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Production of Copper-Complexing Ligands in Response to Elevated Concentrations of Copper in Marine *Synechococcus* spp.

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PRODUCTION OF COPPER-COMPLEXING LIGANDS
IN RESPONSE TO ELEVATED CONCENTRATIONS OF COPPER
IN MARINE SYNECHOCOCCUS SPP.

by

Arunsri Chatkaemorakot Brown
M.Sc. May 1984, Mahidol University, Thailand

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Eastern Virginia Medical School
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May 1999

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ABSTRACT

PRODUCTION OF COPPER-COMPLEXING LIGANDS
IN RESPONSE TO ELEVATED CONCENTRATIONS OF COPPER
IN MARINE SYNECHOCOCCUS SPP.

Arunsri Chatkaemorkot Brown
Eastern Virginia Medical School/Old Dominion University, 1999
Director: Dr. Andrew S. Gordon

Synechococcus spp. (marine cyanobacteria) are extremely sensitive to copper toxicity and can produce high-affinity ligands of unknown structure which form complexes with free cupric ion. These ligands may contribute to the biological control of the levels of free cupric ions in surface seawater. Synechococcus spp. are known to produce metallothioneins (MT) in response to cadmium and zinc stress. In the present study the hypothesis that marine Synechococcus produce MT in response to copper was tested. Three marine Synechococcus spp. i.e., PCC 7001, PCC 73109, and PCC 7003, were exposed to different concentrations of CuSO₄ for various time periods. Size exclusion chromatography, atomic absorption spectrophotometry, and reverse phase HPLC were used to isolate an intracellular copper binding component (CBC) of low molecular weight (< 6,500 Da). Synechococcus PCC 73109 produced an intracellular CBC after exposure to ≥8 μM CuSO₄ for 2 hr. The intracellular CBC was shown not to be MT, phytochelatin or a siderophore. It is not a peptide; it contains lysine and an unidentified UV 254-absorbing component. This compound is a novel copper-binding ligand previously not reported in Synechococcus spp. and may function as a component of a copper export system.
To my parents and my family
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CHAPTER I
INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Inorganic elements required by living cells in small quantities are termed "trace elements". Trace elements include iron, copper, zinc, manganese, cobalt, iodine, molybdenum, vanadium, nickel, chromium, fluorine, selenium, silicon, tin, boron, and arsenic. Trace elements are essential components in the action of certain plant and animal enzymes, in nutrition and in metabolism. Humans and animals require microgram to milligram quantities each day. Trace elements are of interest to scientists in many fields. Trace metals are essential as cofactors for many metabolic enzymes in living organisms. They, also, are essential in every aspect of genetic regulation. A deficiency in metal ions or an insufficient concentration can lead to errors in DNA replication, transcription, and translation (Renault & Deschatrette, 1997; Hoogeveen et al., 1995). Concentrations that are too high or too low can reduce the reproductive rate of microorganisms and can be lethal in animals and humans. For example, two serious illnesses of humans involving copper metabolism are Menkes syndrome and Wilson disease. The clinical symptoms of these two inherited disorders are distinct. The former is an X-linked disorder of copper transport which results in copper deficiency and is fatal to children 3 to 5 years of age (Silver & Phung, 1996). The latter is an autosomal recessive disorder of copper transport resulting in the

The Journal model used is Limnology and Oceanography.
accumulation of hepatic copper (Harris & Gitlin, 1996). High concentrations of trace metals found in humans are derived mainly from food sources, plants, animals, soils, freshwater reservoirs, and marine environments.

Some bacteria are highly resistant to metal ions in their environments. Bacteria have developed resistance mechanisms to toxic inorganic cations and anions in the environment. Bacterial plasmids have been identified which carry genes for specific resistance systems for elements such as Ag, Cd, Cu, Ni, and Zn (Cervantes & Gutierrez-Corona, 1994; Ji & Silver, 1995). Enzymatic detoxification systems have been identified which function by efflux pumping of toxic ions from cells or by bioaccumulation, which may involve the binding of toxic ions to bacterial proteins or polypeptides (Cervantes & Gutierrez-Corona, 1994). Toxicity of metal ions to cell functions is dependent upon the concentrations of metal ions. As reviewed by Fiore & Trevors (1994), in cyanobacteria such as *Nostoc calcicola*, a copper concentration of 5 $\mu$M can completely inhibit the evolution of CO$_2$ and photophosphorylation. A copper concentration of 40 $\mu$M can decrease photosystem I activity by 15.5% and can inhibit photosystem II activity by 50%. Also, 40 $\mu$M copper can inhibit CO$_2$ fixation by 95.4% and can decrease ATP content by 32.3%. A copper concentration of 100 $\mu$M can inhibit 50% of glutamine synthetase activity. Glutamine synthetase is an enzyme which plays a critical role in controlling nitrogen metabolism by conversion of free NH$_4^+$ into glutamine. Also, 100 $\mu$M copper can inhibit 40% of nitrogenase activity.

In oceanic environments, trace metals can influence plankton production
and microbial community structure (Bruland et al., 1991). In surface seawater, the concentration of cupric ions ([Cu$^{2+}$]) is high enough to decrease reproduction of the most sensitive phytoplankton, the cyanobacteria (Brand et al., 1986). These bacteria produce organic ligands of unknown structure, which bind to free Cu$^{2+}$ and form Cu-complexes. The mechanism(s) by which this occurs is unknown. Knowing the structure of these ligands and the mechanism of their production will help to understand how these bacteria respond to toxic levels of free Cu$^{2+}$.

**Trace metals in marine ecosystems**

Determining the concentrations and distributions of trace metals, as well as their biogeochemical cycling is of fundamental importance to understanding the marine ecosystem. Metals including manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), molybdenum (Mo), copper (Cu), and zinc (Zn) are bioactive trace metals required by phytoplankton for various metabolic functions. For example, copper is a cofactor for the enzyme plastocyanin that participates in the photosynthetic electron transport chain; zinc is a cofactor for the enzyme carbonic anhydrase that is involved in hydration and dehydration of CO$_2$; iron and molybdenum are cofactors for the enzyme nitrogenase that is involved in nitrogen fixation (Sunda, 1989). A deficiency of these trace metals may limit oceanic plankton production (Brand et al., 1983) and an excess of these metals may inhibit plankton growth (Brand et al., 1986; Sunda, 1989). Natural concentrations of trace metals in seawater are very low, mostly in the nM to pM.
range. Higher total concentrations of trace metals are found more often in coastal seawater than in oceanic seawater (Brand et al., 1986).

**Sources of trace metals in seawater**

Trace metals in seawater are derived from the atmosphere, weathering processes, the interaction of seawater with newly formed oceanic crustal basalt associated with hydrothermal activity (Donat & Bruland, 1995), waste products from mining activities, industries, (Purves, 1985) and domestic waste.

The relative contribution of atmospheric and riverine input is different for various trace metals (Donat & Bruland, 1995). For example, atmospheric fluxes of lead (Pb) and zinc (Zn) to surface coastal waters are equal to or exceed their riverine fluxes. In contrast, the atmospheric fluxes for Mn, Ni, Co, Cr (chromium) and Cu are an order of magnitude less than their riverine fluxes but the two fluxes are the same order of magnitude for Al (aluminum), Fe and Cd (cadmium). The relative contribution of atmospheric and riverine input also varies regionally. For example, atmospheric deposition has the greatest influence on the surface water of the North Atlantic and the least on that of the South Pacific.

**Removal of trace metals in seawater**

Dissolved trace metals are removed from seawater to marine sediments either by adsorption on to sinking biogenic particles or by being taken up by phytoplankton, followed by sinking of biological detritus. Before their ultimate removal, trace metals undergo various degrees of recycling which involves the
chemical desorption and redissolution reactions in the water column (Donat & Bruland, 1995). The process involved in recycling is biogeochemical; involving biological and geochemical interactions. These reactions are photomediated i.e., photoreduction, photodissolution, and photoinhibition. For example, dissolved Mn$^{2+}$ is removed nightly by Mn-oxidizing bacteria, followed by daily regeneration and maintenance by reductive photodissolution processes and photoinhibition of the Mn-oxidizing bacteria. Such reactions control the concentration of trace metals available for microorganisms present in the water.

**Interaction among trace metals in seawater**

Many metals act synergistically or antagonistically to influence growth or toxicity to microorganisms. Studies using laboratory cultures have demonstrated that the uptake and metabolism of nutrient metal ions are functions of the concentrations of other metals which often act in a competitive or antagonistic manner (Donat & Bruland, 1995). It is not known whether competition occurs at cell surface uptake sites, or at intracellular metabolic sites, or both. Metal antagonisms have been reported for copper and zinc (Rueter & Morel, 1981), copper and manganese (Sunda et al., 1981), copper and iron (Murphy et al., 1984). One study showed that oceanic diatoms could tolerate up to $10^{-7}$ M copper when $10^{-6}$ M iron was present. In contrast, the copper toxicity threshold of the oceanic diatom was reduced to less than $10^{-8}$ M in the absence of added iron (Murphy et al., 1984). The effects of iron on copper toxicity were possibly due to intracellular competition for active sites or to absorption of copper by iron.
precipitates in the medium.

Many studies have shown that when free Cu$^{2+}$ in seawater exceeds a certain critical value, the ratio of two free metal ion concentrations may be more important than the absolute concentration of any one metal alone (Steemann-Nielsen & Wium-Anderson, 1970; Stauber & Florence, 1985). To study the true response of marine microorganisms to high concentrations of free Cu$^{2+}$ ions, the study of an intracellular response should be considered because an extracellular response can be either from the cell itself or from environmental factors such as protozoans or other living organisms (Donat & Bruland, 1995).

**Metal uptake by microorganisms**

The uptake of metals by microorganisms generally comprises two phases: (1) binding of cations to the negatively charged groups on the cell surface, and (2) the subsequent metabolism-dependent intracellular uptake via metal porter molecules. Intracellular uptake can also be a result of permeation and diffusion due to increased membrane permeability (Fiore & Trevors, 1994). When the availability of Fe$^{3+}$ is limited, siderophores, low molecular weight iron chelators that bind to Fe$^{3+}$ with high affinity (up to $10^{32}$), are excreted from most bacteria, cyanobacteria, and fungi, into the surrounding water. Ferric siderophore chelates are then actively taken into the cell by specific permeases and the ferric ions are then released for metabolism (Sunda, 1989). The siderophore-mediated system is essential not only for its solubilization of unavailable ferric hydroxide but also for its specificity to a particular microorganism so that the iron
can be reserved for that species (Butler, 1998).

Siderophores have been detected in freshwater systems, in cyanobacterial algal mats in salt marshes, but have not been detected in bulk seawater (Sunda, 1989). Although laboratory cultures of marine species of coccoid cyanobacteria demonstrated the production of siderophores in iron-deficient culture (Wilhelm & Trick, 1994), the molecular mechanisms oceanic phytoplankton use to sequester iron are unknown (Butler, 1998). Siderophores are capable, also, of binding other metal ions such as copper but with a lower affinity \(10^8\) than for iron. However, marine microorganisms living in surface seawater may have evolved specific uptake systems for metal ions other than iron because these metal ions are complexed to undefined organic ligands distinct from ferric complexing ligands (Butler, 1998).

**Trace metal speciation and toxicity**

The natural concentrations of bioactive metals in seawater are very low. Thus, chemical speciation plays an important role in their oceanic chemistry. Trace metals in seawater can exist in different oxidation states and chemical species i.e., as free ions, as inorganic complexes with \(\text{Cl}^-\), \(\text{SO}_4^{2-}\), as organic complexes with phytoplankton metabolites and as organometallic compounds in which trace metals are covalently bound to carbon as methyl or ethyl forms. These different oxidation states and species undergo different biological and geochemical interactions (Donat & Bruland, 1995).

Several transition metals in their free ionic form are available to
phytoplankton and are toxic when their concentrations are high. The nutrient availability and toxicity of these metals are decreased when they form complexes with ligands i.e., ethylenediaminetetraacetate (EDTA). Among the several trace metals present in seawater, copper is of special interest because the natural concentrations of free Cu$^{2+}$ ($10^{-9}$-$10^{-13}$M) are close to toxic levels ($>10^{-12}$M) for some species of phytoplankton (Brand et al., 1986). The natural concentrations of other trace metals are generally below the toxic level to phytoplankton. In addition, intense upwelling can bring water having Cu$^{2+}$ concentrations approaching $10^{-11}$M into the surface photic zone. This concentration is potentially high enough to limit reproduction of some phytoplankton species, especially cyanobacteria (Sunda, 1989).

In contrast, the toxic levels of free Cd$^{2+}$ can exceed $10^{-10}$ M for the most sensitive phytoplankton, which is well above the range of its natural concentration (Brand et al., 1986). Thus, cadmium toxicity may not be an important selection factor for phytoplankton growth or ecology in oceanic waters (Donat & Bruland, 1995). Requirements of iron, manganese, zinc are habitat related in oceanic and neritic phytoplankton species. They are consistent with the oceanic-neritic distribution of these metals. In general, oceanic phytoplankton species living in an environment having much lower metal concentrations, can tolerate much lower free Fe$^{3+}$, Mn$^{2+}$, and Zn$^{2+}$ concentrations before showing reduced reproductive rates (Bruland et al., 1991).
Cu-complexing ligands

In natural seawater, copper speciation is dominated by complexation with naturally occurring organic ligands that can be determined using a ligand exchange/liquid-liquid partition procedure. In this procedure, acetylacetone (aqueous phase) and toluene (organic phase) were added to seawater (Moffett et al., 1990). At equilibrium, copper distribution between the aqueous and organic phase was determined. An acid-extractable copper acetylacetonate complex was formed in the aqueous phase and naturally occurring copper complexes, which were non-extractable, were determined by atomic absorption spectrophotometry. The fraction of free Cu\textsuperscript{2+} in the absence of acetylacetone was calculated. All data obtained were plotted as Scatchard plots. Conditional stability constants and concentrations of the naturally occurring ligands were estimated from these plots. A single ligand system yields a straight line whereas a multiligand system yields a curved line. Thus, Cu-complexing ligands (L) and the conditional stability constant of the binding strength between Cu\textsuperscript{2+} and the ligand(s) in a complex are determined by computing the side reactions involving metal and ligand present in seawater. In a two-ligand model, copper speciation in the southwestern Sargasso Sea is dominated by two ligand classes, class 1 (L\textsubscript{1}) and class 2 (L\textsubscript{2}). L\textsubscript{1} has a high conditional stability constant of \(10^{13.2}\) and a low concentration at 2 nM. L\textsubscript{2} has a lower conditional stability constant of \(10^{8.7}\) and a higher concentration at 80 nM (Moffett et al., 1990). In surface waters of the central northeast Pacific, the Sargasso Sea, and the South San Francisco Bay, total dissolved Cu\textsuperscript{2+} is bound to strong organic complexes (L\textsubscript{1}) in the upper
200 meters. Binding of copper to L1 causes the free hydrated Cu$^{2+}$ concentrations to vary from approximately $10^{-13.1}$ M at the surface to approximately $10^{-9.9}$ M at 300 meters.

In the central north Pacific, total dissolved copper concentrations increase from 0.5 nM at the surface to $\approx 5$ nM at 4,000 meters (Bruland et al., 1991). The concentration of L1 in the upper 100 meters is $\approx 1.8$ nM which exceeds the dissolved copper concentrations from the surface down to $\approx 200$ meters (Coale & Bruland, 1988, 1990). The excess in [L1] causes the high degree of organic complex formation observed in the upper 200 meters and causes the free Cu$^{2+}$ concentration to increase about 2000-fold from the surface to 300 meter-depth.

Among phytoplankton, only cyanobacteria have been demonstrated to produce a chelator that forms copper complexes of the same strength as those observed in the Sargasso Sea. The study of four marine phytoplankton species indicates that only the cyanobacteria, Synechococcus sp., produce this strong Cu-binding organic ligand (Moffett et al., 1990). However, the chemical structure of L1 is unknown.

*Synechococcus* are widely distributed in the surface waters of the open ocean and play an important role as primary producers in the marine ecosystem. Thus, the chelators produced by this genus may exert a major influence on copper speciation in many oceanic regions (Bruland et al., 1991).

**Biology of cyanobacteria**

Cyanobacteria are gram negative, oxygenic photosynthetic prokaryotes.
They are widespread in freshwater, marine, and terrestrial environments. Their sizes range from < 1 to > 100 \( \mu \text{m} \) in diameter (Fiore & Trevors, 1994). They are found as unicellular, colonial, filamentous, or branched filamentous forms. Motility, if exhibited, is by gliding and not by flagellar movement. Cyanobacteria are the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes (Stanier & Cohen-Bazire, 1977), and are often abundant in metal contaminated freshwater habitats (Say & Whitton, 1980; Whitton & Shehata, 1982).

**Cell composition of Cyanobacteria**

The cell envelope consists of outer and cytoplasmic membrane bilayers. Between the outer and cytoplasmic membrane is the periplasmic space in which a peptidoglycan layer is present (Fiore & Trevors, 1994). Many species have additional layers external to the outer membrane. These layers are called the cell wall layers, the sheath capsules, or the slime layers (Fiore & Trevors, 1994; Golecki, 1977; Stanier & Cohen-Bazire, 1977).

Ultrastructural studies of cyanobacteria suggest that these organisms are gram negative due to their cellular organization (Allen, 1968). However, some cyanobacteria react positively in a gram stain reaction (Jurgens et al., 1985) and have properties more comparable to those of gram positive bacteria than gram negative bacteria i.e., the thicker peptidoglycan layers, the degree of cross-linkage and the presence of polysaccharide covalently linked to muramic acid-6-phosphate of peptidoglycan via phosphodiester bonds (Jurgens et al., 1983,
The outer membrane of the cyanobacteria contains lipopolysaccharide, proteins, lipids, and carotenoids (Jurgens et al., 1985; Murata et al., 1981; Woitzik et al., 1988). Lipopolysaccharides of cyanobacteria do not contain acidic constituents i.e., low or no phosphorous containing acidic sugar derivatives and are neutral or exhibit a low negative charge. In other bacteria, lipopolysaccharides are located on the outer surface and provide a negative charge.

The chemical composition of the sheath and the slime layer of cyanobacteria consists mainly of polysaccharide that is composed of at least one uronic acid and several neutral sugars. Some species contain sulfate and phosphate which may provide binding sites for cations (Fior and Trevors, 1994).

Cyanobacteria contain chlorophyll $a$ in thylakoids that are paired photosynthetic lamellae. The outer surface of thylakoids possess granules called phycobilisomes which are comprised of phycobiliprotein pigments i.e. phycocyanin ($\lambda_{\text{max}}$ 620 nm), allophycocyanin ($\lambda_{\text{max}}$ 650 nm), and allophycocyanin B ($\lambda_{\text{max}}$ 670 nm). Some cyanobacteria contain phycoerythrinoid pigments, i.e. C-phycoerythrin ($\lambda_{\text{max}}$ 550-570 nm) and phycoerythrocyanin ($\lambda_{\text{max}}$ 565-590 nm) (Rippka et al., 1979).

**Multiplication of cyanobacteria**

Unicellular cyanobacteria divide by binary or multiple fission, or by release of apical cells called exosporos. Filamentous cyanobacteria multiply
asexually by fragmentation, a process which involves formation of a transverse wall and breakage of the long filament into an individual cell of shorter length. Some filamentous cyanobacteria produce specialized cells called akinetes and heterocysts. Akinetes constitute a thick-walled resting stage which eventually germinates to produce short chains of cells called trichomes. Heterocysts contain thick cell wall and refractile polar granules. Heterocysts do not function in reproduction but serve as sites for nitrogen fixation (Rippka et al., 1979; Stanier et al., 1971).

Synechococcus spp. are unicellular rod-shaped cyanobacteria in the family Chroococcaceae and multiply by binary fission or budding. Synechococcus spp. possess thylakoids and contain DNA with a G+C ratio of 45-71 mole% (Stanier et al., 1971).

Significance of cyanobacteria

Cyanobacteria are important organisms in marine food chains (Iturriaga & Mitchell, 1986). In addition, they are major primary producers in marine ecosystems. The cyanobacteria, Synechococcus spp., contribute 60% to 95% of the total primary production in some regions (Iturriaga & Marra, 1988). Some species of cyanobacteria can fix nitrogen and are used in soil development for agriculture (Kulik, 1995). A number of cyanobacteria can produce various biologically active compounds that can inhibit bacteria and fungi. Thus they are the potent biocontrol agents of plant pathogenic bacteria and fungi (Kulik, 1995). Because of the abundance of cyanobacteria in natural environments, both
polluted and non-polluted, a significant contribution to metal absorption and desorption has been attributed to these organisms (Fiore & Trevors, 1994).

**Mechanisms of copper detoxification**

Resistance to the toxicity of metals may be achieved by preventing the access of metals to sensitive cellular components or altering them to reduce their sensitivity. To survive under excessive concentration of physiologically required metals, e.g. copper, cells must achieve a balance between the accumulation of sufficient metal for the maintenance of metal-dependent activities and the toxicity of supra-optimal metal concentrations. Thus, rapid alteration of gene expression may be necessary for adequate metal accumulation by the cell (Rouch et al., 1995). Numerous detoxification mechanisms for copper have been reported. Bacterial strains such as marine bacterium *Alteromonas atlantica* and *Klebsiella aerogenes* that contain a capsule as their outermost envelope can tolerate high copper levels by binding of copper to the capsular exopolymers through polysaccharide carboxyl groups (Cervantes & Gutierrez-Corona, 1994). Copper uptake and copper efflux by P-type ATPase is a prominent copper resistance mechanism in humans and prokaryotes i.e., *Escherichia coli*, *Enterococcus hirae* (formerly called *Streptococcus faecalis*) and *Pseudomonas syringae* (Silver & Phung, 1996). In *Escherichia coli*, chromosomal genes for uptake and efflux are involved in metabolic functions and are required for the expression of copper resistance as well as in normal, low-copper situations. When high concentrations of copper are present, the
plasmid encoded gene products will function by interaction with efflux proteins to form an efflux system (Cervantes & Gutierrez-Corona, 1994). In the cyanobacterium *Synechococcus* PCC 7942, the copper uptake ATPase is located on cellular membranes but the efflux ATPase is located on the thylakoid membrane (Kanamaru et al., 1993, 1994).

Complexation of copper with sulfide produced by sulfate-reducing bacteria, *Desulfovibrio* spp. (Temple & LeRoux, 1964) and by marine *Synechococcus* sp. (Walsh et al., 1994) has been reported as another copper resistance mechanism. In *Mycobacterium scrofulaceum*, copper sulfide production, a copper resistance mechanism, is plasmid-encoded (Erardi et al., 1987). Marine bacterium, *Vibrio alginolyticus* produces an extracellular copper-binding protein to detoxify high concentrations of copper (Harwood-Sears & Gordon, 1990). Induction of metallothionein protein (MT) by copper in freshwater *Synechococcus* spp. was thought to be another mechanism of copper detoxification (Robinson et al., 1990). However, deletion of the MT encoding gene did not yield a copper-hypersensitive organism (Turner et al., 1993). An ATPase-dependent copper uptake was suggested as a possible copper resistance mechanism (Silver et al., 1993; Turner & Robinson, 1995).

**Characteristics of metallothionein**

Metallothioneins (MT), small thiol-rich metal binding proteins, have been found in many animals, plants, and higher eukaryotes. A few cyanobacterial strains are the only prokaryotic cells with a well-studied metallothionein system.
MT was first discovered in eukaryotes. MT is a protein of less than 10,000 MW with cysteine comprising one-third of the amino acid residues. MT can detoxify metals by forming mercaptide bonds between the sulfhydryl functional group (-SH) of cysteine residues and reduced metal ions (Hamer, 1986). The metal-thiolate complexes formed are responsible for the characteristic charge transfer bands located in the ultraviolet region (λ ~ 280-300 nm) of the absorption spectrum and give rise to yellow or orange characteristic luminescence in the visible region (near 600 nm) (Beltramini & Lerch, 1981). In animals, MT does not contain aromatic amino acids. MT can not be detected at 280 nm but can be detected at 254 nm due to the lack of aromatic amino acids. The most extensive studies of MT are in eukaryotes and they are used as references in the study of MT in other genera.

Classification of MT

Class I MT is produced in mammals and crustaceans whereas class II MT (which has cysteine residues in different positions than mammalian MT) is produced in yeast and cyanobacteria (Winge et al., 1985; Olafson et al., 1980). In contrast to class I and class II, which are genetically coded, class III MT is synthesized by the enzyme phytochelatin synthetase by repeatedly adding γ-EC (glutamyl-cysteinyl) residues to glutathione (Robinson & Jackson, 1986). All glutamate residues are linked to adjacent amino acids through γ-carboximide linkages (Reddy & Prasad, 1990). The synthesis of phytochelatins can be inhibited by buthionine-SR-sulfoximine (BSO), a specific and potent inhibitor of
phytochelatin synthetase (Reese & Wagner, 1987). Class III MT is found in some yeast as cadystins and in some plants as phytochelatins.

Structures of MT

The structure of MT consists of two distinct clusters of amino acids. The A cluster contains 11 cysteines and is contained within the carboxy-terminal α domain extending from amino acid 31 to 61. The B cluster contains 9 cysteines and is contained in the amino-terminal β domain extending from amino acid 1 to 30 (Hamer, 1986). The structure of Cu-MT is quite different from that of Cd or Zn-MT due to stoichiometry of metal binding (Nielson et al., 1985). In mammalian cells, MT binds to cuprous ion and the ratio of cysteine to cuprous ion is <2. Thus, the stoichiometry of Cu(I)-MT is M\textsubscript{12}-MT and the coordination of Cu(I) to MT is trigonal three-coordination or linear two-coordination. In contrast, the ratio of cysteine to cadmium or zinc is about 3 and the stoichiometry of Cd/Zn-MT is M\textsubscript{7}-MT which forms the tetrahedral four-coordination (Nielson et al., 1985; Robbins & Stout, 1992).

This structural difference causes the properties of Cu-MT to be more complex than Cd,Zn-MT. As a result, polyacrylamide gel electrophoresis (PAGE) which is effective for the isolation and quantification of Cd, Zn-MT, can not be successfully applied to Cu-MT, probably due to the presence of copper that causes a smear in the gel (McCormick & Lin, 1991); the Cu-protein molecule was misidentified earlier as distinct from metallothionein due to its physical properties (Winge et al., 1975).
Induction of MT

In animals, Cu-MT exists at elevated concentrations during early development as well as in diseases affecting copper homeostasis (Bremner, 1987), and in animals treated with copper (Sternlieb, 1980). Many studies have shown that copper is less effective than cadmium or zinc for MT induction, only high concentrations of copper (i.e. those which exceed a high threshold value) induce an increased level of Cu-MT (Durnam & Palmiter, 1981; Bremner et al., 1986; Blalock et al., 1988).

In mammals, metal ions sequester into cells and bind to the small pool of thionein. At higher concentrations of metals, the excess metal ions bind to other proteins that bind to the \textit{mt} genes. This binding induces the additional synthesis of thionein to bind to the excess metal ions. Consequently, the concentration of free metal ions is reduced and the stimulus for increased thionein production is decreased to basal level (Bremner, 1991).

The ability to incorporate copper into MT is markedly different among species of mammals (Mehra & Bremner, 1984). A small proportion of copper being associated with MT in the liver of copper-loaded sheep (Mehra & Bremner, 1984) and reports of copper as a poor inducer of MT in sheep (Peterson & Mercer, 1988) imply that binding of copper to MT may be crucial in the induction of MT. The administration of zinc before or after copper loading enhances the binding of hepatic copper to MT in rats (Day et al., 1981).
Turnover rate of MT

The half-life of copper-induced MT in rat liver is about 17 hr which is similar to that of Zn-MT but much less than that of Cd-MT and other cytosolic proteins (Bremner et al., 1978). The disappearance of Cu-MT may result from degradation of the protein, although some may be secreted into plasma and bile (Sato & Bremner, 1984).

MT in prokaryotes

In prokaryotes, MT was first discovered in marine cyanobacteria, *Synechococcus* sp. Naegeli 1849 (strain RRIMP NI which is the same species as PCC 7003) (Olafson et al., 1979). Olafson has shown that cadmium and zinc, but not copper, induce MT production in this marine *Synechococcus* strain (Olafson et al., 1980). Additional studies have been performed in freshwater cyanobacteria, *Synechococcus* strain UTEX-625 and strain Tx-20. These studies indicate that amino acid compositions of MT in the marine strains and those of freshwater strains are comparable, although they are substantially different from eukaryotic MT (Olafson, 1984). Studies in freshwater cyanobacteria have progressed to the point of determination of the DNA sequence coding MT protein (Robinson et al., 1990). In freshwater *Synechococcus* strain PCC 6301 (= TX-20), copper induces MT production but less effectively than cadmium or zinc (Robinson et al., 1990). In that study, the cultures were sampled 4 hr after exposure to metals. In another study in which marine *Synechococcus* strain RRIMP NI (the same species as PCC 7003) did
not induce production of Cu-MT (Olafson et al., 1980), cultures were sampled at early stationary phase in the presence of copper, which was about 12-15 days after exposure to copper. Since the structure and properties of Cu-MT are different from Cd, Zn-MT and the binding abilities of copper to MT are different among mammalian species, the difference in sampling times and/or in species may explain the apparent lack of Cu-MT in the marine cyanobacteria studied to date.

PROJECT GOALS AND OBJECTIVES

The current project was designed to study the mechanism used by marine Synechococcus spp. to overcome the toxicity of high copper concentrations. Because MT is induced by copper in freshwater Synechococcus spp. and the turnover rate of Cu-MT is rapid, the hypothesis to be examined was that marine Synechococcus spp. produce Cu-MT that can be detected intracellularly at a certain time in response to copper toxicity. In order to test this hypothesis, the following specific aims were examined.

1. To determine whether marine Synechococcus have the same or similar MT as freshwater Synechococcus. To test this specific aim, the Southern blot hybridization technique was applied to genomic DNA isolated from marine Synechococcus using a DNA fragment encoding MT from freshwater Synechococcus as a probe.

2. To determine whether different strains of marine Synechococcus produce different MT's. It has been shown by others that marine Synechococcus
RRIMP N1 do not produce Cu-MT under conditions of copper toxicity. Thus, three different strains of marine *Synechococcus* were included in this study to determine the sensitivity and the response to elevated concentrations of CuSO₄.

3. To determine whether time of sampling is critical (due to a rapid turnover rate of Cu-MT) for detection of Cu-MT in *Synechococcus* spp., intracellular proteins were isolated from cultures of marine *Synechococcus* spp. exposed to CuSO₄ for different time periods.

4. To determine whether MT is produced during copper stress in marine *Synechococcus* spp. and whether it has unique characteristics. The experimental methods that determined the characteristics unique to MT were applied to the components produced during copper stress. The experimental methods included: size exclusion chromatography to determine molecular weight, atomic absorption spectrophotometry to determine the high content of total copper, and the Ellman reaction to determine the content of sulfhydryl groups.

Understanding the copper resistance mechanisms in marine cyanobacteria will contribute to our general understanding of the effect of elevated copper on marine cyanobacteria, and their influence on the biogeochemical cycling of copper in the oceans.

In this study, one of three marine strains demonstrated an increased amount of the copper binding component (CBC), that was not MT, in response to high concentrations (≥ 8 μM) of CuSO₄ after 2 hr exposure. The CBC was not a peptide but contained lysine and an unidentified uv 254-absorbing constituent.
CHAPTER II
MATERIALS & METHODS

MATERIALS

Cyanobacterial cultures

Sources of marine cyanobacteria

Cyanobacteria used in this study are listed below. All *Synechococcus* spp. were purchased from American Type Culture Collection (ATCC), Rockville, Maryland. *Synechococcus* ATCC 27194 (the same strain as *Synechococcus* PCC 7001, and *Anacystis marina*) was originally collected from intertidal mud. *Synechococcus* ATCC 29404 (the same strain as *Synechococcus* PCC 73109, and *Agmenellum quadruplicatum*) was originally collected from sea water. *Synechococcus* ATCC 27265 was originally collected from clam bed sand. This strain is identical to *Synechococcus* PCC 7003, *Coccolithus elabens*, and *Synechococcus* RRIMP NI.

Cultivation of marine cyanobacteria

All three strains used in this study were grown in BG-11 medium (Rippka, 1988), under fluorescent light 32-66 μmol quanta / cm² / sec (3,000-3,600 lux), dark/light cycle (12/12 hr), at 28°C. Strain PCC 7003 was grown in BG-11 medium with the addition of 1% NaCl and 1 μg/liter of vitamin B₁₂ (Stanier et al.,
Medium BG-11 contains (per liter): NaNO₃, 1500 mg; K₂HPO₄·3 H₂O, 40 mg; MgSO₄·7 H₂O, 75 mg; CaCl₂·2 H₂O, 36 mg; Na₂CO₃, 20 mg; citric acid, 6 mg; ferric ammonium citrate, 6 mg; disodium-magnesium EDTA, 1 mg; Micronutrients, 1 ml, which contain (per liter) H₃BO₃, 2.86 g; MnCl₂·4 H₂O, 1.81 g; ZnSO₄·7 H₂O, 0.22 g; CuSO₄·5 H₂O, 0.08 g; Na₂MoO₄·2 H₂O, 0.39 g; Co(NO₃)₂·6 H₂O, 0.049 g. For subculturing, 5 ml of stationary phase organisms (OD₄₅₀ >1 and cell density ~10⁸-10⁹ cells/ml) were inoculated aseptically into a 125 ml-flask containing 75 ml of sterile fresh BG-11 medium.

*Synechococcus* PCC 7001 and PCC 73109 were used in most studies. Strain PCC 7003 was used, also, in the studies involving the maximum tolerated concentration (MTC). Due to its promising results, only strain PCC 73109 was used in studies to characterize the copper-binding components.

**Source of reagents**

All chemical reagents used in the experiments were an analytical grade, except acetonitrile and trifluoroacetic acid were the HPLC grade. Most chemical reagents were purchased from Sigma if they were not specified in the methods.

**CuSO₄ solution**

Sterile stock solution of CuSO₄ (1mM) was prepared in Milli-Q water. Three working concentrations of CuSO₄ were used in this study in an attempt to induce the production of MT. These concentrations were selected based upon
the response of the organism to each concentration. Low concentrations of CuSO₄ used in the study were determined by stepwise adaptation as described in the methods section. Maximum tolerated concentrations (MTC) of CuSO₄ were selected from an assay in which the cultures were grown in various concentrations of CuSO₄. The extreme concentration of CuSO₄ (i.e. 50 μM) which exceeded the threshold of toxicity to Synechococcus spp. was chosen as a lethal dose. Control samples consisted of each strain grown without the addition of CuSO₄.

All other media and glassware preparation

Media was prepared by using Milli-Q water. All solutions and buffers were prepared in Milli-Q water and run through a Chelex-100 (Bio-Rad Laboratory) column to remove trace metal contamination. All glassware was rinsed several times with deionized water after soaking overnight in 10% nitric acid.

METHODS

To reduce the extraneous variation, the conditions of each assay were kept constant i.e., the volume of buffer, the amount of glass beads.

Determination of Cell density

Cell number was determined from the optical density (450 nm) of Synechococcus cultures. In order to calibrate optical density, cells were counted
under the fluorescence microscope (Vanox, Olympus) after staining with acridine orange (Francisco et al., 1973). In addition, the green pigment of each strain was used as a visual indication of cell viability. For each experimental procedure, the optical density of each strain of Synechococcus was monitored in order to obtain approximately the same number of cells.

Response(s) of marine Synechococcus spp. to different concentrations of CuSO₄

Stepwise adaptation

Using a stepwise adaptation method described by Gupta et al. (1992), two strains of marine Synechococcus (PCC 7001, and PCC 73109) were subcultured in liquid BG-11 medium containing an initial concentration of 0.5 µM CuSO₄. Cells which grew in the initial concentration of CuSO₄ were further subcultured to fresh medium for five times with the increasing concentrations of CuSO₄ added to the medium at each time i.e., 0.8, 1.2, 1.5, 2.0, 2.2 µM. After subculturing five times, cultures were maintained at 2.2 µM CuSO₄ for subsequent studies.

Maximum tolerated concentration (MTC) assay

Different concentrations of CuSO₄ (0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 8.0, 10.0, 20.0 µM) were added to tubes containing fresh subcultures (OD₄₅₀ = 0.06-0.07) of marine Synechococcus PCC 7001, PCC 73109, and PCC 7003. Optical density was determined daily (450 nm) until constant. The
highest concentration of CuSO₄ in which cells survive was designated as the MTC. The survival of cells was determined by the optical density and the green color of the liquid culture. The MTC of CuSO₄ was used to induce the cell response examined in the present study. A lethal concentration of CuSO₄ was indicated by a clear and colorless culture. The selected MTC of CuSO₄ for each strain was added to log phase cultures in subsequent experiments i.e., the study of the cell response to MTC of CuSO₄. In this study, cultures were grown to log phase (OD₄₅₀ = 0.2-0.3). Cultures were then divided into two flasks (80 ml each). MTC of CuSO₄ was added to one of these flasks. The growth of both cultures was monitored by reading the absorbance at 450 nm every 15 min for 2 hr, every 2 hr for 24 hr after the first 2 hr, and every day after the first 24 hr.

Southern blot hybridization of genomic DNA from marine Synechococcus spp. to MT encoding DNA from freshwater Synechococcus strain

Isolation of genomic DNA

Genomic DNA was isolated from strains PCC 7001 and PCC 73109, which were maintained in the medium containing 2.2 μM CuSO₄. Genomic DNA was isolated using a modification of the method described by Porter (1988). Cells were harvested at the stationary phase of growth (cell density ~ 1×10⁸ cells /ml). After centrifugation of the cell culture at 5,000 x g for 10 min, the cell pellet was resuspended in a solution containing 25% sucrose, 50 mM Tris, 100 mM EDTA, pH 8.0. The cells were then lysed by repeated freeze (-80°C)/ thaw (37°C)
cycles. Lysozyme (10 mg/ml) and RNase (5 mg/ml) were added and the lysate was incubated at 37°C for 30 min. Sodium dodecyl sulfate (1% final concentration) was added. The lysate was incubated at 50°C after adding proteinase K (100 ug/ml). After phenol/chloroform extraction, the DNA was dialyzed in 1X TE (10 mM Tris and 1 mM EDTA) buffer.

**Probing for MT encoded genes**

Equal concentrations of genomic DNA isolated from marine *Synechococcus* PCC 7001 and PCC 73109 grown in the presence or absence of CuSO₄ were digested with restriction enzymes (Hind III, Sal I, EcoR I, BamH I) followed by Southern blot DNA transfer using a rapid downward alkaline capillary transfer technique described by Chomczynski (1992). The blot was hybridized with a non-radioisotope probe of a *smt* gene fragment. The *smt* gene probe was prepared by labeling a 1.8 Kb Hind III-Sal I DNA fragment isolated from a freshwater *Synechococcus* PCC 6301. The plasmid containing the *smt* fragment was provided by Dr. Nigel Robinson, University of Durham, UK. The probe was labeled using a Rad-Free System kit for the labeling and detection of nucleic acids (Schleicher & Schuell). The hybridization and washing conditions were performed according to the instructions from the manufacturer. The map of a freshwater *smt* DNA fragment used for preparing a probe is shown in Fig. 1.
Fig. 1. The map of freshwater smt gene. A 1.8 Kb Hind III-Sal I DNA fragment of freshwater smt gene was used as a DNA probe to detect smt gene in genomic DNA isolated from marine Synechococcus spp. The arrows indicate directions of transcription.
Induction of MT in marine Synechococcus spp. under different concentrations of CuSO₄

Exposure of marine Synechococcus to 2.2 μM CuSO₄ (determined by stepwise adaptation)

Cultures of marine Synechococcus PCC 7001 and PCC 73109 were grown to log phase. Cultures of each strain were divided into 2 flasks of 80 ml each. A low concentration of CuSO₄ (2.2 μM) was added to one of these flasks. The other flask was a control culture which was grown without addition of CuSO₄. Cells were harvested at early stationary phase (cell densities of 10⁷-10⁸ cells/ml as determined by direct counts) for intracellular protein isolation. The exposure period to added CuSO₄ was 7 to 10 d.

Exposure of marine Synechococcus spp. to the MTC of CuSO₄ and to a lethal concentration (50 μM) of CuSO₄ for different time periods

For each strain of Synechococcus spp. (PCC 7001, PCC 73109, and PCC 7003), twelve flasks containing 80 ml of a log phase-culture were pooled. The pooled culture was transferred, 80 ml each, to twelve new flasks. Sets of two flasks of cultures were labelled as 0 min, 30 min, 2 hr, 24 hr, 3 d and stationary phase. The MTC of CuSO₄ determined for each strain was added to one flask of each set. Controls were flasks without an addition of CuSO₄. After the cells were exposed to CuSO₄ for a given time period i.e., 0 min, 30 min, 2 hr, 24 hr, 72 hr (3 d), and until the cultures reached the stationary phase of growth (OD₄₅₀ >1
and cell number ~10⁸-10⁹ cells/ml), cells for each exposure period were harvested for intracellular protein isolation. Similarly, CuSO₄ (50 μM) was added to log phase cultures of marine Synechococcus spp. (PCC 7001, and PCC 73109). After 2, 24 and 72 hr (3 d) of exposure to CuSO₄, cells were harvested for intracellular protein isolation.

**Intracellular protein isolation**

After exposure to different concentrations of CuSO₄, the absorbance (450 nm) of each culture was read. The cultures were placed on ice for 30 min. Cells were then centrifuged at 3,000 rpm, at 4°C for 15 min. Cell pellets were washed in ice-cold sterile Milli-Q water three times before being suspended in 10 ml of ice-cold Tris Cl, 0.5 M, pH 8.6. The cells were ruptured with an ice cold “Bead-Beater” (Biospec Products) containing 27.5 g of acid washed glass beads, 0.1 mm diameter, for 3 min. The lysate was centrifuged at 3,000 rpm, 4°C for 15 min. The supernatant was obtained as a crude extract containing intracellular proteins. The crude extract was filtered through a 0.45 micron membrane before injection to size exclusion column.

**Determination of intracellular protein concentrations**

The concentrations of intracellular proteins contained in crude extracts were determined by using a BCA Protein Assay and reading the optical density at 562 nm as described in the manufacturer's manual (Pierce).
Identification of the intracellular copper-binding components produced by marine *Synechococcus* under copper stress

**Separation of intracellular components by size exclusion chromatography**

Proteins in the crude extract were separated by injecting 200 µl of crude extract onto a size exclusion (Superose 12 HR 10/30) column connected to an FPLC (Fast Protein Liquid Chromatography) system (Pharmacia). Column effluent was monitored by absorbance at 254 nm. The elution buffer used was 30 mM Tris Cl with 5 mM mercaptoethanol, pH 8.6, at a flow rate of 1 ml/min. The chromatogram was recorded at chart speed of 0.5 cm/ml. The following molecular size standards were used to calibrate the column: bovine serum albumin (BSA, MW 66,000), egg albumin (MW 45,000), carbonic anhydrase (MW 29,000), cytochrome C (MW 12,000), and vitamin B$_{12}$ (MW 1,355). A graph of log of MW vs. retention volume of these standards was plotted as shown in the result (chapter III). After an injection of crude extract onto the column, the molecular weights of peaks appearing in the size exclusion-FPLC chromatogram were determined by reading the retention volumes of the peaks from the chromatogram and then determining the molecular weights of the peaks from the graph mentioned above. The relative molecular weight of the distinct peak <10,000 Da (hereafter referred to as peak A) was identified. The area of peak A was measured using an electronic graphic calculator (Numonics Corp) and was normalized to total intracellular protein in the crude extract.
Collection of eluate from a Superose column

Fractions (3 ml) of eluate from a Superose column were collected using a fraction collector (Fraction-100, Pharmacia). The fractions were used for further analysis including the determination of total copper, scanning spectrophotometry, scanning spectrofluorimetry, and protein purification by reversed phase HPLC.

Determination of total copper concentration

The concentration of total copper in crude extracts and in eluate fractions from the Superose column was determined by Polarized Zeeman graphite furnace atomic absorption spectroscopy (Hitachi, Z-8100) equipped with an autosampling system as described in the manufacturer's instruction manual. The following optimal conditions were set: wavelength at 324.8 nm with 0.4 nm bandpass, lamp current at 10.0 mA (PMT voltage was maintained at 411-413 V) and auto sampler volume at 10 microliters. Pyrolytic cuvettes were used for all assays. An aliquot of copper reference standard (1.000 mg/ml, Fisher Atomic Absorption Reference Standard) was diluted to 1:100 in the sample buffer (30 mM Tris HCl with 5 mM mercaptoethanol, pH 8.6). This diluted standard was used as a working standard. Standards containing 2.5, 5.0, 7.5 and 10.0 microliters of the working standard were prepared in 1.00 ml of buffer. Copper concentrations of these standard were 25, 50 75 and 100 ppb, respectively. The sample buffer without addition of copper standard was used as blank. The standard curve (standard concentrations vs. their absorbances) of each
analytical run was plotted to monitor the linearity of the range of standard concentrations (Fig. 2). Trace Elements in Water (No. 284), obtained from the Environmental Protection Agency (EPA), was used as a control for each analytical run. This control was diluted fourfold by mixing the EPA stock (50 µl) with deionized water (50 µl) and the sample buffer (100 µl). Control values were between the reported range of 300-380 micrograms/L. Samples that were crude extracts and that were eluted from the size exclusion column were diluted with buffer, as required, to maintain assay concentrations within the limits of the standard curve. The copper concentrations were measured in duplicate and the average copper concentrations were then normalized to total intracellular protein in the crude extracts.

Spectrophotometry and spectrofluorimetry scanning

Only strain PCC 73109 of Synechococcus spp. was used in studies to characterize the copper-binding components. Absorbance and emission characteristics of copper-binding components were determined with a spectrophotometer and a spectrofluorimeter. Eluate fraction containing peak A, collected from a Superose column, was scanned (200-900 nm) using an UV-visible spectrophotometer (Varian, Cary 3 Bio). The same fraction, also, was scanned for absorption (200-400 nm) and emission (200-700 nm) spectra using a spectrofluorimeter (Shimadzu, RF 5000 U).
Fig. 2. A typical standard curve for determination of total copper concentration. Total copper concentrations in the samples (the crude extracts and the eluate fractions from the size exclusion column) were determined from the standard curve. Samples with total copper concentrations higher than 100 ppb were diluted with the same buffer and reassayed. The absorbance at Y-intercept represents absorption due to copper contamination in buffer, which was automatically subtracted from the reported concentrations.
Purification of the MW <10,000 components by using a reverse-phase HPLC column

From strain PCC 73109, the fraction (3 ml) containing peak A was collected from a Superose column. Three of the fractions from three different runs were pooled and concentrated with a lyophilizer. The dry component was resuspended in 150 µl of Milli-Q water. The suspension (100 µl) was injected to the reversed-phase HPLC column (Macrosephere 300 RP C8 7U, Alltech) using the gradient profile described by Klauser et al. (1983). The equilibration buffer (buffer A) used was 0.1% trifluoroacetic acid (TFA, Sigma) in Milli-Q water and the elution buffer (buffer B) used was 0.1% TFA containing 60% acetonitrile (Fisher, HPLC grade). The flow rate of buffer was 1 ml/min. The gradient profile was obtained using buffer B, 0-30% for 10 min, and 30-45% for 60 min. The column was washed in 100% buffer B for 5 min, and then was equilibrated in buffer A for 10 min before injecting the next sample. In some experiments, 0.01 M tetrabutylammonium bromide (TBA) was added to the mobile phase as an ion pairing agent. Fractions containing 1 ml of eluate were collected from the column using a fraction collector (Fraction-100, Pharmacia). The fractions of eluate were used for further studies i.e. determination of the concentrations of -SH side chain, CAS assay, mass spectrometry, and amino acid analysis.

Determination of the concentration of -SH side chain

The concentration of -SH side chains were determined in the PCC 73109 HPLC sample by the method of Ellman (1959) with the reaction volume scaled
down by 1/3. The mixture containing 0.9 ml eluate collected from RP-HPLC, 0.6 ml of 30 mM Na₂HPO₄, pH 7.0, and 1.5 ml of Milli-Q water, was added to 0.02 ml of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma; 39.6 mg of DTNB was dissolved in 10 ml of 30 mM Na₂HPO₄, pH 7.0). The color developed was measured at 412 nm. The concentration of -SH was calculated using the following equation:

\[ C₀ = \frac{AD}{ɛ} \]

where \( C₀ \) = molar concentration, \( A \) = absorbance at 412 nm, \( D \) = dilution factor, \( ɛ \) = extinction coefficient which is 13,000/M/cm.

**CAS (Chrome azurol S) assay to detect siderophores**

Intracellular crude extract and eluate fractions of strain PCC 73109 collected from RP-HPLC were used to determine the presence of siderophores using a universal chemical assay developed by Schwyn & Neilands (1987). The CAS assay solution was prepared as follows:

A volume of 6 ml of 10 mM hexadecyltrimethylammonium bromide (HDTMA, Sigma) was pipetted into a 100 ml volumetric flask containing 10 ml of Milli-Q water. A mixture of 1.5 ml iron solution (1 mM FeCl₃·6H₂O, 10 mM HCl) and 7.5 ml of 2 mM aqueous CAS (Chrome azurol S) solution was slowly added with constant stirring. Anhydrous piperazine (4.307 g) was dissolved in 50 ml of Milli-Q water and 6.25 ml of 12 M HCl was added slowly to obtain pH 5.6. This buffer, then, was added to the flask containing the reagents above. The volume was adjusted to 100 ml with Milli-Q water to obtain 100 ml of CAS assay
solution.

Intracellular crude extract (0.5 ml) or eluate fractions collected from RP-HPLC were mixed with 0.5 ml of CAS assay solution. The reference control for intracellular crude extract was 0.5 M Tris Cl, pH 8.6. The reference control for the eluate fractions from RP-HPLC, was 0.1% TFA in Milli-Q water. EDTA (0.5 M) was used as the positive control. The existence of siderophores in the sample caused a change of color from blue to orange.

Mass spectrometry analysis and amino acid analysis

A purified component in eluate fractions collected from RP-HPLC was submitted for the mass spectrometry and amino acid analysis at the Biomolecular Research Facility, University of Virginia, Charlottesville, Virginia.

Statistical analysis

A two sample t-test was applied to determine the significance of differences in peak A area measured from FPLC chromatogram of control and copper treated cultures. The t-test was also used to determine the significance of differences between total copper concentrations in FPLC fractions and crude extracts between the control and copper treated cultures. The peak A area ratio as well as total copper concentration ratio between control and copper treated cultures was used for statistical comparison of replicate experiments. Duplicate samples were used in most experiments. Peak A area of each sample was determined from the average of the quadruplicate injections to the Superose-
Electron microscopy of *Synechococcus* PCC 73109

Scanning electron microscopy was performed on a culture of PCC 73109. The preparation of the sample for scanning electron microscopy and the picture were provided by Mr. Bill Dashek from Electron Microscopy Facility, Old Dominion University, Norfolk, Virginia. Also, a sample was prepared for transmission electron microscopic study. The *Synechococcus* culture was centrifuged at 3,000 rpm, fixed with 2.5% glutaraldehyde and post-fixed with 2% osmium tetroxide. The preparation of the sample for the transmission electron microscope and the picture were provided by Dr. Francine Marciano-Cabral, Virginia Commonwealth University, Richmond, Virginia.
CHAPTER III

RESULTS

Marine *Synechococcus* spp. used in the present study contain a green pigment which can be used as a visual indication of viable cells in addition to the optical density to determine growth. A clear and colorless culture signifies a dead culture whereas a green color indicates viability of the culture.

**Stepwise adaptation**

The initial concentration of CuSO$_4$ added to all cultures was 0.5 μM. After subculturing five times with increasing copper concentrations, a CuSO$_4$ concentration of 2.2 μM was selected as the concentration at which cultures were maintained. The cultures were prepared for the genomic study and the isolation of intracellular protein. After the third subculture, when 1.2 μM CuSO$_4$ was added, the growth of *Synechococcus* PCC 7001 was slower than the growth of PCC 73109 on the same day as determined by visual observation. The culture of strain PCC 7001 was clear to light green in comparison to dark green cultures of strains PCC 73109. The cultures were maintained by repeated subculturing in medium containing 2.2 μM CuSO$_4$ until they were used for experimental studies.

**Determination of maximum tolerated concentrations (MTC) of CuSO$_4$ in marine *Synechococcus* spp.**

The MTC of CuSO$_4$ was determined in all three strains of marine
*Synechococcus* i.e., PCC 7001, PCC 73109, and PCC 7003. Three distinct types of growth were observed with varying copper levels in all three strains (Fig. 3): 1) At low copper concentrations (1.5 μM), the growth rate was similar to the control (no copper added); 2) When excessive copper concentrations (10-20 μM) were added, the cells were killed; 3) When the copper concentrations were between 1.5 to 8 μM, growth was inhibited but cells were viable (lower OD450 and green color culture).

Although various concentrations of CuSO₄ (1.5-20 μM) were added to the cultures, only certain copper concentrations are shown in the figures for clarity. In *Synechococcus* PCC 7001 (Fig. 3a), in the presence of 1.5 μM CuSO₄, cell density (determined by absorbance at 450 nm and a change in color of the cultures) was not significantly different from the control (no additional CuSO₄ added) at every time point and the growth rate (~0.06/d) was the same as the control. In the presence of 2.0-3.0 μM CuSO₄, cell growth was inhibited as demonstrated by the lower cell density at every time point and the lower growth rate (~0.02/d for 2.0 μM CuSO₄) than those in the presence of 1.5 μM CuSO₄ and negative growth rate (~ -0.002/d for 3.0 μM CuSO₄). Cells were viable as the cultures were green in color. In the presence of ≥5 μM CuSO₄, cells were killed as evidenced by the lower absorbance of the culture at later time points. Also, the cultures were colorless. In this study, the MTC of CuSO₄ for strain PCC 7001 was 3.0 μM and was chosen as the effective CuSO₄ concentration to induce the cell response.

When cultures of *Synechococcus* PCC 73109 were treated with 1.5-4.5 μM CuSO₄, cell densities of these cultures were lower than the cell density of the
control at every time point (Fig. 3b). However, the growth rate (~0.06/d) of these cultures was similar to that of the control. In the presence of 5.0-8.0 μM CuSO₄, cell densities were lower than those of cultures challenged with 1.5 μM CuSO₄. Among the 1.5-8.0 μM CuSO₄ challenged cultures, the 8.0 μM CuSO₄ challenged culture demonstrated a very low cell density up to day 20 and cells were still viable as the cultures appeared green in color. After day 20, the increased cell densities were observed in the 5.5, 6.0 (data of both were not shown) and 8.0 μM CuSO₄ cultures. At higher concentrations of CuSO₄ (10 and 20 μM), the absorbance at later time points was lower than the absorbance at day 0, and the cultures were colorless. The addition of 8.0 μM CuSO₄ to the culture resulted in the lowest cell density during the first 20 days of incubation (compared to 1.5 μM-6.0 μM CuSO₄), but cells were still alive (green color of the culture vs. the colorless culture when 10-20 μM CuSO₄ was added). Consequently, 8.0 μM of CuSO₄ was selected as the copper concentration for the study of the induction of copper-complexing compounds for strain PCC 73109.

In PCC 7003 (Fig. 3c), the growth rate of the control culture was 0.08/d while the growth rate of cultures with 1.5-4.5 μM CuSO₄ was ~0.09/d. The growth rates were decreased to ~0.07-0.05/d in the presence of 5.0-8.0 μM CuSO₄. The cultures were colorless when 10-20 μM CuSO₄ were added. A CuSO₄ concentration of 5 μM was chosen as the concentration for further studies in strain PCC 7003, although 8.0 μM CuSO₄ would be the MTC of CuSO₄. However, when 8.0 μM CuSO₄ was added to exponentially growing cultures, the cells died. The cell density decreased and the culture became
Fig. 3. Maximum tolerated concentrations (MTCs) of copper for marine Synechococcus spp. Growth of Synechococcus PCC 7001 (a), PCC 73109 (b), and PCC 7003 (c) in cultures containing varying concentrations of copper. CuSO₄ was added to cultures on day 0. The highest concentrations of CuSO₄, i.e. 3 μM for PCC 7001, 8 μM for PCC 73109, and 5 μM for PCC 7003 (the reason of not selecting 8 μM CuSO₄ is mentioned in the results) that were toxic but non-lethal to the cells were selected for further studies of induction of copper-complexing ligands. Error bars represent the standard deviations of absorbances.
Fig. 3, continued.
colorless (data not shown). Therefore, 5.0 μM CuSO₄ was selected for the induction study. The lethal effects were seen also in the cells at concentrations of CuSO₄ at 10 and 20 μM.

Response of *Synechococcus* spp. to the MTC of CuSO₄

The selected concentrations of CuSO₄ i.e., 3, 5, and 8 μM, were added to log phase cultures (OD₄₅₀ = 0.2-0.3) of *Synechococcus* PCC 7001, PCC 7003, and PCC 73109, respectively. The response of cells to these concentrations of CuSO₄ was determined by a comparison of the absorbance of CuSO₄ challenged cultures and the control cultures in which additional CuSO₄ was not added. In strain PCC 7001 (Fig. 4a), after adding 3 μM CuSO₄, the absorbance did not change for approximately 22 hr before an increase in absorbance was observed. An increase in the absorbance of the same culture without additional CuSO₄ added (the control) was observed after day 8. The cell density of the culture in the presence of 3 μM CuSO₄ was higher than the control culture in which additional CuSO₄ was not added. The higher cell density was observed also in the culture with the addition of 2 μM CuSO₄. These cultures were started from the same-pooled cultures.

In strain PCC 73109 (Fig. 4b), the absorbance of the cultures with and without 8 μM CuSO₄ was the same until hour 26. After 26 hr, the absorbance of the control culture (no additional CuSO₄ added) increased, whereas the absorbance of the culture with 8 μM CuSO₄ decreased. The absorbance of the copper-stressed culture increased again after day 30 and reached the original level at day 35.
In strain PCC 7003 (Fig. 4c), after adding CuSO$_4$, the absorbance of the culture was lower than the control (without an additional CuSO$_4$) until day 23. After 23 days, the culture grew exponentially. In contrast, the control culture grew initially, then cell densities dropped on day 8 and 26 after which the cell density was increased. This study was repeated and the similar profile was demonstrated (Fig. 4d).

**Detection of the smt genes in marine *Synechococcus* spp.**

Southern blot hybridization was performed using genomic DNA isolated from cultures of PCC 7001 and 73109 maintained in 2.2 μM CuSO$_4$. An equal amount of genomic DNA (10 μg) was loaded in each well (Fig. 5). However, because of the impurity of the DNA, lane 8 of the ethidium bromide stained gel (Fig. 5a) showed less DNA. The experiments were repeated with volumes of DNA solution which produced bands of equal intensity. In both strains (PCC 7001 and 73109), no hybridizing band was found which hybridized to the freshwater smt gene (Fig. 5b). However, hybridizing bands (Fig. 5b, lane 4 and 5) were readily observed in genomic DNA extracted from a freshwater strain PCC 6301 (from which the smt probe was derived). This freshwater strain was reported by Gupta et al. (1992) to amplify its smt gene after repeated exposure to CdCl$_2$. Therefore, this freshwater strain was used in the present study as the positive control. The result of the positive control was the same as the previous report. The density of a hybridizing band was higher when the freshwater culture was exposed to 2.2 μM CdCl$_2$. 

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Fig. 4. Response of marine *Synechococcus* spp. to the MTCs of CuSO$_4$. CuSO$_4$ at 3 μM, 8 μM, and 5 μM was added to growing cultures of PCC 7001 (a), PCC 73109 (b) and PCC 7003 (c and the replicate, d) respectively. CuSO$_4$ was added to the cultures on day 0 (OD$_{450}$=0.2-0.3).
c) 

**Synechococcus PCC 7003**

![Graph showing absorbance at 450 nm over days with and without CuSO₄ addition.]

- Solid line: No CuSO₄ added
- Dotted line: 5 uM CuSO₄ added

Fig. 4, continued.

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Fig. 5. Southern blot hybridization of genomic DNA of marine *Synechococcus* spp. to the freshwater *smt* DNA probe. Visualization of ethidium bromide-stained gel (a) and autoradiograph of southern blot of the same gel (b). Drosophila DNA (a gift from Dr. Christopher Osgood, Old Dominion University, Norfolk, Virginia) as a negative control (lane 1). *smt* fragment (lane 2). λ HindIII DNA marker (lane 3). Freshwater *Synechococcus* PCC 6301; no CdCl$_2$ added (lane 4) and 0.3 μM CdCl$_2$ added (lane 5). Marine *Synechococcus* PCC7001 (lane 6-8) and PCC 73109 (lane 9-14) with no CuSO$_4$ added (lane 6, 9 and 12), with 2.2 μM CuSO$_4$ added (lane 7, 10 and 13), and with 0.3 CdCl$_2$ added (lane 8, 11 and 14). The amplification of *smt* gene was seen as a more intense band (lane 4 vs. lane 5). No hybridizing band was seen in the negative control and in marine strains.
Calibration of a size exclusion -FPLC column

A calibration curve of molecular weights of standard proteins vs. the retention volumes of the eluate fractions (from the size exclusion-FPLC column used in the present study) containing the peak of standard proteins is shown in Fig. 6. The molecular weight of components <10,000 kDa retained in the fractions of a retention volume >16 ml.

Intracellular copper-binding components produced by marine Synechococcus spp. under different concentrations of CuSO₄

Different concentrations (2.2, 3, 5, 8 and 50 μM) of CuSO₄ were used to induce the production of copper-complexing metabolites. The low CuSO₄ concentration (2.2 μM) was the concentration at which Synechococcus could be maintained readily after stepwise adaptation to CuSO₄. The MTCs of CuSO₄ were higher than 2.2 μM but were not lethal to the cells. The lethal CuSO₄ concentration (50 μM) was an extremely high concentration above the threshold of toxicity. Intracellular molecules isolated from these cells were injected into a size exclusion Superose-FPLC column.

Chromatograms (from the Superose column) of intracellular proteins isolated from each culture tested showed a distinct peak A which eluted from the column at the retention volume ~16-17 ml (Fig. 7). The molecular weight of peak A determined from the calibration curve (molecular weight of the standard proteins vs. their retention volumes) was 4,000-6,000 Da (Fig. 6). The concentration of total copper was highest in the fraction containing this peak.
Fig. 6. Molecular weight of protein standards vs. retention volume in the Superose column. Standard proteins were injected and the retention volume of each standard and its molecular weight was plotted.
Fig. 7. Typical chromatogram of intracellular protein and total copper concentrations in eluate fractions of *Synechococcus* spp. The figure shown is representative of strain PCC 73109 with an addition of 50 μM CuSO₄. Intracellular protein (200 μl) extracted from cultures with or without additional CuSO₄ added was injected into a Superose size exclusion column connected to an FPLC system. The eluent buffer was 30 mM Tris Cl, pH 8.6 and 5 mM mercaptoethanol with a flow rate of 1 ml/min. Fractions (3 ml) were collected and total copper concentration was determined in each fraction by graphite furnace atomic absorption spectrometry (AAS). Peak A (retention volume ~16-17 ml) presents the component(s) of MW 4,000-6,000 Da.
When PCC 7001 was challenged with 2.2 μM CuSO₄, peak A area was similar to, and the total copper concentration was lower than, those of the control sample (Fig. 8a). The smaller peak A area and lower total copper concentration were demonstrated, also, in PCC 73109 challenged with 2.2 μM CuSO₄ (Fig. 8b). The total protein concentrations were not determined so peak A areas and total copper concentrations were not normalized to total protein. However, the cultures were adjusted to the same optical density before extraction of the intracellular protein. The intracellular protein isolation protocol and volume of crude extract injected to the FPLC-size exclusion column were identical in both samples. SDS-PAGE of the same volume of crude extract from the control cultures and CuSO₄ treated cultures and staining with a silver stain, demonstrated the similar protein profiles and band intensities (data not shown). Therefore an approximately equal amount of intracellular protein was injected in each case onto a size exclusion-FPLC column.

When MTCs of CuSO₄ i.e., 3 μM for PCC 7001, 5 μM for PCC 7003, and 8 μM for PCC 73109 were used as the inducing concentrations, CuSO₄ was added to log phase cultures (OD₄₅₀=0.4-0.5). Cultures were exposed to CuSO₄ for different time periods i.e., 0 min, 30 min, 2 hr, 24 hr, and until cells reached the stationary phase of growth i.e., 35 d for PCC 7001, 41 d for PCC 7003, and 42 d for PCC 73109. The absorbance at 450 nm and the protein concentrations in intracellular crude extracts from each strain were determined with and without copper addition (Table 1a, b and c).
Fig. 8. Peak A areas and total copper concentrations in intracellular extracts from cultures of marine *Synechococcus* spp. with and without exposure to 2.2 uM CuSO₄. *Synechococcus* PCC 7001 (a) and PCC 73109 (b).
exposure to CuSO₄, cell densities and total protein concentrations in the control cultures and CuSO₄ treated cultures were the same or varied slightly. After 3 d of exposure to CuSO₄, cell densities and total protein concentrations of control cultures and CuSO₄ treated cultures were essentially comparable to those of shorter exposure time periods. When the cultures reached the stationary phase, cell densities of both control cultures and CuSO₄ added cultures were much higher than those of the shorter exposure time periods. However, at the stationary phase, cell densities were different in the CuSO₄ treated cultures and the control cultures. Cell densities in CuSO₄ treated cultures were the same as control PCC 7001 cultures, lower than control cultures of PCC 73109 and higher than control cultures of PCC 7003. Total protein in CuSO₄ treated cultures of strain PCC 7001 and PCC 73109 were lower than those in the control cultures.

Intracellular molecules isolated from cultures exposed to the MTCs of CuSO₄ were injected to the size exclusion Superose-FPLC column. A similar chromatogram profile was seen in control and copper-treated cultures of PCC 73109 after 2 hr exposure and of PCC 7001 after 30 min exposure to CuSO₄. A larger peak A area and a higher total copper concentration was observed in the eluate fraction from the CuSO₄ treated samples (Fig. 9b) than those in the control sample (no additional CuSO₄ added) (Fig. 9a). To assure that the larger peak area and higher total copper concentration was not due to CuSO₄, CuSO₄ solution at the same concentration in crude extracts was injected to the column. The peak area of the CuSO₄ solution was smaller and the total copper concentration was lower than that of the CuSO₄ treated culture extract (data not
Table 1a. Absorbance of liquid cultures prior to cell lysis and concentrations of intracellular protein in crude extracts isolated from *Synechococcus* PCC 7001 after exposure to 3 μM CuSO₄ for different time periods

<table>
<thead>
<tr>
<th></th>
<th>0 min</th>
<th>30 min</th>
<th>2hr</th>
<th>24hr</th>
<th>3d</th>
<th>35d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs. [protein]</td>
<td>0.45</td>
<td>0.49</td>
<td>0.45</td>
<td>0.38</td>
<td>0.46</td>
<td>0.52</td>
</tr>
<tr>
<td>CuSO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>Abs. [protein]</td>
<td>0.45</td>
<td>0.43</td>
<td>0.45</td>
<td>0.48</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>0 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Abs. [protein]</td>
<td>0.45</td>
<td>0.39</td>
<td>0.45</td>
<td>0.43</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>3 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.43</td>
<td>0.58</td>
</tr>
</tbody>
</table>

* Liquid cultures (80 ml each) of *Synechococcus* PCC 7001 were exposed to 3 μM CuSO₄ for 0 min, 30 min, 2hr, 24 hr, 3 d, and until the culture reached the stationary phase (35 d). Control cultures were grown without adding CuSO₄. After each time period, the absorbance (450 nm) of cultures was read and then the cultures were placed on ice. Cells were washed three times in ice-cold Milli-Q water. Cell pellets were resuspended in 0.5 M Tris Cl, pH 8.6 and were ruptured by using a bead beater with 27.5 g of glass beads (1 mm) for 3 min. Cell lysates were centrifuged at 3,000xg, 4°C, for 15 min and supernatant fluid was obtained as crude extracts. Total protein concentrations (mg) in crude extracts were determined by the Pierce BCA protein assay.
Table 1b. Absorbance of liquid cultures prior to cell lysis and concentrations of intracellular protein in crude extracts isolated from *Synechococcus* PCC 73109 after exposure to 8 μM CuSO₄ for different time periods

<table>
<thead>
<tr>
<th>CuSO₄</th>
<th>0 min</th>
<th>30 min</th>
<th>2hr</th>
<th>24hr</th>
<th>3d</th>
<th>42d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.48 ±0.04</td>
<td>0.54 ±0.08</td>
<td>0.48 ±0.04</td>
<td>0.43 ±0.04</td>
<td>0.5 ±0.01</td>
<td>0.6 ±0.04</td>
</tr>
<tr>
<td>8 μM</td>
<td>0.48 ±0.04</td>
<td>0.48 ±0.04</td>
<td>0.48 ±0.04</td>
<td>0.46 ±0.04</td>
<td>0.48 ±0.01</td>
<td>0.48 ±0.02</td>
</tr>
</tbody>
</table>

* Liquid cultures (80 ml each) of *Synechococcus* PCC 73109 were exposed to 8 μM CuSO₄ for 0 min, 30 min, 2hr, 24 hr, 3 d, and until the culture reached the stationary phase (42 d). Control cultures were grown without adding CuSO₄. After each time period, the absorbance (450 nm) of cultures was read and then the cultures were placed on ice. Cells were washed three times in ice-cold Milli-Q water. Cell pellets were resuspended in 0.5 M Tris Cl, pH 8.6 and were ruptured by using a bead beater with 27.5 g of glass beads (1 mm) for 3 min. Cell lysates were centrifuged at 3,000xg, 4°C, for 15 min and supernatant fluid was obtained as crude extracts. Total protein concentrations (mg) in crude extracts were determined by the Pierce BCA protein assay.
Table 1c. Absorbance of liquid cultures prior to cell lysis and concentrations of intracellular protein in crude extracts isolated from *Synechococcus* PCC 7003 after exposure to 5 μM CuSO₄ for different time periods

<table>
<thead>
<tr>
<th>CuSO₄</th>
<th>0 min</th>
<th>30 min</th>
<th>2 hr</th>
<th>24 hr</th>
<th>3 d</th>
<th>41 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>0.43</td>
<td>0.07</td>
<td>0.43</td>
<td>0.08</td>
<td>0.46</td>
<td>0.08</td>
</tr>
<tr>
<td>5 μM</td>
<td>0.43</td>
<td>0.09</td>
<td>0.43</td>
<td>0.06</td>
<td>0.47</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*a* Liquid cultures (80 ml each) of *Synechococcus* PCC 7003 were exposed to 5 μM CuSO₄ for 0 min, 30 min, 2 hr, 24 hr, 3 d, and until the culture reached the stationary phase (41 d). Control cultures were grown without adding CuSO₄. After each time period, the absorbance (450 nm) of cultures was read and then the cultures were placed on ice. Cells were washed three times in ice-cold Milli-Q water. Cell pellets were resuspended in 0.5 M Tris Cl, pH 8.6 and were ruptured by using a bead beater with 27.5 g of glass beads (1 mm) for 3 min. Cell lysates were centrifuged at 3,000xg, 4°C, for 15 min and supernatant fluid was obtained as crude extracts. Total protein concentrations (mg) in crude extracts were determined by the Pierce BCA protein assay.
Fig. 9. Chromatograms of protein and total copper concentrations in fractions from marine Synechococcus PCC 73109 with and without exposure to CuSO$_4$. No additional CuSO$_4$ was added to the culture (a). The culture was challenged with 8 $\mu$M (MTC) CuSO$_4$ for 2 hr (b).
Fig. 9, continued.
For each marine strain exposed to the MTC of CuSO₄, peak A area, the total copper concentration (in the fraction containing peak A) and the total intracellular copper concentration in the crude extract were normalized to total intracellular protein in the crude extract. Comparison of normalized peak A area and normalized total intracellular copper between the control samples and CuSO₄ treated samples at different time periods, is shown in Fig. 10a, b, and c.

In strain PCC 7001 (Fig. 10a.1), peak A area of the sample treated with 3 μM CuSO₄ was similar to or smaller than that in the control sample of the same time period, except the exposure time of 35 d (stationary phase) which demonstrated the larger peak A area in the sample treated with 3 μM CuSO₄. The similar or smaller total intracellular copper concentrations were demonstrated, also, in CuSO₄ treated samples of every time period except the 35 day exposure time (Fig. 10a.2).

When PCC 73109, was exposed to 8 μM CuSO₄ for different time periods from 0 min to stationary phase, peak A area from the CuSO₄ amended cultures was larger than the control after 30 min and 2 hr of exposure. Total copper concentration in the fraction containing peak A was also increased. Most of the copper was detected in the fraction containing peak A. A comparison of the area of peak A (normalized to total protein) between the control samples and CuSO₄ (8 μM) treated samples at different time periods is shown in Fig. 10b.1. Larger peak area was observed in CuSO₄ (8 μM) treated samples at 0 min, 30 min, 2 hr, and 42 d (stationary phase) sampling times. At 30 min and 2 hr exposure time,
the peak A areas of CuSO₄ (8 μM) treated samples were significantly larger ($p<0.05$) than the control. Total intracellular copper concentrations per mg protein in crude extracts of CuSO₄ (8 μM) treated samples at every time period were similar to or higher than those of the control without additional CuSO₄ (Figure 10b.2). The ratios of total intracellular copper concentrations in control sample (no additional CuSO₄) to CuSO₄ (8 μM) treated samples were: 1:1.24±0.78, 1:2.79±0.45, 1:1.96±0.67, 1:1.39±0.38, 1:1.12±0.18 and 1:1.7±0.34 for 0 min, 30 min, 2 hr, 24 hr, 3d and 42 d, respectively. Statistical analysis of the ratios of total intracellular copper concentrations demonstrated that total intracellular copper concentrations of 2.79 (30 min exposure), 1.96 (2 hr exposure) and 1.7 (42 d exposure) fold higher in CuSO₄ treated samples than the controls were significant ($p<0.025$, $p<0.1$, $p=0.05$, respectively). However, in 8 μM treated samples, total intracellular copper concentrations decreased after 2hr. In contrast, total intracellular copper concentrations per mg protein in crude extract of control samples increased until day3, after which they decreased.

In PCC 7003 (Fig. 10c.1&2), peak A area and total intracellular copper concentrations per mg protein of samples treated with CuSO₄ were similar to or smaller than the controls at every time period except at the 24 hr exposure time.

When 50 μM CuSO₄ was added to cultures of PCC 7001 and PCC 73109, all cultures changed color from green to yellowish green after 18 hr exposure and then became clear and colorless after 10 d exposure to CuSO₄. Intracellular proteins isolated from the cultures at 2 hr, 24hr, and 3 d exposure to CuSO₄ were injected to the size exclusion Superose-FPLC column. In both strains, after 2 hr
Fig. 10. Peak A area in control cultures (no additional CuSO₄ added) vs. the MTC of CuSO₄ added cultures. PCC 7001 with and without 3 µM CuSO₄ (a), PCC 73109 with and without 8 µM CuSO₄ (b), PCC 7003 with and without 5 µM CuSO₄ (c). A comparison was demonstrated by peak A areas normalized to total protein in crude extract (1) and total intracellular copper concentrations (in crude extract) normalized to total protein concentrations (2). Error bars shown in b. represent the standard deviations.
**b)**

*Synechococcus PCC 73109*

(1)

![Graph showing Peak Area per mg total protein vs. exposure time for Synechococcus PCC 73109 with and without CuSO₄ addition.](image)

(2)

![Graph showing [Cu] in crude extr. (ppb x1000) per mg total protein vs. CuSO₄-exposure time for Synechococcus PCC 73109 with and without CuSO₄ addition.](image)

Fig. 10, continued
Fig. 10, continued.
of exposure to CuSO₄, the chromatogram and total copper concentrations in eluate fractions, were similar to that shown in Fig. 9a and b. The comparison of normalized peak A area and normalized total copper concentration between the CuSO₄ treated sample and the control sample in each marine strain is shown in Fig. 11a and b.

In PCC 7001, peak A areas (Fig. 11a.1) and total intracellular copper concentrations (Fig. 11a.2) per mg total protein in CuSO₄ treated samples were lower than the controls at each time point. At 24 hr exposure to CuSO₄, total intracellular protein concentration was too low to be detected by the method used. Therefore, a normalized value could not be generated.

In PCC 73109, normalized peak A area was larger in CuSO₄ treated samples than that in control samples at 2 hr and 24 hr exposure times (Fig. 11b.1). The ratios of peak A area of the control samples to CuSO₄ treated samples were: 1:4.26±1.36, 1:6.36±2.51 and 1:0.795±1.12 for 2 hr, 24 hr and 3d, respectively. Statistical analysis of the ratios of peak A areas which was 4.26 fold increased at 2 hr and 6.36 fold increased at 24 hr in CuSO₄ treated samples were significant (p<0.05). Total intracellular copper concentrations in 50 μM CuSO₄ treated samples were increased at 2 hr and 24 hr and then decreased at 3 d (Fig. 11b.2). The ratios of total intracellular copper concentration of the control samples to CuSO₄ treated samples were: 1:7.02±5.73, 1:6.41±4.07 and 1:0.975±1.38 for 2 hr, 24 hr and 3d, respectively. Statistical analysis of the ratios of total intracellular copper concentrations which were 7.02 fold increased at 2 hr and 6.41 fold increased at 24 hr in CuSO₄
Fig. 11. Control cultures (no additional CuSO$_4$ added) vs. 50 μM CuSO$_4$ added cultures of marine *Synechococcus* spp. *Synechococcus* PCC 7001 (a), PCC 73109 (b). A comparison demonstrated by peak A areas normalized to total protein in crude extract (1) and total intracellular copper concentrations normalized to total protein concentrations (2). Error bars shown in b. represent the standard deviations.
**Synechococcus PCC 73109**

(1)  

![Graph 1](image1)

(2)  

![Graph 2](image2)

Fig. 11, continued.
treated samples, were not significant.

Because of the apparent induction of components contained within peak A in PCC 73109 under copper stress, the material in peak A was further characterized to identify the intracellular component(s) induced by the addition of CuSO₄.

**Characterization of the intracellular components induced by the addition of ≥8 µM CuSO₄ in PCC 73109**

A number of small molecular weight Cu (I)-complexes display charge transfer transitions as characteristic absorbance maxima in the ultraviolet region of the absorption spectrum and characteristic luminescence in the visible region (Lytle, 1970). The eluate fraction containing peak A was scanned using a UV-visible spectrophotometer (wavelength 200-900 nm) and a spectrofluorimeter. The absorption maximum was observed at 230 nm in the CuSO₄ treated sample, the control sample, and the CuSO₄ solution of the same concentration as that present in the sample. However, the sample treated with 50 µM CuSO₄ for 2 hr demonstrated the highest peak (Fig. 12).

The samples with and without CuSO₄ added as well as the control buffer were scanned through the excitation wavelength of 200-400 nm using a spectrofluorimeter, the spectra shown in Figure 13 were observed. Maximum emission at wavelength 450 was demonstrated when the samples were excited at 250 nm and 350 nm. Maximum emission at 450 nm was not demonstrated in buffer itself or the CuSO₄ solution (Fig. 13a and b). The emission peaks of

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Fig. 12. UV-visible spectrophotometer scanning of Peak A material. An eluate fraction (3 ml) containing Peak A material was scanned (200-900 nm) by using a UV-visible spectrophotometer. (a) eluent buffer (30 mM Tris Cl pH 8.6 and 5 mM mercaptoethanol) used as a mobile phase of a Superose-FPLC column, (b) an eluate fraction of a CuSO₄ solution (the same concentration as total copper concentration in the culture), (c) an eluate fraction of a control culture (no CuSO₄ added), and (d) an eluate fraction from a 50 μM CuSO₄ treated culture harvested after 2 hr.
sample treated with 50 \( \mu M \) \( \text{CuSO}_4 \) for 2 hr or treated with 8 \( \mu M \) \( \text{CuSO}_4 \) for 42 days were smaller than the peak of the control sample (Fig. 13f, e and c, respectively). However, when the sample was treated with 8 \( \mu M \) \( \text{CuSO}_4 \) for 2 hr, the emission peak was the same as the control (Fig. 13d and c).

The RP-HPLC chromatogram of components contained within peak A of 8 \( \mu M \) \( \text{CuSO}_4 \) treated sample is shown in Fig. 14. The components which did not bind to the column (void volume) were eluted at approximately 4 min. The areas of the peaks at the retention time 6-7 min (shown as peak a and b in RP-HPLC chromatogram) were different between \( \text{CuSO}_4 \) treated sample and the control sample when normalized to total protein in the FPLC fraction containing peak A. The sample treated with \( \text{CuSO}_4 \) showed a larger peak area (266.57/\( \mu g \) total protein) than the control (104.3/\( \mu g \) total protein). This difference was similar to the peak area of peak A in chromatogram from a size exclusion-FPLC column. The intracellular component was retained about 2 min. longer in the RP-HPLC column when tetrabutylammonium bromide (TBA) was added to the mobile phase buffer as an ion pair reagent.

The -SH concentration (normalized to total protein) in the fraction containing peak a and b from RP-HPLC column was similar in \( \text{CuSO}_4 \) treated samples and the control samples i.e., 14.2 \( \mu M \) per mg total protein vs. 19.9 \( \mu M \) per mg total protein, respectively.

In the CAS assay, the qualitative analysis (using 0.03 mg total protein in crude extract or 1.25 \( \mu g \) protein in purified fraction from RP-HPLC) demonstrated no change in color in either crude extract nor any fraction collected from FPLC.
Fig. 13. Spectrofluorimeter scanning of Peak A material. The eluate fraction (3 ml) containing Peak A was scanned through the excitation wavelength range 200-400 nm and through the emission wavelength range 200-700 nm by using a spectrofluorimeter. The maximum emission wavelength at 450 nm was demonstrated when the sample was excited at 250 nm and 350 nm.
Fig. 14. Reversed phase-HPLC chromatogram of an eluate fraction containing Peak A of *Synechococcus* PCC 73109. Three, 3 ml-fractions containing Peak A eluted from a Superose-FPLC column were pooled, lyophilized, and resuspended in 150 μl of Milli-Q water. 100μl (~78μg protein) was injected into C₈-RP-HPLC column with a gradient buffer (1ml/min) formed between buffer A (0.1% trifluoroacetic acid) and buffer B (0.1% trifluoroacetic acid containing 60% acetonitrile). Peak a and b were shown as a single band of <6,500 MW in SDS-PAGE gel (data not shown).
The blue color became orange in the tube containing EDTA which was used as the positive control for iron chelation.

Ion/mass ratio from mass spectrometry analysis indicated that the compound in the fraction eluting at 6-7 min from RP-HPLC was not a peptide (Fig. 15). Amino acid analysis of this compound revealed lysine and a large peak of unknown identity which absorbed at 254 nm (Table 2). Reports from the Biomolecular Research Facility are shown (Appendix A and B).

*Synechococcus* PCC 73109 was the only strain observed to produce intracellular CBC. Scanning and transmission electron microscopy was used to reveal the structural characteristics of this strain (Fig. 16a and b). Compared to the intracellular structure of *Anacystis nidulans* (Allen, 1968) which is the same species of rod shaped freshwater *Synechococcus* from which *smt* gene probe was derived, marine *Synechococcus* PCC 73109 contains thylakoid lamella as the main organelle in this rod-shape marine cyanobacteria. The intracellular structure of marine *Synechococcus* PCC 73109 in the present study is more similar to *Gleocapsa alpicola*, which is the coccoid *Synechocystis* sp., (Allen, 1968) than the freshwater *A. nidulans*.
Fig. 15. Electrospray mass spectrogram of intracellular CBC. The fraction containing peak a and b from a RP-HPLC was submitted to Biomolecular Research Facility, University of Virginia, Charlottesville, Virginia.
Table 2. Amino acid composition of intracellular CBC

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Sample 7802 pmol/20 μl</th>
<th>% composition</th>
<th>Sample 7803 pmol/20 μl</th>
<th>% composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>80.3</td>
<td>1.4</td>
<td>158.1</td>
<td>2.4</td>
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<tr>
<td>Glx</td>
<td>102.6</td>
<td>1.8</td>
<td>309.0</td>
<td>4.7</td>
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<tr>
<td>Ser</td>
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<td>3.9</td>
<td>469.2</td>
<td>7.2</td>
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<tr>
<td>Gly</td>
<td>268.5</td>
<td>4.7</td>
<td>569.0</td>
<td>8.7</td>
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<tr>
<td>His</td>
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<td>122.7</td>
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<tr>
<td>Arg</td>
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<td>Ala</td>
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<td>Pro</td>
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<td>Val</td>
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<td>Ile</td>
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<tr>
<td>Leu</td>
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<td>513.2</td>
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<tr>
<td>Phe</td>
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<tr>
<td>Lys</td>
<td>3021.0</td>
<td>52.3</td>
<td>2608.4</td>
<td>39.9</td>
</tr>
<tr>
<td>Total</td>
<td>5769.2</td>
<td>100.0</td>
<td>6529.4</td>
<td>100.0</td>
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</table>
Fig. 16. Electron micrographs of marine *Synechococcus* PCC 73109. Scanning electronmicrograph (a) and transmission electronmicrograph (b).
CHAPTER IV
DISCUSSION & CONCLUSION

In marine environments, copper detoxification by marine *Synechococcus* spp. is of great interest to marine chemists and biogeochemists because this marine phytoplankton can produce a mysterious ligand (L₁) of unknown structure which has a possible role in biological control of the level of free Cu²⁺ in the ocean. Knowing how marine *Synechococcus* spp. survive in the presence of toxic concentrations of free Cu²⁺ can be a clue to the structure of L₁ and the biogeochemical cycle of copper in marine environments. However, unlike freshwater *Synechococcus*, the resistance mechanisms to copper in marine *Synechococcus* sp. have not been well studied. MT production in freshwater *Synechococcus* spp. was induced by exposure to copper but hypersensitivity to copper was not observed in a mutant lacking the gene coding for MT (Turner et al., 1993). On the basis of this observation, it was concluded that MT had no role in copper detoxification in freshwater *Synechococcus*. It has been reported that P-type ATPase is involved in copper uptake because the disruption of the gene encoding for ATPase causes an increase in copper resistance (Silver et al., 1993). Thus, it is likely that a mechanism other than MT was used by freshwater *Synechococcus* to detoxify high concentrations of copper. Alternatively, there is possibly more than one mechanism for copper detoxification. The possibility of multiple ligands with affinity for free Cu²⁺ was reported in marine *Synechococcus* spp. by Moffett & Brand (1996). A study by
Walsh et al., (1994) suggested that a marine *Synechococcus* sp. forms copper sulfide complexes extracellularly to detoxify high concentrations of copper. However, these copper sulfide complexes are not the same strong ligands as L₁ (Moffett & Brand, 1996). To date, no marine strain of *Synechococcus* has been reported to produce MT in response to copper stress, although some strains produce MT in response to cadmium and zinc (Olafson et al., 1980).

Marine *Synechococcus* was reported to be unable to produce MT in response to copper induction but to use an exclusion mechanism to survive under copper stress (Olafson et al., 1980). However, it was initially postulated in the present study that marine *Synechococcus* produced Cu-MT under copper stress. This hypothesis was based on the similarity between marine and freshwater *Synechococcus*, the similarity of MT in marine and freshwater *Synechococcus*, and the fact that the final metabolism of MT yields sulfide. The sulfide derived from MT can possibly be one of many sources of extracellular sulfide reported in marine *Synechococcus* by Walsh et al. (1994). The incubation time selected prior to measurement of intracellular MT as well as the strain of marine *Synechococcus* was hypothesized to be critical. In the study of freshwater strains, the cultures were sampled 4 hr after exposure to metals (Robinson et al., 1990). In the study of a marine strain, cultures were sampled at early stationary phase in the presence of copper, which was about 12-15 days after exposure to copper (Olafson et al., 1980). Copper binds to MT with a different stoichiometry than the binding of cadmium and zinc to MT. This difference causes the structure and properties of Cu-MT, including its turnover.
rate, to be different than Cd, Zn-MT. Due to the faster turnover rate of Cu-MT, it was hypothesized that copper induced MT was undetected in the marine strain because it degraded before isolation.

In previous studies with marine and freshwater *Synechococcus*, different concentrations of copper were used to induce MT production (Olafson et al., 1980; Robinson et al., 1990). In freshwater *Synechococcus*, amplification of the *smt* gene coding for MT was evident after repeated subculturing of the organisms in the selected concentration (determined by a stepwise adaptation technique) of cadmium (Gupta et al., 1992). Likewise, if marine *Synechococcus* spp. used in the present study contained genes coding for MT, the amplification of the MT encoded genes would be expected after repeated subculturing in the presence of CuSO$_4$ (2.2 μM) determined by a stepwise adaptation. The molecular genetics study of MT in freshwater strains indicated that the same MT was induced in the presence of cadmium, zinc, or copper of higher concentration than cadmium and zinc (Robinson et al., 1990). In a strain of marine *Synechococcus*, cadmium and zinc, but not copper, induced MT of comparable amino acid composition as the freshwater MT (Olafson, 1984). The freshwater and marine *Synechococcus* used in these studies are from the same genus and the similarity of the MT they produced suggests the possibility of the induction of similar MT by copper in marine strains. Therefore, the Southern blot hybridization technique was applied to test for MT production in marine strains under copper stress. The negative results of Southern blot hybridization indicated that MT in marine *Synechococcus* spp. (if present) was different from
the freshwater strains. In this study, no similar MT sequence homologous to the
smt gene found in the freshwater strain was detected. Possible explanations for
this observation were: the marine strains in this study produced class II MT of
different amino acid sequence from the freshwater strain; the marine strains in
this study produced only class III MT (phytochelatin); or they produced no MT at
all. In regard to the first explanation, class II MT was demonstrated in freshwater
strains under cadmium, zinc and copper stress and in a marine strain under
cadmium and zinc stress. Although the number of cysteine residues are similar,
the marine MT contains 81 total amino acid residues whereas freshwater MT
contains 56-60 total amino acid residues. Moreover, there was a high content of
Glx (either Glu or Gln) residues in the marine strain. These differences indicate
different amino acid sequences and DNA sequences between freshwater and
marine strains.

In regard to the second explanation, class III MT (phytochelatin) contains
repeating residues of Glu, and Cys and a residue of Ala or Gly at the C-terminus
and is not genetically coded. It is synthesized by the enzyme phytochelatin
synthase. Consequently, this non-genetically coded MT would yield the
negative result in the Southern blot hybridization. Also, it is possible that marine
strains produce class III MT in response to copper, but produce class II MT in
response to cadmium and zinc. There are several reports of organisms
producing different MTs in response to different metals (Mehra et al., 1988;
Dallinger et al., 1997). However, the sequence of class II MT in marine strains
had to be different from the freshwater strain because of the negative result in

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the Southern blot hybridization. The possibility that there is no MT in these marine strains was tested by isolation of intracellular protein from the marine strains after exposure to the same concentration (2.2 μM) of CuSO₄. By comparison to control cultures, there was no production of putative MT in these marine strains as the amount of component in peak A was not increased under copper challenge. This negative result could be due to an insufficient concentration of copper as others reported that excess concentrations of copper were more efficient in inducing MT production. Many studies both in eukaryotes and prokaryotes concluded that copper was not a potent MT-inducer (Bremner et al., 1986; Robinson et al., 1990). In addition, the isolation of MT was performed after the cultures reached the stationary growth phase in which MT might not exist intracellularly. Therefore, an excess concentration of CuSO₄ (50 μM) above the threshold of copper toxicity in these marine strains, as well as varied time intervals for intracellular protein isolation were tested. However, 50 μM CuSO₄ was too high a concentration for this marine strain to tolerate. Therefore the concentration of CuSO₄ that was high enough to inhibit growth but that the cells could tolerate (the maximum tolerated concentrations, MTC) were utilized for induction of copper-complexing compounds.

Results from the MTC study indicated that marine *Synechococcus* used in the study have a similar sensitivity to CuSO₄ i.e., 3 μM, 5 μM, and 8 μM for PCC 7001, PCC 7003, PCC 73109, respectively. *Synechococcus* PCC 7001 is the most sensitive among these three strains. The sensitivities of these marine *Synechococcus* to CuSO₄ observed in this study were similar to published...
studies which reported that marine *Synechococcus* was an extremely sensitive organism to free Cu$^{2+}$ (Brand et al., 1986; Walsh et al., 1994). After an addition of CuSO$_4$, the free Cu$^{2+}$ concentrations in the medium were lower than 3, 5, or 8 μM due to complexation of copper by media constituents such as EDTA. The measurement of free Cu$^{2+}$ concentrations was attempted but the concentrations were too low to be detected by a Cu$^{2+}$ ion electrode which can measure cupric ion concentration $\approx 10^{-8}$ M. The low concentration of free Cu$^{2+}$ was due to the presence of chelators in the BG-11 medium such as EDTA, and citrate. This low concentration of free Cu$^{2+}$ demonstrated the highly sensitive nature of marine *Synechococcus* spp. to copper toxicity.

It has been observed that there is covariation of population density of ligand producing phytoplankton and ligand concentration (Gordon, 1998). This observation may explain why exponentially-growing cultures of PCC 7001 were not inhibited by 3 μM CuSO$_4$ while the same copper concentration inhibited low density culture in the MTC study. Absorbance of cultures when CuSO$_4$ was added in the study of response to copper was 0.3, while that in the MTC study was 0.07. However, the survival of cells at higher cell density may be either from the high concentration of copper-complexing ligand produced by high numbers of cells, or from the decrease of copper toxicity due to the lower amount of copper per cell.

In the study of the response to copper, CuSO$_4$ was added to log phase cultures in order to observe an immediate response in growing cells to toxic concentrations of CuSO$_4$. An immediate response, such as a sudden drop of
cell density and the recovery of the cell density was expected. Such responses were observed although not immediately. In PCC 7003, the cell density of the control culture was unusually decreased. The experiment was repeated and the similar result was obtained. The decrease of cell density in the control culture was not seen in the MTC study, however. This drop of the cell density was possibly due to the deficiency of an essential nutrient e.g. vitamin B12 or iron in the medium which may not have occurred in the MTC study because the initial optical density was much lower.

The increased cell density of the cultures grown in the presence of CuSO₄ after a prolonged lag of growth was possibly caused by the decrease of the concentration of free Cu²⁺ to the non-toxic level during the lag period and cells, thus, regained their ability to grow. Formation of Cu-complexes during the prolonged stabilization of growth was possibly one of the mechanisms to decrease the concentrations of free Cu²⁺. However, the complexation of copper in these marine strains was not due to the same MT as in freshwater strains as shown by the results of Southern blot hybridization. Therefore, to determine what component the marine strains produce to form complexes with free Cu²⁺, the isolation of intracellular molecules from cells exposed to CuSO₄ and the determination of the component highly bound to copper was undertaken.

The present study demonstrated the production of a low molecular weight intracellular (<6,500 Da) component that bound to copper. The highest concentrations of intracellular copper were observed in the eluate fraction (from size exclusion-FPLC column) containing this component. The production of an
intracellular copper binding component (hereafter referred to as CBC) was observed in PCC 73109 within 2 hr exposure to CuSO₄ under extreme concentrations (50 μM) above the threshold of copper toxicity. The amount of CBC produced was possibly able to partially protect the cells since the green color of cultures was maintained up to 18 hr. In addition, the very low concentrations of total intracellular protein (nearly undetectable) suggests that the detoxification system may be overwhelmed. At lower concentrations (the MTC) of CuSO₄, the production of CBC was seen in PCC 73109 within 2 hr of exposure to CuSO₄. The present study indicated that exposure time to CuSO₄ as well as the concentration of CuSO₄ is significant to the production of this CBC. Toxic concentrations of CuSO₄, i.e., the maximum tolerated concentration (8 μM) or above the threshold concentration (50 μM), induced the increase of this component above the basal level. The possibility that the increase of CBC was not from the induction by high concentrations of copper but, instead, the CBC existed naturally as an endogenous pool waiting for sequestered copper ions, was addressed. If it was the case, the larger peak A area and the higher concentration of total copper would be always detected in the cultures with the addition of CuSO₄. In the present study, peak A areas and total copper concentrations were not increased when 2.2 μM CuSO₄ was added to the cultures. The observed copper-complexing capacity of CBC suggests a possible role in copper detoxification. The fact that CBC was detectable in control cultures indicates the basal production at the low (non-toxic) copper concentration present in the culture medium. As an extremely sensitive
organism to copper toxicity, the basal level could maintain cell viability in situations where copper concentrations rise rapidly or, alternately, it may have an additional cellular function. Increased intracellular concentration of the CBC may allow *Synechococcus* to survive at these elevated copper concentrations. A decrease of intracellular concentration of the CBC, although above the basal level, observed after exposure to CuSO$_4$ longer than 2 hr, may be due to secretion of the component after the intracellular saturation. A decrease in total cellular copper corresponding with a decrease in peak A area is consistent with the idea of an excretion mechanism. An excretion mechanism was reported in marine *Synechococcus* WH 7803, however, it was not clear that copper was secreted as copper-ligand complexes or as free copper ion (Moffett & Brand, 1996). In Cu-resistant freshwater cyanobacteria, an export of intracellular copper into the medium was reported by Verma & Singh (1991). Their study showed that the rate of Cu$^{2+}$-efflux was rapid during the first 5 min and, after 60 min of efflux, there was a significant reduction in the intracellular level of Cu$^{2+}$.

Because of the unique structure of phytochelatins (a 800-2,000 Da of a 2-11 repeat of glutamylcysteinyldipeptides added to glutathione) and their rapid production (within 5 min after exposure to metals), the possibility that CBC was phytochelatin was considered. However, phytochelatins have been reported only in plants, and fungi. The content of Cys residues and the amino acid analysis showed that CBC was not phytochelatin. Absence of high concentrations of sulfhydryl groups in intracellular proteins isolated from cultures exposed to CuSO$_4$ at different time periods supports the null hypothesis that Cu-
MT (class II or class III, phytochelatin) is not present in PCC 73109. In addition, the analysis of the purified component by mass spectrometry and amino acid analysis did not confirm the component as phytochelatin. A high content of lysine and an unidentified component that absorbed at 254 nm was demonstrated in the amino acid analysis. CBC is not a peptide while both MT and phytochelatin are.

Natural chromophores are contained in all proteins. These chromophores absorb light near UV wavelength and also are responsible for protein fluorescence. For example, the maximum absorption for aromatic residues (phenylalanine, tyrosine and tryptophan) is 260-290 nm and the emission wavelength of tryptophan in most protein is 340 nm. Absorbance and fluorescence of these residues may change on protein binding to a ligand. The change can be demonstrated by either a shift in emission wavelength or an increasing absorbance at a given wavelength (Bagshaw & Harris, 1987). In the present study, UV-visible scanning demonstrated a maximum absorption of CBC at 230 nm which indicated that CBC did not contain aromatic amino acid residues. The maximum excitation wavelength of 250 nm and a maximum emission wavelength at 450 nm by spectrofluorimetry scanning possibly indicated the presence of compound with cyclic or aromatic structure. The emission wavelength of nucleic acid is 450 nm. Also, these results confirmed that the component was neither class II MT nor class III MT (phytochelatins). A typical excitation wavelength of class II MT was 305 nm and maximum emission wavelength was 565 nm (Beltrami & Lerch, 1981).

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The possibility of the intracellular copper-binding component as a siderophore was considered, although a siderophore is an extracellular component, because of its low molecular weight (range from 229 to 1,400 Da depending on the class of siderophore) and its ability to chelate copper. Biosynthesis of siderophores is regulated by exogenous Fe\(^{3+}\) level i.e. repressed in high Fe\(^{3+}\) concentration. In marine bacteria, under conditions of iron limitation, siderophore production is rapid during the exponential growth phase, with the synthesis decreasing sharply during stationary phase for the catechol-type siderophores (Reid & Butler, 1991). In contrast, the accumulation of hydroxamate-type siderophores, i.e schizokinen, in the growth medium was found to be proportional to cell density throughout growth phase and to continue well into stationary phase (Clarke et al., 1987). The amino acid analysis of catechol siderophores in marine bacterium Alteromonas luteoviolaceus showed the presence of serine, glycine and arginine in a ratio of 1:2:1 (Reid & Butler, 1991). Serine and glycine are well known components of siderophores. The exact mass of siderophores determined by fast atom bombardment (FAB) mass spectral analysis in this marine bacterium were 945.3961 and 927.3954. The maximum UV absorbance was at 320 nm. By the characteristics of existing siderophores, the intracellular CBC in the present study did not appear to be a siderophore. Results of CAS assay performed in the intracellular crude extract and HPLC fraction containing CBC were also negative for iron-binding compounds. Consequently, the blue color of the Fe\(^{3+}\)-CAS-HDTMA complex was not changed to orange color because Fe\(^{3+}\) was not removed from the
complexes. Unchanged color due to an insufficient amount of iron-binding compounds or siderophores (if there were any) was considered. Because of the high sensitivity of the assay which measured the affinity of iron to chelator (1:1 ratio), <7.5 nmol of chelator with high affinity to iron, i.e. siderophores, can be detected. The assay has been applied to detect siderophores directly from the supernatants of culture fluids by a paper electrophoresis chromatography or in a culture agar plate due to its high sensitivity (Schwyn & Neilands, 1987; Reid & Butler, 1991). The molecular weight of the purified fraction from HPLC in this study was <6,500 Da (observation from SDS-PAGE which is not shown). The molecular weight of siderophores reported in the literature ranges from 229-1400 Da (Reid & Butler, 1991). In this study, if the CBC was a siderophore, its concentration in crude extract or in the purified fraction from HPLC should be sufficient to remove Fe\(^{3+}\) from the dye complexes. The negative results were confirmed after allowing the reaction to proceed overnight or using the CAS shuttle solution to accelerate the iron exchange rate. The negative results indicate that the intracellular copper binding component did not bind to iron or had a low affinity to iron and only high concentrations (>7.5 nmol) of the component was required to give the positive test. In the present study, the result was negative when a crude extract was also tested. Thus it was not a siderophore. In addition, the negative results confirmed that the CBC had higher affinity to copper than iron (if it could bind to iron).

Further study of the structure of the intracellular copper-binding component isolated in this study is needed. A small molecular weight (MW 500)
extracellular copper binding ligand (CBL) has been reported in methanotrophic bacteria (Tellez et al., 1998). This CBL is a hydrophobic molecule with some aromatic character and has high affinity and selectivity for copper. The ligand is produced constitutively during periods of growth and is reinternalized when it is bound to copper. In the present study, the observation of pH influence on retention by RP-HPLC suggests the intracellular component is an ionic solute of which the ionic charge changes as a function of pH of mobile phase. Data from the present study shows that the copper-complexing component is of low molecular weight (<6,500) contains lysine, probably contains a cyclic or aromatic functionality, and has ionic charge characteristics. Lysine contains \( \alpha \)-carboxylate, \( \alpha \)-amino, and \( \varepsilon \)-amino group that can chelate free Cu\(^{2+}\) (Martin, 1979). These data suggest that the CBC production may be a mechanism by which marine Synechococcus PCC 73109 alleviates copper toxicity.

Among three strains of marine Synechococcus studied, only PCC 73109 demonstrated the production of intracellular CBC. In strain PCC 7001 and 7003 with addition of CuSO\(_4\), peak A or intracellular "CBC" was lower than basal level and intracellular copper concentration was also lower than the control. Other mechanisms were probably used to detoxify high copper concentrations in these two strains. The fact that toxic concentrations of CuSO\(_4\) were added to the cultures and intracellular copper concentrations were lower than the control suggests that most of the copper was trapped extracellularly or effluxed from the cells. The production of extracellular chelators by freshwater cyanobacteria in response to copper stress has been reported (Jardim and Pearson, 1984).
When intracellular copper concentration in the copper stressed culture became higher than the control, the intracellular CBC was produced as indicated by a larger peak A in PCC 7001 at 35 d and in PCC 7003 at 24 hr compared to the control. Thus, multiple mechanisms to detoxify high copper concentration may be involved in PCC 7001 and PCC 7003. When a lethal concentration (50 μM) of CuSO₄ was added to the culture of PCC 7001, the decrease in intracellular copper concentration at 24 hr caused by the very low concentrations of total intracellular protein and then its increase at day 3 indicated the detoxification being overwhelmed.

The results from the present study (i.e., induction of CBC in PCC 73109 and possible different mechanisms in PCC 7001 and PCC 7003 to detoxify high concentrations of copper) suggests that different strains utilize different copper-detoxification mechanisms. Cyanobacteria are the most diversified microorganisms. Three strains of marine Synechococcus used in this study as well as the freshwater strain from which the probe was derived are classified as the same genus of Synechococcus based on cellular characteristics i.e., the dimensions of the cell, reproductive characteristics and DNA base composition. There are, still, many characteristics that have not been considered which may separate these cyanobacteria into different genera. The visual comparison of the electronmicrograph of PCC 73109 in this study to the electronmicrographs of Gleocapsa alpicola (a Synechocystis sp.) and Anacystis nidulans (a freshwater Synechococcus sp.) reported by Allen (1968), demonstrated more similarity of intracellular structures of PCC 73109 to G. alpicola than to A. nidulans which is
classified as the same genus as PCC 73109. This evidence addresses a question whether marine *Synechococcus* is truly the same genus as freshwater *Synechococcus*.

Variation of the cultures from batch to batch was clearly seen. For example, in the induction experiments, peak A area and total intracellular copper concentrations from an individual set (consists of a control culture and a CuSO$_4$ added culture) of cultures were greatly increased in the copper challenged cultures. However, the average of those results from the two sets of cultures (a set of culture consists of a control culture and a CuSO$_4$ added culture, the two sets were experimented on the different day) yielded a high standard deviation which caused an insignificant statistic test. To reduce the variations between two sets, the ratios of control samples to CuSO$_4$ added samples for peak A area and total intracellular copper concentration were used for the statistic test.

It is clear that the intracellular copper concentrations per mg protein in these three marine strains are controlled to be less than 140 ppm by producing intracellular CBC or by some other mechanisms. Thus, this concentration of copper is possibly the saturated concentration for these marine *Synechococcus*.

Studies by Moffett et. al. (1990, 1996) of Cu-complexing ligands in marine *Synechococcus* showed that this marine genus can produce a strong Cu-complexing ligand (a conditional stability constant $\approx 10^{13}$, Moffett et. al. 1990) similar to $L_1$ ligand found in natural seawater. It was proposed that this marine genus effected biological control of free Cu$^{2+}$ concentrations in natural seawater. Many metal-binding compounds have been detected in cultures of marine and
freshwater cyanobacteria. However, these compounds are not strong ligands like L₁ reported in marine *Synechococcus*. Further study by nuclear magnetic resonance spectroscopy (NMR) may reveal the dynamic three-dimensional structure of the intracellular CBC isolated in the present study. In addition, high resolution of NMR can provide the biological function of a molecule, i.e., ligand binding, protonation (Case & Wright, 1993) which can be used to determine the affinity of CBC for copper and whether it is a strong L₁-like ligand. However, NMR studies will require milligram quantities of purified CBC. Preparative scale culture volumes (about 500-fold increase in volume), size exclusion chromatography and reversed phase chromatography are necessary to yield about 1 mg of purified CBC.
REFERENCES


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APPENDIX A

MASS SPECTROMETRY ANALYSIS

Reported by Dr. Michael Kinter, Ph.D.
Director, W.M. Keck Biomedical Mass Spectrometry Laboratory

The chromatogram obtained from this analysis (Fig. 1) showed no peptide peaks. The breakthrough of the solvent was observed at the beginning of the analysis, this material gave the electrospray spectrum showed in Fig. 2. The low intensity peaks seen at approximately scan number 300 gave the electrospray spectrum shown in Fig. 3. Neither of these spectra are consistent with electrospray spectra of peptides. Specifically they do not show the charging pattern characteristic of peptide mass spectra. Similarly, no peptide ion are seen in the matrix assisted laser desorption time-of-flight mass spectrum shown in Fig. 4. Therefore, it would appear that the HPLC fraction submitted is either a peptide with no basic sites (no N-terminus and no histidine, lysine or arginine) or it is not a peptide but rather some other non-peptide, UV-absorbing material. I would also add that two aspects of the HPLC analysis would appear inconsistent with this fraction being a peptide. First, the fraction of interest elutes from a C18 column at an extremely low acetronitrile content. In my experience, peptides either are not retained on a C18 column, elute at >30% acetronitrile, or do not elute. I have not seen any peptides that elute at 5% to 10% acetronitrile. Second, based on the chromatographic peak intensity, this very simple isolation protocol produced nmole quantities of a single peptide without detectable amounts of any other peptides. Although our experience is exclusively with mammalian cells, we would expect a far greater number of peptides in such an extract.

Note: The result in chapter III showed only Figure 4 mentioned above.
APPENDIX B

AMINO ACID ANALYSIS

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The results of the amino acid analyses are shown in Table 1. The chromatograms obtained in these analyses are shown in Fig. 1 and 2. Three observations of these data:

1. The amount of peptide expected, based on the chromatographic fractionation of Dr. Brown, was 20 nmol. Therefore at least 20 nmol of all amino acids in the peptide would be expected. Only lysine was present in nmol amounts of the other amino acids are approaching baseline.

2. If this is a peptide then the amino acid composition is approximately 50% lysine.

3. As seen in the figures there is a large non-amino acid contaminant eluting at approximately 13.6 min. The identity of this contaminant is not known but the peak intensity is orders-of-magnitude greater than any of the amino acids seen in the chromatogram.

Note: The result in chapter III showed only Table 1 mentioned above.
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