CYANOBACTERIAL CLASS II METALLOTHIONEINS

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To my beloved mother, Annicleta Francisco for her unselfish love and for supporting me in any endeavor I undertook. And to my dear grandmother, Maria Francisco, for her unending love and guidance. Mama, ki ora ku mi tabatin mester di bo judansa bo tabata kla pa judami. Masha danki pa bosnan karinjo i amor. Dios Bendishona bosonan.

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ABSTRACT

Cyanobacteria, like many other bacterial species, manifest various metal resistance mechanisms. Some cyanobacteria have been shown to be resistant to arsenate at levels exceeding that found in natural waters. They are also capable of nickel uptake, a metal necessary for hydrogenase and urease activity. Cyanobacterial metal resistance mechanisms range from plasmid encoded mechanisms, the production of siderophores, to the excretion of other intracellular components (through cell lysis) which bind metal ions in order to offset their availability in the medium. A potentially very important metal resistance mechanism employed by the cyanobacteria is the production of metallothionein proteins. Metallothioneins are low-molecular-weight proteins or polypeptides which bind metal ions predominantly via metal-thiolate clusters. To date, the presence of prokaryotic metallothionein has been only demonstrated in *Synechococcus* species. Seven different cyanobacterial species surveyed in this study by polymerase chain reaction (PCR) gave a PCR product comparable to the size of the metallothionein gene PCR product observed for *Synechococcus*, except *Anabaena* and *Fischerella* which give a larger PCR product. RFLP's were performed to demonstrate the integrity of the DNA isolated from the various strains and to rule out DNA contamination. *E. coli* strain B ultra pure DNA yielded a PCR product of equivalent size to that of *Synechococcus* PCC 7942. BLAST search of protein databases led to the match of an unclassified *E. coli* K12 protein with the *Synechococcus* PCC 7942 *smt*A primers and protein sequence. Further analysis of this protein leads to its classification as a class II metallothionein protein. We have also shown the presence of plasmids in cyanobacterial strains isolated from metal contaminated soils from the El Paso area.

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INTRODUCTION

1.1 The Cyanobacteria

Cyanobacteria are gram-negative prokaryotes capable of oxygenic photosynthesis. Fossil evidence for the existence of cyanobacteria on earth dates back approximately 2.5 billion years. With the appearance of the cyanobacteria, a new element was introduced into the atmospheric equation, oxygen. The release of oxygen, through cyanobacterial oxygenic photosynthesis, lead to the gradual transformation of earth's primarily reducing atmosphere into an oxidizing one. Oxygen was then available for the evolution of an aerobic mode of heterotrophic metabolism on earth (Fay, 1992). The major distinguishing trait between the cyanobacteria and other prokaryotes, except *Prochlorales*, is their dual photosystem which allows the use of H_2O as photoreductant with the consequent liberation of O_2. This process is associated with chlorophyll a, both as a light-harvesting pigment and as a reaction center pigment (Hensyl, 1989).

Cyanobacteria are the most thoroughly studied and understood group of the oxygenic photosynthetic prokaryotes. They range from < 1 to >100 μm in diameter. Only a small percentage of the cyanobacteria are presently in culture due to the difficulty in growing a large variety of them using the present culture methods. The cyanobacteria can appear in three different forms: 1) unicellular, 2) colonial, and 3) filamentous. Cyanobacteria are found in a wide array of environments. Their distinctive properties have allowed them to exist and predominate in soil and bodies of fresh and marine water (Hensyl, 1989).

Most cyanobacteria multiply through binary fission by constricting all envelope layers, to include the outer membrane and sheath, inward until cell separation is complete

or nearly complete. Some filamentous strains divide by constriction of cytoplasmic membrane and peptidoglycan layer without involvement of the outer membrane and sheath.

Even though pili are abundant in cyanobacteria, motility has been limited to a swimming type of motion perceived in some unicellular strains and a gliding motion observed in most strains, primarily in the filamentous ones (Hensyl, 1989).

Thylakoid membranes, absent in other eubacteria, are found in the cyanobacteria. These membranes serve as pigment-bearing apparatus. Hemispherical phycobilisomes are found on both surfaces of the thylakoids in an orderly row. Phycobilisomes are complex protein-pigment aggregates found in most cyanobacteria except in the*Prochlorrales* order.

Beside their oxygenic photosynthetic capabilities, some strains of cyanobacteria are also capable of fixing atmospheric nitrogen (Brahamsha *et al.*, 1991, Buikema *et al.*, 1991). Members of the *Nostocales* and *Stigonematales* produce heterocysts, at intervals in trichomes, through vegetative cell differentiation under combined inorganic nitrogen deprivation. This process compensates for the lack of combined nitrogen from the biosphere which results from the continuous process of bacterial de-nitrification (Fay, 1992, Hensyl, 1989). Heterocysts are only found in the cyanobacteria. Cyanobacteria also produce akinetes which are unique to them. Akinetes are granular cells which differentiate from vegetative cells and accumulate cyanophycin, glycogen, lipids and carotenoid pigments (Hensyl 1989).

The cyanobacteria can be divided into five different orders:

- **Subsection I.** Order *Chroococcales*; this order consists of unicellular or nonfilamentous aggregates of cells held together by outer walls or gel-like matrix (colonies) or filamentous strains; trichome of cells branched and unbranched, uniseriate

or multiseriate, which undergo binary fission in one, two ore three planes, symmetric or asymmetric or multiply by budding.

- **Subsection II.** Order *Pleurocapsales*, these strains reproduce by internal multiple fissions with production of daughter cells smaller than the parent or by multiple and binary fission.
- **Subsection III.** Order *Oscillatoriales*, these strains' trichomes are composed of cells which do not differentiate into heterocysts or akinetes.
- **Subsection IV.** Order *Nostocales*, one or more cells of each trichome of the strains in this order differentiate into a heterocyst when concentration of combined nitrogen is low; some also produce akinetes.
- **Subsection V.** Order *Stigonematales*, some strains found in this order undergo binary fission in one plane only giving rise to uniseriate, unbranched trichomes, although false branching may occur; others undergo binary fission periodically or commonly in more than one plane, giving rise to multiseriate trichomes or trichomes with true branches or both.

The above mentioned orders can be further sub-divided into a variety of genera, group and species. The cyanobacterial strains used in this study belong to four of these orders, namely the *Chroococcales*, the *Nostocales*, the *Oscillatorias*, and the *Stigonematales*. One other strain pertaining to an order not considered by all scientist as cyanobacterial, *Prochlorothrix hollandica*, was also used (Hensyl 1989).

From the order *Chroococcales* two different genera were used, genus II (*Gloebacter*) and genus III (*Gloethece*). From the genus *Gloebacter* the group *Synechococcus*, strain PCC 7942 was used. *Synechococcus* PCC 7942 are unicellular coccoid to rod-shaped cyanobacteria that are less than 3 μm in diameter. These

cyanobacteria contain photosynthetic thylakoids which are located peripherally, and lack structured sheaths. They were isolated from hot springs and do not possess any gliding or swimming motility. The two other strains from the order *Chroococcales* that were used belong to the genus *Gloethece*, the group *Synechocystis*, strain PCC 6714 and ATCC 22663. *Synechocystis* PCC 6714 pertains to the high GC-cluster. Their cells are unicellular and range between 2-3 μm in diameter and are spherical to oval in shape. The major light-harvesting pigment found in this cluster is the phycocyanin. No gliding or swimming motility have been observed in this strain. This strain was isolated from freshwater. *Synechocystis* ATCC 22663 belongs to the microcystis-cluster. Cells in this cluster are unicellular, spherical to oval in shape and between 3 to 8 μm in diameter. They occur singly or in pairs and some of them form loose aggregates. They possess gas vacuoles and produce toxins (Appendix A). They occur in freshwater and do not display any type of motility (Hensyl 1989).

From the Order *Nostocales* cyanobacterial strains from genus I and V were used. From genus I the group *Anabeana*, strain ATCC 22664 was used. *Anabaena* ATCC 22664 are filamentous, with trichomes that are straight, curved or helically (spherically) formed. The cells can be cylindrical, spherical, or ovoid. The width ranges from 2 to 10 μm. Some species have displayed widths over 20 μm. This strain produces heterocysts and is capable of nitrogen fixation. They are mostly found in brackish marine water and possess both phycoerythrocyanin and phycocyanin. All vegetative trichomes in the *Anabaena* species display gliding motility. It is this gliding motility of vegetative cells that primarily distinguishes *Anabaena* from the *Nostoc* group. *Nostoc* belongs to genus V and the strain ATCC 29105 was used in this study. Their cells are cylindrical, spherical, or ovoid and their trichomes appear in confluent gel holding masses to form massive thalli which may be

spherical, ovoid or of less discernible shape. Motility is seen in trichomes near or between heterocysts. *Nostoc* strains can be found in freshwater or in moist terrestrial environments. Furthermore, *Nostoc* display a developmental cycle which involves differentiated hormogonia which is not seen in *Anabaena* (Hensyl 1989).

The next strain used, group *Spirulina*, strain ATCC 53843, is part of the order *Ocillatoriales*, genus I. *Spirulina* are filamentous organisms that divide exclusively by binary fission and grow in tight to nearly tight coiled right- or left-handed helices. Their trichomes show a width range between < 1 μm to about 5 μm. They possess gliding motility in the form of transverse movement with little forward motion. These strains have been isolated from a variety of fresh, marine, and brackish waters worldwide.

From the order *Stigonematales*, genus II, the group *Fischerella*, strain ATCC 27929 was used. *Fischerella*'s true branches are uniseriate and composed of cells that are ordinarily longer than they're broad. The branches arise from axes which are mainly uniseriate as well but can become multiseriate, in part by divisions in more than one plane. The axes in the division regions rarely get thicker than 2-3 cells. *Fischerella* are filamentous cyanobacteria. *Fischerella* strains are commonly found in flowing hot springs and display a gliding, rotating trichome motility and form heterocysts that are different from the ones found in the order *Nostocales* (Hensyl 1989).

Another group of oxygenic bacteria that was also used in this study belongs to the order *Prochlorales*. *Prochlorales* are not considered by all scientists as being members of the cyanobacteria group. The oxygenic bacteria in this group can be either unicellular or filamentous, branched or unbranched prokaryotes that only differ from the cyanobacteria in that they form chlorophylls a and b and fail to express accessory red or blue bilin pigments. The member of this order that was used in the present study is from the genus

Prochlorothrix, strain *hollandica.* This strain is the only representative of this genus. It is composed of straight, sheathless trichomes of variable length. Its cells are cylinder-shaped and are 3-15 μm long and 0.5-3 μm in diameter. Until now no cell differentiation has been observed and no nitrogen fixation activity has been detected in this strain. Their habitat is freshwater and they are free living. This strain does not display any motility on agar plates (Hensyl 1989).

1.2 Mechanisms For Resistance to Metals

1.2.1 Bacterial metal resistance

1. Prevalence of Metal Resistances among the bacteria

In addition to the above mentioned, very distinctive characteristics, cyanobacteria, like many other bacterial species, manifest metal resistance properties. Metal resistance is a very valuable trait in microorganisms considering the role of environmental pollution in today's society. In recent years, some of these metals have been targeted as major causes, and others suspected of being possible causes, of environmental pollution (Nakahara *et al.*, 1977).

Since resistance to metals is widespread among microorganisms, experiments have been performed using a variety of organisms like*Escherichia coli, Klebsiella Pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* to determine the frequency of this resistance. Results indicated that the frequency of metal resistance in these strains was equal to, or higher than that of antibiotic resistance. Antibiotic resistance is often expressed on plasmids (Hardy, 1981, Nakahara *et al.*, 1977).

Some metals, although in low concentrations, are essential for the growth of both prokaryotic and eukaryotic cells since they serve as enzyme cofactors (Trevors *et al.*,

1986). Metals are thought to enter cells by means of the same transport systems as the nutrients that are structurally related to them (Silver *et al.*, 1981). For instance, arsenate enters cells through the less specific phosphate uptake mechanism, the Pit system, of the bacterial cell (Silver *et al.*, 1981).

Even though chromosomal metal resistance does manifest itself in many plasmidless bacteria, heavy metal resistances in microorganisms are still widely associated with the presence of plasmids (Nakahara *et al.*, 1977). Plasmid genes are thought to confer added resistance to the cells through reduced uptake and accelerated ATP dependent efflux of the heavy metal. Chromosomally regulated resistance in some organisms is hypothesized to arise as a consequence of mutations in chromosomal genes. For example, in the arsenate resistance mechanism, elimination of the uptake mechanism less specific to the nutrient (phosphate) through a mutation can convey resistance for a particular substance (arsenate) (Silver *et al.*, 1981).

Other metal resistance mechanisms are employed by different bacterial species. The general understanding, is that metals enter the cells and are subsequently exported by means of accelerated efflux (Mobley *et al.*, 1982). The transport systems that have been implicated in these processes in bacteria can be categorized into three classes: (1) group translocation systems, (2) secondary porters linked directly to the proton motive force, and (3) systems coupled to phosphate bond energy (Mobley *et al.*, 1982).

a. Arsenic Resistance As a Model of Metal Resistance Mechanisms

Transport systems coupled to phosphate bond energy have been a recurrent theme in various articles (Silver *et al.*, 1981, Mobley *et al., 1982*, Chen *et al.*, 1985, Ji *et al., 1992*, Rosenstein *et al., 1992*, Broer *et al., 1993*) since this system is thought to be employed by the arsenate-arsenite-antimonite resistance mechanism. This mechanism is

one of the most thoroughly studied and consequently one of the better understood mechanisms of heavy metal resistance. Arsenate resistance is very common in a variety of strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Nakahara *et al.*, 1977), and *Staphylococcus xylosus* (Rosenstein *et al.* 1992).

Arsenate, arsenite and antimonite resistances have been determined to be encoded by the same operon. The arsenical resistance genes of this operon have been suggested to posses at least two loci, one encoding arsenate resistance and the other arsenite and antimonite resistance (Rosenstein *et al.*, 1992). The arsenical operon found on the plasmid R773 from *Escherichia coli* consists of five genes designated arsR, arsD, arsA, arsB and arsC while on plasmid pI258 from *Staphylococcus aureus* and plasmid pSX267 from *Staphylococcus xylosus* three genes are found, arsR, arsB and ArsC (Broer *et al.*, 1993, Rosenstein *et al.*, 1992).

The arsR, arsB and arsC genes in all three organisms show similarities both in sequence and function (Rosenstein *et al.*, 1992, Broer *et al.*, 1993). The arsR gene appears to encode for a repressor protein which regulates the system (Ji *et al.*, 1992) in all three of these organisms. arsB encodes for a membrane protein (Rosenstein *et al.*, 1992) which functions as the channel of an arsenite pump in *E. coli* . This same function is suspected in *Staphylococcal* strains considering the degree of homology (58%) exhibited by the proteins (Rosenstein *et al.* 1992). Even though the arsC protein displayed a very low degree of homology (18%) between the *Staphylococcal* and *E. coli* strains it is believed to fulfill the same function, namely, reduction of arsenate to arsenite in the presence of DTT and thioredoxin, in all three of the organisms. The arsA protein which is expressed only in the *E. coli* strains is thought to be an arsenite or antimonite-stimulated ATPase subunit

which, in conjunction with arsB, carry out an energy dependent efflux of oxyanions of arsenic leading to its reduced cellular accumulation (Ji *et al.*, 1992). The arsD protein, expressed in *E. coli*, encodes a secondary down-regulatory protein functioning independently from the repressor arsR protein.

To determine the exact role of the different proteins in the arsenate resistance mechanism, a variety of experiments were done. In 1985 Chen *et al.* reported that insertional mutations in the ars operon of R773 produced a variety of phenotypically different plasmids. Insertion within the proximal-distal region of the operon lead to the inactivation of arsenate resistance but not arsenite resistance, strengthening earlier arguments that the arsenic operon consisted of two separate loci for arsenate and arsenite resistance. This insertion caused the disappearance of a 16 kDa polypeptide suggesting the involvement of this protein in the regulation of arsenate resistance. It was interesting to note that according to an expression study of the arsR, B, and C genes in *E. coli*, arsC expressed a polypeptide of approximately 15,500 kDa (Rosenstein *et al.*, 1992). These observations strengthen the conclusions that Chen et al. arrived at; insertion in this gene probably leads to the distruction of the arsC locus and, in so doing, impairs or eliminates, the process of arsenate reduction into arsenite. As a result arsenate accumulates in the cells and will not be effluxed through the arsB protein, since this protein is thought to be arsenite specific (Broer *et al.*, 1993).

In 1982 Mobley *et al.* demonstrated that arsenate efflux in *E. coli* cells required a source of energy both with and against a concentration gradient. At the same time they showed a positive correlation between ATP generation and arsenate efflux. Sources of proton motive force, like succinate, did not accelerate the rate of arsenate efflux. When the proton motive force was inhibited by a blocker of the respiratory process (cyanide), in the

presence of glucose as a source of energy, the rate of arsenate efflux was not noticeably reduced. Following these experiments they proceeded to osmotically shock the *E. coli* cells. Though osmotic shock usually leads to the loss of phosphate binding proteins in the cell membrane, there was no such loss in these cells. There was a loss of ATP pools, but this loss by itself was not enough to account for the decrease in arsenate efflux from the cells. The proton motive force appeared to be unaffected since the uptake of proline, a shock resistant system driven by proton motive force, remained unaltered. The experiments failed to provide an airtight arguments for the need of ATP in the rate of arsenate efflux. Resynthesized ATP after recovery from the osmotic shock also appeared insufficient to drive the arsenate efflux. The findings did provide enough evidence to rule out proton motive force as a source of energy in arsenate efflux. Results of these experiments point in the direction of ATP or a closely related phosphorylated compound as the driving force of energy.

Much less is known about the mechanism of arsenite uptake and efflux in bacteria. It has been determined that arsB functions as the membrane protein through which arsenite is exported (Broer *et al.*, 1993). The arsenite and antimonite uptake mechanisms are largely unknown. These two metals show similar resistance patterns and are therefore treated the same experimentally, considering the fact that it is still unknown whether there are two separate genes encoding for resistance to these two metals (Silver *et al.*, 1981). In 1981 Silver *et al.* demonstrated that the mechanism of resistance to these two metals does not involve their oxidation to less toxic forms nor their binding by soluble thiols excreted by resistant cells.

b. Resistances to Other Metals

Accelerated efflux is not specific to arsenic resistance. This system is widely employed in a variety of metal resistance mechanisms in bacteria. *Alcaligenes eutrophus* CH34, *Alcaligenes xylosoxidans* 31A and *Alcaligenes eutrophus* KT02 possess plasmid determined metal resistances for nickel, cobalt, zinc, cadmium, and copper. *Alcaligenes eutrophus* CH34 contains two plasmids which confer resistance to an assortment of metal ions. Three of these resistance mechanism have been characterized in detail. The chr system encodes resistance to chromate, the cnr to nickel and cobalt while the czc encodes resistance to cobalt, zinc, and cadmium. The metal resistance properties of all three of these systems rely on active transport of these metals out of the cell. Co^{2+}, Zn^{2+} and Cd^{2+} enter the cells of *Alcaligenes eutrophus* via the magnesium uptake systems (Nies, 1995). These cations are actively extruded from the cell in the presence of the czc determinant. The czc determinant codes for five polypeptides: CzcR and CzcD which act as regulatory proteins in czc expression, and CzcA, CzcB and CzcC which together form an efflux complex through which the cations are exported. Nies determined that the CzcA, CzcB and CzcC complex formed a cation-proton antiporter which did not require ATP, since inhibition of the proton motive force by the uncoupler FCCP lead to inhibition of cation/proton exchange.

In 1994 Schmidt and co-workers determined that *Alcaligenes xylosoxidans* 31A possessed two distinct loci, ncc and nre, which conferred nickel resistance. Little is still known about the nre locus except that it confers low-level nickel resistance to *A. eutrophus* and *E. coli*. The ncc locus seems to code for seven polypeptides designated, NccA, NccB, NccC, NccN, NccX, NccY and NccH. Through comparisons it was determined that the different proteins had possibly the same functions in *A. xylosoxidans* as the cnr and czc

loci in *A. eutrophus* CH34. The sequencing of the ncc operon has not been completed and all the regulatory genes have not been identified. The emphasis at the present time is on identification of the genes and their functions, considering that, besides similarities, this operon also shows differences that should be taken into account when classifying the systems.

1.2.2 Cyanobacterial metal resistance

It has been difficult to analyze cyanobacterial genetics by conventional methods of bacterial genetics (Van den Hondel *et al.*, 1980). This has led to difficulty in the isolation, mapping and analysis of genes at the DNA level and consequently contributes to the mystery surrounding the functions of plasmids isolated from cyanobacterial strains.

Plasmids are double stranded DNA molecules which are not part of the main chromosome. Plasmids can also be inherited separately from the chromosome. The molecular weight of identified bacterial plasmids ranges from $1x10^6$ to $200x10^6$ daltons. Most plasmids exist as covalently closed circles. Plasmids can be found in three different conformations in bacteria: a) *Supercoiled*, double stranded covalently closed circle plasmid twists to form supercoils, b) *Relaxed*, one of the polynucleotide strands in a closed circular plasmid is broken to form a relaxed circle, and c) *Linear*, both polynucleotide strands are broken (Hardy *et al.*, 1981).

Genes found on plasmids code for a wide variety of metabolic activities and often enable bacteria to degrade compounds which would accumulate as toxicants or pollutants if they were not degraded by micro-organisms. Furthermore, bacterial plasmids enable some bacteria to fix nitrogen or encode antibiotics (Hardy *et al.*, 1981, Rebiere *et al.*, 1986). Plasmids are very important in medicine and agriculture because they are capable of