong>- ACG TGC AAA CAC ACT GGG TA <strong>R</strong>- GCC TCA CCC ACT TGA ATT GT</td>
<td><em>S. elongatus</em> PCC 6301, chromosomal <em>merA</em> gene</td>
<td>NC_008578</td>
<td>198 bp</td>
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</table>

* F, forward; R, reverse

Table 2. Primers used in molecular analysis of *Synechococcus* sp. IU 625 via Quantitative Polymerase Chain Reaction. Plasmid-specific (pANL36 and NR1merA) and chromosomal specific (rpsL and CmerA) were designed to examine the expression levels of mercury resistance genes in *Syn. sp.* IU 625.
Results and Discussion

I. Effect of mercuric chloride in combination with ferric chloride on the growth of *Synechococcus* sp. IU 625

A large number of studies demonstrated the effects of various heavy metals on microorganisms such as bacteria, cyanobacteria and algae (29-32,34). The way in which different organisms respond to toxic heavy metals in the environment depends on the particular heavy metals that they are exposed to. The study of toxic heavy metals is important because they negatively affect marine ecosystems and thus contaminate sources of water and food that humans consume. Microorganisms have an inherent ability to adapt to their surroundings stemming from their evolutionary maturity. This adaptability can be very helpful in processes such as bioremediation if the organisms are used properly. However, the adaptability can actually hinder the environment and the biological entities within the particular ecosystem if ignored and not studied thoroughly.

A study conducted by Lee *et al.* (2000) to determine whether a protective effect could occur if constant mercury concentrations were placed in combination with varying concentrations of selenium showed that selenium did not produce a significantly beneficial effect on the toxicity of mercury in *Synechococcus* sp. IU 625. A quantitative approach was taken to determine the percentage of mercury and selenium that remained in the media alone of the inoculated cultures. Only 4.61% of the mercury was found in the media, compared with approximately 82.77% of the selenium. This suggested that the mercury had a greater affinity to the cells than the selenium. These results also indicated that *Syn.* sp. IU 625 cells were much less permeable to the selenium since most of it remained in the media. The authors hypothesized that this phenomenon could
potentially be occurring in other organisms, and thus suggested that it is necessary to investigate this probable phenomenon using other organisms and other combinations of heavy metals with mercury in order to see if there is a protective effect occurring in comparison to cyanobacteria growing in the presence of only a single toxic heavy metal.

In order to further investigate the potential protective effects of other heavy metals to the toxicity of mercury, the combined effects of iron and mercury were studied. A previous study looking at the effects of ferric chloride ($\text{FeCl}_3$) alone on $\text{Syn. sp. IU 625}$ revealed that the cells were able to grow in concentrations of up to 100 mg/L $\text{FeCl}_3$.
(Suzen Awad’s Thesis). The study also suggested that a concentration of 10 mg/L FeCl₃ might actually enhance the growth of the IU 625 cells and could be beneficial for the cells in response to stress from other heavy metals. Unlike the previous study where the concentrations of mercury were kept constant while varying the concentrations of selenium, three different concentrations of mercury (0.1, 0.5, and 1.0 mg/L) and a constant concentration of iron (10 mg/L) were used in this study (Figure 4). Control cells illustrate the standard growth curve of untreated *Synechococcus sp.* IU 625 grown in 3M medium. The growth of cells in the presence of 0.1 mg/L mercury did not appear to be inhibited as the growth curve for these samples was similar to that of control cells. Cell growth was significantly inhibited in the presence of 1.0 mg/L mercury. Interestingly, cells grown in the presence of 0.5 mg/L mercury exhibited a lag period of approximately 14 days before exponential growth began to occur. A previous study looking at the effects of mercury alone on the green algae *Chlamydomonas reinhardtii* showed that the algae were able to grow in the presence of up to 7.5 mg/L mercury (58). The difference in tolerance to mercury between cyanobacteria and the green algae appears to stem from the ability of each organism to metabolize mercury. Eukaryotic organisms such as *C. reinhardtii* have more complex systems involving many proteins and other structures involved in the metabolism of heavy metals when compared to prokaryotic cells like cyanobacteria (41).

After a period of 23 days, aliquots from control and mercury-treated samples were subsequently passaged to begin a new cycle of treatment (Figure 5). As was seen in passage 1, cells in the presence of 0.1 mg/L mercury from the second passage did not exhibit any signs of growth inhibition when compared to the control. Although the initial
lag period was again observed in cells grown in the presence of 0.5mg/L, however, the key difference in cells from the second passage was the shortening of the lag period from about 14 days from the first passage down to 4 days in the second. This data suggests that heavy metal resistance pathways to deal with the mercury stress may have become activated during the first passage. The data also suggest that the resistance may have remained activated when the cells were passaged and were reintroduced to the mercury stress. In correlation with this hypothesis, cells grown in the presence of 1.0mg/L mercury from the second passage exhibited an initial lag period of approximately 18 days followed by exponential growth that was not seen in the first passage. Therefore, these cells seem to have acquired a mechanism to combat the heavy metal stress that was introduced in the second passage.

Figure 5. Growth of Syn. sp. IU 625 post-inoculation with varying concentrations of HgCl₂ (2nd Passage).
Cells grown in the presence of 1.0mg/L from the second passage were exposed to a final round of treatment with 1.0mg/L HgCl₂ (Figure 6). The purpose of this was to determine if a decrease in the initial lag period would be observed as it was with 0.5 mg/L HgCl₂-treated cells. As was observed with the 0.5mg/L samples from passage 1 to 2, samples treated with 1.0mg/L mercury also exhibited a decreased initial lag period from about 18 days in passage 2 down to approximately 11 days in passage 3. The results from this study indicated that Syn. sp. IU 625 could acquire tolerance to the HgCl₂ as they are passaged. The results further suggest that there is some tolerance mechanism that is being induced in the presence of the mercury stress.

Figure 6. Growth of Syn. sp. IU 625 post-inoculation with 1.0 mg/L HgCl₂ (3rd Passage).
II. The uptake profile of HgCl$_2$ during the growth of *Synechococcus* sp. IU 625

One of the most important questions that we have to address when conducting research on toxic heavy metals is exactly how much of the metal is going into the cells. To further investigate the effects of mercury stress on *Syn. sp.* IU 625, mercury-treated cells were centrifuged; the media was removed and saved, and the cells were resuspended in fresh 3M media. Then, the metal content of the original media and the resuspended cells were analyzed. Samples were collected to determine the levels during short-term (24-hr) and long-term (17-day) mercury stress (Figures 7 and 8, respectively). After a period of 24 hours, the intracellular amount of mercury in samples treated with a concentration of 0.1 mg/L mercuric chloride was approximately equal to the amount of mercury in the growth medium (Figure 7A). These results were similar to those seen in the samples treated with 0.5 mg/L mercuric chloride (Figure 7B). The amount of mercuric chloride from the cells was slightly greater than the amount from the media after the 24-hour incubation period. Interestingly, the amount of mercury chloride from cells treated with 1.0 mg/L mercuric chloride was much greater than the amount from the media of those samples after the 24-hour period, approximately 69% compared to 31% in the growth media (Figure 7C). The results from the long-term exposure to mercury stress were quite surprising. After only three days of exposure, the amount of mercury from *Syn. sp.* IU 625 at all three treatment concentrations of mercuric chloride was 100% (Figure 8). Not only was all of the mercury inside the cells, it actually stayed inside the cells for the remainder of the 17 days. Only the cells treated with 0.5 mg/L mercuric...
chloride appeared to release some of the mercury back into the growth media by day 17 (Figure 8B).

The combined data from the long-term and short-term exposure studies suggest that when *Syn. sp.* IU 625 cells are treated with mercury, it appears as if they are taken by surprise because of how quickly the mercury is able to make itself into the cells. The results also suggest that the permeability for the HgCl₂ to get into the IU 625 cells is extremely high. Mercuric chloride had the highest permeability in IU 625 cells when compared to other metals such as copper (CuCl₂), zinc (ZnCl₂), and cadmium (CdCl₂) (data not shown). In *Syn. sp.* IU 625, copper is very impermeable (approx. 87-100%), while zinc and cadmium have higher levels of permeability (approx. 50% and 30%, respectively). There appears to be no significant transport mechanism in place for the cells to get the mercury out, and thus are very sensitive to mercury stress. The fact that the mercury is getting in so quickly also suggests that the cells do not have a specific defense system in place at the level of the plasma membrane to combat the mercury. Therefore, they have no way of blocking the mercury from making its way into the cells. This is why we believe there may be some other tolerance mechanism within the IU 625 cells that needs to be expressed for the cells to work on getting the mercury out.
Figure 7. Concentrations of mercury within *Syn. sp.* IU 625 cells and growth medium after 24-hour exposure to HgCl₂. *Syn. sp.* IU 625 cells were collected at 0, 2, 4, and 24 hours after treatment with 0.1, 0.5, and 1.0 mg/L HgCl₂ and the amount of mercury was determined by the Cold Vapor Technique. (A-C) Plots of the concentrations of HgCl₂ within the cells and the growth media. (D) Data tables of the concentrations plotted in the graphs along with the percent of HgCl₂ in and out of *Syn. sp.* IU 625 cells.
Figure 8. Concentrations of mercury within *Syn. sp.* IU 625 cells and growth medium after 17-Day exposure to *HgCl₂*. *Syn. sp.* IU 625 cells were collected at 0, 3, 8, and 14 days after treatment with 0.1, 0.5, and 1.0 mg/L *HgCl₂* and the amount of mercury was determined by the Cold Vapor Technique. (A-C) Plots of the concentrations of *HgCl₂* within the cells and the growth media. (D) Data tables of the concentrations plotted in the graphs along with the percent of *HgCl₂* in and out of *Syn. sp.* IU 625 cells.
III. Presence of high molecular weight plasmid in *Synechococcus sp.* IU 625

One of the few molecular studies for *Synechococcus sp.* IU 625 reported that this organism contains plasmids. In 1978, Simon isolated plasmids of different sizes, 4.7-MDa and 37-MDa, respectively (51). To correlate the findings from this study, mercury-treated cells were collected at an optical density equal to 1, were lysed, plasmid DNA isolated, and visualized via gel electrophoresis (Figure 9). The results indicated the presence of bands larger than 12kb at all concentrations suggesting the presence of a high molecular weight plasmid in HgCl$_2$-treated samples. The results also indicated that the higher the concentration of the HgCl$_2$, the denser the band is. This suggested that the higher the concentration of mercury in the samples leads to an increase in the amount of plasmid DNA. More quantitative studies should be carried out to determine the concentrations of the plasmid at these concentrations of mercury.

![Figure 9. Detection of high molecular weight plasmid via QIAprep Miniprep Plasmid Isolation. Mercury treated cells were lysed and plasmid DNA isolated. Isolated plasmid DNA was visualized on a 1% agarose gel.](image)

![Figure 10. Priming for gene 1 of *Syn. elongatus* PCC 7942 plasmid pUH24 with isolated plasmid DNA from *Syn. sp.* IU 625 cells. PCR analysis shows positive priming of isolated plasmid DNA with pUH24.1 primers.](image)
IV. Molecular comparison between plasmids of *Synechococcus elongatus* PCC 7942 and sp. IU 625.

The relationship between the plasmids isolated by Simon and the 7.6-kb (pUH24) and 46.6-kb (pANL) plasmids of strain PCC 7942 is unknown. Therefore, one of the goals of this study was to provide preliminary findings on the relationship between the plasmids. Primers designed to detect gene 1 of plasmid pUH24 showed positive priming in plasmid DNA extracts from mercury-treated *Syn. sp*. IU 625 (Figure 10). A PCR product of approximately 500 bp was visualized as expected. Primers designed to detect gene 21 of plasmid pANL also showed positive priming in all mercury treated samples (Figure 11A). PCR products of approximately 500 bp in length were visualized for each sample as expected. Sequence analysis of the PCR products using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) showed the sequence was 96% identical to that of gene 21 from pANL (Figure 11B and 11C). Interestingly, one of the BLAST results showed that gene 21 is flanked by sequences for a NarL homolog and a histidine protein kinase. Therefore, it possible that gene 21 may potentially be involved in signal transduction. Primers designed to detect gene 36 of plasmid pANL showed positive priming in plasmid DNA extracts from mercury-treated *Syn. sp*. IU 625 (Figure 12A). PCR products of approximately 600 bp in length were visualized for each sample as expected. Analysis of the sequenced PCR products (Figure 12B) exhibited 91% identity to the sequence from plasmid pANL of *Syn. elongatus* PCC 7942 (Figure 12C). Similar results were also observed with primers designed to detect gene 53 of plasmid pANL (Figure 13A-C).
Figure 11. Priming for gene 21 of *Syn. elongatus* PCC 7942 plasmid pANL with isolated plasmid DNA from *Syn. sp.* IU 625 cells. A) PCR analysis shows positive priming of isolated plasmid DNA with pANL21 primers. B) Sequence retrieved from overlapping the forward and reverse sequences of the sequenced PCR products. C) BLAST search results for retrieved sequence.
Figure 12. Priming for gene 36 of *Synechococcus elongatus* PCC 7942 plasmid pANL with isolated plasmid DNA from *Synechococcus* sp. IU 625 cells. A) PCR analysis shows positive priming of isolated plasmid DNA with pANL36 primers. B) Sequence retrieved from overlapping the forward and reverse sequences of the sequenced PCR products. C) BLAST search results for retrieved sequence.
Figure 13. Priming for gene 53 of Syn. elongatus PCC 7942 plasmid pANL with isolated plasmid DNA from Syn. sp. IU 625 cells. A) PCR analysis shows positive priming of isolated plasmid DNA with pANL53 primers. B) Sequence retrieved from overlapping the forward and reverse sequences of the sequenced PCR products. C) BLAST search results for retrieved sequence.
Figure 14. Mercury resistance via both plasmid and chromosomal pathways. Pure genomic DNA (A) and isolated plasmid DNA (B) extracts from Syn. sp. IU 625 cells were primed with chromosomal-specific (CmerA) and plasmid-specific (pANL36 and merAl&A5) primers. Genomic DNA shows positive priming with only CmerA while plasmid DNA shows positive priming with pANL36 and merAl&A5.

PCR products of approximately 600 bp in length were visualized for each sample as expected. A BLAST search of the sequence revealed 99% identity to that of pANL. The search also revealed that the sequence codes for an ATP-binding protein of the ATP-binding cassette (ABC) transporter SrpK. SR protein kinases are kinases that are specific for the serine/arginine family of splicing factors. The results from this study indicated that the three regions from the isolated DNA obtained from Syn. sp. IU 625 cells have high homology to the respective regions in plasmid pANL of related strain Syn. elongatus PCC 7942. Therefore, the data suggests that the high molecular weight bands that were visualized from the miniprep experiments were plasmid in origin.
V. Mercury resistance via both plasmid and chromosomal mediated pathways.

Previous studies have shown that mercury resistance (mer) operons are found commonly in plasmids (33,41). However, the possibility remains that mercury resistance determinants may also be encoded by chromosomal genes as reported in many bacteria. In order to determine the location of mercury resistance in *Synechococcus sp.* IU 625 at the level of DNA, plasmid-specific and chromosomal-specific primers were used to prime isolated plasmid DNA and pure *Syn. sp.* IU 625 genomic DNA, respectively. In pure genomic DNA, positive priming was seen only with *CmerA* primers (Figure 14A). Conversely, positive priming was seen with both pANL36 and *merA1&A5* primers but not with *CmerA* primers in isolated plasmid DNA (Figure 14B). The PCR products of *CmerA* and *merA1&A5* were sequenced and analyzed using BLAST (Figures 15A-B and 16A-B). BLAST search results for the sequence of *CmerA* exhibited 98% identity to the genomic sequences of related strains PCC 6301 and PCC 7942 (Figure 15B). Interestingly, BLAST search results for the overlapping sequence of *merA1&A5* exhibited 90-95% identity to plasmid sequences with aberrant mercury transposons from other organisms (Figure 16B). Therefore, the data suggests that the location of the *CmerA* is genomic and *merA1&A5* and pANL36 genes are plasmid in origin. Moreover, the results also suggest that mercury resistance in *Synechococcus sp.* IU 625 may be located in both plasmid and genomic DNA.
A

**Synechococcus elongatus PCC 6301 merA complete sequence**

GTTAGCTCTGCGATTTCACAACAAATATCCTGACCTGCCATCACCACCATCCCAACGATTGATATGAAACCCCG
AGCCCTGCTATTGCTGATGATCCGCTGCTGGATGTTGGGGGAGAACACTCGGATGCTGCTGCTGGGAAGCC
GACAGGATAGGTTGGCTTTAAAGGTTGCTTAAATGTGAAAAGCAACTATGCGGGTGGTAGATCTGCTCATAT
TTGGTTGTGTTGCCTCTATGAAAGCTATGCTATGCTATGCTATGCTATGCTATGCTATGCTATGCTATGCTATG
GCCGCAGCTCTGCTTTTGTGATGGTAAACCGAACAATGTCACGCGGAGGGCAGCCACCTACGAAGTTGAGATG
TGATTCGAACCTGGCAGGGAGAGGTATCCATCTCGATCTCGATCTCGATCTCGATCTCGATCTCGATCTCGA
TGAGACTGTTTCTCTCTGTATTTGGTACCTGGCCGCCATTGCTATTGGGGAGAGGCAGCCGACTCGTTGAC
GAGTTAGCTGCAAGCTTCCCCAACATGCAGCTGAACGTCGCGTTTTTAAACAGGCTAGCCGCAACTGTTTAC
CTAAAGGAGGATTAGCGGCTAAGGTCGCTGCTGGTACCCGGAAGATTCATACATACATATATATATATATATAT
CTGAAATTTATTGCGGCTCGGGTAATGCTGCTGCTGCCATGACCCGGAAGGGCAGCCTCGGCTGCCATGACCCG
CGATGGTGATTAAAAATGCGCTTTTTCACCCGTTGGCATTGCAAAACTTAAAGTGTCGCTGCTGCTGCTGCTG
TTCCGCGTTAATTTTACGATCGGAGAACGCGGCAATGCGGCAATGCGGCAATGCGGCAATGCGGCAATGCGG
AGGAATTTCGACTGCACAAATTTCACTCCTTCGATCAAGTGACGCTCAGCGCTTATGTTGGTCCTGACAACTGAA
GCCGGGTATTAGGAAATTCATCATCAGGAAATCCACGATAATTATTCGGCGCAGAGGTTGGTCCCTGAC
ACCGAGGTTAAATGATTTCAGGTCGACTACCGGCTACGGGAAATGCTGAGGCTAGCTGCTGCTGCTGCTGCTG
TGTCATTAGCTTTCGATCAGGGAAATAGATGACAGCTGACTTAAACCGCGCTGACTCTTACCTAGAACAAGCTATGCTA
CTAACAACCCATGCAAAACAGCTACTGAAAACTTAAATGCTAAAATAAA

F primer: CAAATAATGGAAGGCTGAA
R primer: ACTCGAAGCAGCTCAGGCTA

B

**Distribution of 2 Blast Hits on the Query Sequence**

Mouse over to see the define, click to show alignments

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Figure 15. Priming for *CmerA* of *Synechococcus elongatus* PCC 7942 with pure DNA from *Syn. sp.* IU 625 cells. A) Sequence retrieved from overlapping the forward and reverse sequences of the sequenced PCR products. B) BLAST search results for retrieved sequence.
**A**

Shigella flexneri merA gene of R1 plasmid complete sequence

ATGAGCACT CTCAAAAT CACC35GCATGACT TGCGACTCGT GCGCAGTGCAT GT CAAGGACGOCCTGGA

Figure 16. Priming for merAI & A5 of Syn. elongatus PCC 7942 with pure DNA from Syn. sp. IU 625 cells. A) Sequence retrieved from overlapping the forward and reverse sequences of the sequenced PCR products. B) BLAST search results for retrieved sequence.
VI. Induction of high molecular weight plasmid in *Synechococcus sp.* IU 625 in response to mercury.

Because the previous experiment suggested that mercury resistance genes are located on the high molecular weight plasmid of *Syn. sp.* IU 625, the next step was to determine whether or not production of the plasmid was being induced in response to the mercury stress. Plasmid DNA was isolated at 0, 3, 7, 10 and 14 days from *Syn. sp.* IU 625 after treatment with HgCl$_2$ and primed with pANL36 primers during real-time PCR (Figure 17). The process of real-time PCR works by collecting fluorescence data at the end of each cycle of the reaction. The SYBR Green dye used in the experiment binds to the double-stranded PCR products, causing the dye to fluoresce. As the reaction continues, the instrument records the threshold cycle for each sample. The threshold cycle ($C_t$) is the critical cycle at which the first significant increase in fluorescence is detected. Once the PCR reaction ended, the data was collected the $C_t$ values for all samples were analyzed. The standard for comparing $C_t$ values is that a difference in one $C_t$ is equivalent to a two-fold difference in the amount of DNA. Results showed an eight-fold difference in the amount of pANL36 from Day 0 to Day 3 (Figure 17B). The levels of pANL36 appeared to decline after Day 3, returning approximately to the same levels as before treatment with HgCl$_2$. This data suggests that the plasmid is being induced early on after the introduction of the mercury stress.
Figure 17. Long Term Induction of High Molecular Weight Plasmid in Syn. sp. IU 625. A) Standard Curve of plasmid DNA. B) Cycle threshold of pANL36 DNA. C) Samples visualized on a 1% agarose gel.
VII. Short-Term expression levels of heavy metal resistance genes in *Synechococcus sp. IU 625*.

A previous study by Liu *et al.* (2004) examined the expression levels of a metal transporter and a metallothionein in the cyanobacterium *Oscillatoria brevis*. The findings from their study revealed that at fixed concentrations of zinc, copper, cadmium, and manganese, there are temporal expression of both the metal transporter and the metallothionein. The temporal expression of these genes was seen very early on after the introduction of the metal. Since *Synechococcus sp. IU 625* have a metallothionein and maybe genes that are heavy metal specific, it would be quite interesting to examine the expression levels of the general and specific heavy metal responses of this cyanobacterium. Moreover, a study of this sort has never been done for *Synechococcus sp. IU 625*.

In order to determine the expression levels of metallothionein and mercury resistance genes, samples were obtained from control and mercury-treated samples at 0, 0.5, 1, 2, 4, 6, 8, 24, and 48 hours and the total RNA was isolated and used for cDNA synthesis with random primers. Before real-time PCR was performed, the primers designed for the study were tested with both DNA and the cDNA to ensure that accurate priming could be achieved. Traditional PCR results showed positive priming with all primer sets with both DNA and cDNA expect the set designed to prime the plasmid-specific *merA* (Figure 18A and 18B, respectively). Since there were no visible signs of primer-dimer formation in any of the samples, it was suggested that the non-specific binding was not occurring. It was hypothesized that the reason for this was because the primer set for the plasmid-specific *merA* had a higher melting temperature (Tm) than the
Figure 18. QPCR Primers Test for Short-Term Expression of Heavy Metal Resistance Genes. Primers designed for plasmid-specific (NR1merA) and chromosomal-specific (CmerA) mercury resistance genes, along with pANL36, and 16S ribosomal subunit (rpsL) primers were tested on DNA (A) and cDNA (B) extracts from *Syn. sp.* IU 625 cells. Samples were visualized on 1% agarose gels.

rest of the primer sets. Another trial run was performed the primer annealing temperature was increased from 56°C to 60°C. However, raising the melting temperature still proved to be ineffective. It is possible that the temperature may actually be higher than 60°C because the primer annealing temperature for the plasmid-specific merA primers that were used to detect the presence of the gene in plasmid DNA extracts was actually 64°C.

After the primers were tested, a real-time PCR reaction was set up to determine the levels of expression of the respective plasmid and chromosomal genes. However, there was an issue with the run and unfortunately results could not be retrieved. There are a few possibilities as to what may have gone wrong during the run. First, the plate
setup in the Mx3000P software may have been set up incorrectly and therefore data was
not collected properly. However, samples were visualized on a 1% agarose gel, and none
of the samples exhibited any amplification (data not shown). Thus, an error that could
have been made was that the concentration of either cDNA or primers used was too low.
This too seems unlikely because the standard protocol used in real-time PCR states that
final concentrations of 10-100ng of cDNA and 50nM primers should be used per sample.
The concentrations used during the run were 100ng cDNA and 100nM primers.
Therefore, it is unlikely that this was the issue. Future studies should be carried out to
working with one set of primers at a time to provide positive and more accurate results.
Conclusions

The study of toxic heavy metals and how they contaminate sources of water and food that humans consume is an ongoing process. The ability of cyanobacteria such as Syn. sp. IU 625 to thrive in the presence of certain heavy metals can be a tremendous disadvantage to higher organisms. As humanity becomes more aware of the negative effects toxic pollutants have on ecosystems, it is necessary to develop our understanding of this ability and turn it into something advantageous for the environment. An excellent way to make use of this ability in microorganisms like Syn. sp. IU 625 is in the process of bioremediation to help remove heavy metals like mercury from the environment.

A ubiquitous cyanobacterium, Syn. sp. IU 625 has been used in many studies as an indicator of heavy metal toxicity. In this study of heavy metal resistance in Syn. sp. IU 625, we concluded that as the cells are moved from passage to passage, they become more tolerant to concentrations of 0.1, 0.5, and 1.0 mg/L of HgCl₂. The shortening of the lag period from passage 1 to 2 that is seen in samples treated with 0.5 mg/L HgCl₂ indicates that there is or are mechanisms of resistance that these cells are developing. The data findings from this study also suggest that the resistance mechanisms are being set in motion by the end of the first passage that when the cells enter the second passage and are reintroduced to the heavy metal stress, they are able to adapt to the stress a faster and more efficiently than before.

Molecular comparison of the high molecular weight plasmid of Syn. sp. IU 625 to plasmid pANL of related strain Syn. elongatus PCC 7942 revealed that at least three genes (21, 36, and 53) have a high degree of sequence similarity. This data suggests that there may be a high degree of similarity between the plasmids of these two strains.
Molecular analysis of *Syn. sp.* IU 625 cells also revealed that these microorganisms have mercury-specific resistance genes both in the genome and in the high molecular weight plasmid that they harbor. It is unknown as to how *Syn. sp.* IU 625 may have acquired resistance at both the genomic and plasmid levels.

In studying the induction of the high molecular weight plasmid of *Syn. sp.* IU 625 in response to toxic concentrations of mercury, we concluded that the plasmid is induced very early on after the introduction of the mercury stress. Higher levels of the plasmids were identified after a period of only three days. Therefore, we hypothesized that the resistance mechanisms in cells from the second passage respond very quickly to the stress and are able to deal with the problem in a very efficient manner. It is unfortunate that the study on the expression of the resistance genes did not go as planned because it truly would have given a great representation as to what is really occurring in *Syn. sp.* IU 625 cells when they are confronted with toxic levels of mercury. Fortunately, the study was not a total loss. All of the primers with the exception of the plasmid-specific *merA* have been shown to amplify cDNA that was synthesized from total RNA extracts. So even though we were unsuccessful in getting the real-time PCR reaction to work, we are only a step away from gaining a deeper understanding of the mechanisms of mercury tolerance in *Synechococcus sp.* IU 625.
Future Studies

The findings of this study have provided a path for future research into the mechanisms of heavy metal tolerance in *Synechococcus sp.* IU 625. One of the next steps is to continue where this research left off. Almost everything is in place to begin the analysis of expression levels of the genes designed for heavy metal tolerance. The primers have been designed and tested, but only one set must be analyzed further or possibly redesigned. Whether each gene is studied individually or all are done together, we believe that the data from such a study would greatly enhance our understanding of the how this microorganism is responding to stress from heavy metals.

The mercury resistance operon is composed of eight different genes. Now that we have identified one of the key components of the mercury resistance operon, mercuric reductase (*merA*), it would be very interesting to probe for the remaining components of the operon. A follow-up study could involve analyzing the interplay between the genes and how one affects the expression of one or more of the others. Since we also know that *merA* is located on both the high molecular weight plasmid and genome of *Syn. sp.* IU 625, another project could be to determine if transposons played a role in bringing about this type of resistance.

Since the only sequence data available for *Syn. sp.* IU 625 is the metallothionein gene, a sequencing project could be done to map out the genome and/or the high and low molecular weight plasmids of the microorganism. Data from this study would allow us to determine the exact degree of similarity between *Syn. sp.* IU 625 and related strains *Syn. elongatus* PCC 7942 and PCC 6301 at the DNA level.
References


APPENDIX 1

Protocol: Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 μg of high-copy plasmid DNA from 1-5 ml overnight cultures of E. coli in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 44.

Please read “Important Notes” on pages 15-21 before starting.

Note: All protocol steps should be carried out at room temperature.

Procedure
1. Resuspend pelleted bacterial cells in 250 μl Buffer P1 and transfer to a microcentrifuge tube.
   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
   If lyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure lyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. Add 250 μl Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lys reaction to proceed for more than 5 min.
   If lyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. Add 350 μl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g., 5 ml) may require inverting up to 10 times. The solution should become cloudy.
   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. Centrifuge for 10 min at 13,000 rpm (17,900 x g) in a tabletop microcentrifuge.
   A compact white pellet will form.

5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

6. Centrifuge for 30-60 s. Discard the flow-through.

7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30-60 s. Discard the flow-through.
   This step is necessary to remove trace nuclease activity when using endA strains such as the JM series, HB101 and its derivatives, or any wildtype strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α do not require this additional wash step.

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30-60 s.

9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μl Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

Protocol: Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and 5 ml Collection Tubes

The QIAprep Spin Miniprep procedure can be performed using 5 ml centrifuge tubes (e.g., Greiner, cat. no. 115101 or 115261) as collection tubes to decrease handling. The standard protocol on pages 22-23 should be followed with the following modifications:

Step 4: Place a QIAprep spin column in a 5 ml centrifuge tube instead of a 2 ml collection tube.

Step 6: Centrifuge at 3000 x g for 1 min using a suitable rotor (e.g., Beckman GS-6R centrifuge at ~4000 rpm). (The flow-through does not need to be discarded.)

Steps 7-8: For washing steps, centrifugation should be performed at 3000 x g for 1 min.
   (The flow-through does not need to be discarded.)

Step 9: Transfer the QIAprep spin column to a microcentrifuge tube. Centrifuge at maximum speed for 1 min. Continue with step 10 of the protocol.
QIAquick PCR Purification Kit Protocol
using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 4). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MiniElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 60 µl pH indicator I to 1.50 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of 5.7-5.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure
1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or xylene.
   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH indicator I has been added to Buffer PB, check that the color of the mixture is yellow.
   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.
   Collection tubes are re-used to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30-60 s.
7. Discard flow-through and place the QIAquick column back in the same tube.
   Centrifuge the column for an additional 1 min.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average elution volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.
   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at -20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.
   Loading dye contains 3 marker dyes (bronzophorol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

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