Virginia Journal of Science
Volume 52, Number 4
Winter 2001

Production of a Novel Copper-Binding Ligand by Marine *Synechococcus* (Cyanobacteria) in Response to Toxic Concentrations of Copper

Arunsri C Brown[1] and **Andrew S Gordon**[2]
Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529-0266

ABSTRACT

Marine *Synechococcus* spp. are extremely sensitive to copper toxicity. Some strains have been shown to produce high-affinity, extracellular ligands of unknown structure which form complexes with free cupric ion. They are also known to produce metallothioneins (MT) in response to cadmium and zinc stress. In the present study, marine *Synechococcus* PCC 73109 (*Agmenellum quadruplicatum* BG-1) (Van Baalen) was exposed to three concentrations of $CuSO_4$ for various times. Size exclusion chromatography, atomic absorption spectrophotometry, and reverse phase HPLC were used to isolate an intracellular copper binding ligand of low molecular weight (< 6,500 Da). The ligand was detected after exposure to ≥ 8 μM $CuSO_4$ for 2 hr in BG-11 medium. The intracellular ligand was characterized by electrospray mass spectrometry, amino acid analysis and a universal assay for siderophores. The ligand was not MT, phytochelatin or a siderophore. It is not a peptide but it contains lysine and an unidentified UV 254-absorbing constituent. This compound is a novel copper-binding ligand previously not reported in *Synechococcus* spp.

Key Index Words: copper toxicity, intracellular copper binding ligand, lysine-contained ligand, marine *Synechococcus*, low molecular weight non-peptide ligand.

Abbreviations: FPLC, Fast Protein Liquid Chromatography; MT, Metallothionein; OD_{450}, Optical density at 450 nm; *smt*, *Synechococcus* metallothionein encoded gene.

INTRODUCTION

Trace metals found in oceanic surface waters can control phytoplankton production and species composition (Bruland *et al.*, 1991). Some trace metals, such as copper, are essential but are toxic at high levels. Excessive copper concentrations can inhibit growth and reproductive rates of phytoplankton in the ocean (Brand *et al.*, 1986; Walsh *et al.*, 1994; Gledhill *et al.*, 1997). A number of marine bacteria, fungi and protozoa have evolved methods to control cellular metal concentrations. Intracellular metal

1 Present address: Philips Institute-School of Dentistry, Virginia Commonwealth University, Richmond, VA 23298-0566
2 Corresponding author: (Phone) 757-683-3594, (E-mail) Agordon@odu.edu

detoxifying chelators known as phytochelatins, found in algae and higher plants, can effectively reduce toxic levels of free copper to nontoxic levels (Grill *et al.* 1985). Phytochelatins are produced by marine phytoplankton (Ahner *et al.*, 1995; 1997) and their concentration has been observed to vary systematically with free copper concentration in coastal waters (Ahner *et al.*, 1997). Microbial oxidation reactions also reduce the toxicity of some metals (Emerson *et al.*, 1979; Tebo, 1995). Extracellular copper complexing ligands may also function in copper detoxification (Harwood-Sears and Gordon, 1990; Harwood and Gordon, 1994; Moffett and Zika, 1983; Bruland *et al.*, 1991). *Synechococcus spp.* (WH7803 and PCC 73109) have been shown to produce a strong extracellular copper chelator of unknown structure in response to copper stress (Moffett and Brand, 1996; Gordon *et al.* 2000). Furthermore, McKnight and Morel (1979) detected a strong chelator in freshwater *Synechococcus* cultures. The principal ligand in their studies was shown to be a siderophore (McKnight and Morel, 1980). Various metal-binding compounds including H_2S, siderophores, and metallothionein, have been detected in *Synechococcus* cultures and possibly protect *Synechococcus* from copper toxicity. There is no evidence that these compounds are the strong extracellular ligands detected in cultures (Moffett and Brand, 1996). MT has been reported to be induced by cadmium and zinc, but not copper, in marine *Synechococcus* (Olafson *et al.*, 1980). In contrast, those three metals induced MT in a freshwater strain, but a mutant strain lacking the MT gene (*smt*) was sensitive to high concentrations of cadmium and zinc but not copper (Robinson *et al.*, 1990; Turner *et al.*, 1993). This observation suggests that MT is not important in determining copper tolerance in these microorganisms.

The purpose of the present study was to test the hypothesis that MT is produced in response to elevated concentrations of copper by marine *Synechococcus.* Metallothionein, phytochelatin, or siderophores were not produced by Cu-stressed cells of the strain we examined. However, a low molecular weight (<6,500 Da) molecule that bound the majority of copper contained in cellular extracts of marine *Synechococcus* exposed to copper was isolated. This component, which is not a peptide but which contains lysine, was produced in significantly higher amounts when the organisms were stressed by copper.

MATERIALS & METHODS

Source of marine *cyanobacteria.*

The strain of cyanobacteria used in this study was *Synechococcus* PCC 73109 (American Type Culture Collection ATCC 29404; *Agmenellum quadruplicatum* BG-1), that was originally collected from seawater (Rippka *et al.*, 1979).

Cultivation of marine *cyanobacteria.*

Axenic cultures of PCC 73109 were grown in BG-11 medium (Rippka, 1988), under fluorescent light (30-60 µmol quanta (par)'cm^{-2}'s^{-1}, dark/light cycle [12/12 hr]), at 28°C. Cell number was determined from optical density (450 nm) and cultures were routinely checked for bacterial contamination by streak-plating on Tryptic Soy agar (Difco) plates. Green pigmentation of cultures was used as a visual indication of cell viability. All glassware was rinsed several times with deionized water after soaking overnight in 10% nitric acid. Medium was prepared using Milli-Q water. Solutions

and buffers were prepared in Milli-Q water and run through a chelex -100 (Bio-Rad Laboratory) column to remove trace metal contamination.

Induction of copper-complexing ligands in marine *Synechococcus*.

Three concentrations (2.2, 8 and 50 μM) of $CuSO_4$ were used in this study to induce the production of copper-complexing metabolites. These concentrations were selected based upon toxic response of the organism to each concentration in BG-11 medium. 2.2 μM was the concentration at which *Synechococcus* could be maintained readily after stepwise adaptation to $CuSO_4$ (described below). Eight μM was selected based upon the results of toxicity assays as an inhibitory but non-lethal concentration and 50 μM was used as an excessive, lethal dose. Control samples consisted of the organism grown without the addition of $CuSO_4$.

Stepwise adaptation. Using a stepwise adaptation method described by Gupta *et al.* (1992), marine *Synechococcus* PCC 73109 was subcultured in liquid BG-11 medium containing an initial concentration of 0.5 μM $CuSO_4$. Cells that grew in the initial concentration of copper were further subcultured to fresh medium containing higher concentrations of $CuSO_4$ (1, 1.5, 2.2 μM). After adaptation, cultures were maintained in 2.2 μM $CuSO_4$ for subsequent studies.

Inhibitory concentrations of CuSO4.

Different concentrations of $CuSO_4$ (0-20 μM) were added to tubes containing fresh subcultures (OD_{450} = 0.06-0.07) of PCC 73109. Optical density was determined daily (450 nm) until constant. A non-lethal concentration of $CuSO_4$ that caused significant growth inhibition was selected as the inhibitory concentration. The inhibitory concentration (8μM) was added to log phase cultures in subsequent induction experiments. For these experiments, cultures (80 mL) of strain PCC 73109 were grown to log phase (OD_{450} = 0.4-0.5 nm). Cultures were pooled and transferred (80 mL each) to two flasks. Eight μM $CuSO_4$ 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.

Lethal concentration of CuSO4.

Fifty μM copper addition was lethal as indicated by a clear and colorless culture after ten days. This concentration was also utilized in short-term induction experiments for comparison with the inhibitory concentration.

Isolation of genomic DNA and Southern blot hybridization.

Genomic DNA was isolated from *Synechococcus* PCC 73109 that had been maintained in medium containing 2.2 μM $CuSO_4$. Genomic DNA was isolated using a modification of the method described by Porter (1988). 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^{-1}) and RNase (5 mg•mL^{-1}) were added and the lysate was incubated at 37°C for 30 min. Sodium dodecyl sulfate (1%) was added. The lysate was incubated at 50°C after adding proteinase K (100 μg/mL). After phenol/chloroform extraction, the DNA was dialysed in 1X TE (10 mM Tris and 1 mM EDTA) buffer. Equal concentrations of genomic DNA isolated from marine *Synechococcus* PCC 73109 grown in the presence

or absence of $CuSO_4$ 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* strain (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.

Isolation of intracellular components from PCC 73109 after exposure to $CuSO_4$.

Three different concentrations (2.2, 8, 50 μM) of $CuSO_4$ were added to cultures of marine *Synechococcus* PCC 73109 during the log phase of growth. Cells were harvested at early stationary phase (cell densities of 10^7-10^8 cells•mL^{-1} as determined from acridine orange direct counts) for intracellular protein isolation when 2.2 μM $CuSO_4$ was added. The exposure period to added $CuSO_4$ was 7 to 10 d. When 8μM $CuSO_4$ was added, cells were sampled at 0 min, 30 min, 2 hr, 24 hr, 72 hr (3 d), and when the cultures reached the stationary phase of growth (OD_{450} >1 and cell number ~10^8-10^9 cells•mL^{-1}). When 50 μM $CuSO_4$ was added, cells were harvested for intracellular protein isolation after 2 hr, 24 hr, and 72 hr (3 d) of exposure.

At the time of culture harvest, the absorbance (450 nm) of each culture was read and the cultures were placed on ice for 30 min. Cells were then centrifuged at 3,000xg, 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 0.5 M Tris Cl, pH 8.6. The cells were ruptured by using an ice cold "Bead-Beater" (Biospec Products) with 27.5 g of acid washed glass beads (0.1 mm diameter) for 3 min and the lysate was centrifuged at 3,000xg, 4°C for 15 min. The supernatant obtained was a crude cellular extract. The concentration of intracellular protein contained in crude extracts was determined by using a BCA Protein Assay (Pierce).

Separation of intracellular components produced by copper-stressed marine *Synechococcus* .

Crude extracts were separated by 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 was 30 mM Tris Cl with 5 mM mercaptoethanol, pH 8.6, at a flow rate of 1 mL•min^{-1}. Peak area was measured with an electronic graphic calculator (Numonics Corp). Molecular weight standards used for column calibration were 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). The concentration of total copper in crude extracts and in eluate fractions from the Superose column was determined with Polarized Zeeman graphite furnace atomic absorption spectroscopy (Hitachi, Z-8100). Copper concentrations were normalized to total intracellular protein in the extracts.

Absorbance and emission characteristics of copper-binding components were determined with a spectrophotometer and a spectrofluorimeter. Eluate fractions con-

taining the copper-complexing material collected from a Superose column were scanned (200-900 nm) using a UV-visible spectrophotometer (Varian, Cary 3 Bio). The same fractions were also scanned for absorption (200-400 nm) and emission (200-700 nm) using a spectrofluorimeter (Shimadzu, RF 5000 U).

Three-mL fractions containing the copper-complexing components of interest were collected from a Superose column, pooled and concentrated by lyophilization. The dry material was resuspended in 150 µL of Milli-Q water. The suspension (100 µL) was injected into a reversed-phase HPLC column (Macrosphere 300 RP C8 7U, Alltech) and eluted (1 $mL \cdot min^{-1}$) using the gradient profile described by Klauser *et al.* (1983). The equilibration buffer (buffer A) was 0.1% trifluoroacetic acid (TFA, Sigma) and the elution buffer (buffer B) was 0.1% TFA containing 60% acetonitrile (Fisher, HPLC grade). The gradient profile was buffer B, 0-30% over 10 min, and 30-45% over 60 min. The column was washed in 100% buffer B for 5 min, and then was equilibrated in buffer A for 10 min prior to a second injection. 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 (Fraction-100, Pharmacia). The eluate fractions were used for determination of -SH concentrations, Chrome azurol S (CAS) assay for siderophores, electrospray mass spectrometry, and amino acid analysis.

Determination of the concentrations of sulfhydryl groups.

-SH concentrations were determined in RP-HPLC eluate fractions by the method of Ellman (1959). The reaction volume was scaled down by 1/3.

CAS (Chrome azurol S) assay.

Intracellular crude extract and eluate fractions collected from RP-HPLC were used to determined the presence of siderophores using a universal chemical assay developed by Schwyn & Neilands (1987). 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 HCl, pH 8.6. The reference control for the eluate fractions was 0.1% TFA. EDTA (0.5 M) was used as the positive control.

Mass spectrometry analysis and amino acid analysis.

A purified component in fractions collected from RP-HPLC was submitted for electrospray mass spectrometry and amino acid analysis at the W.M. Keck Biomolecular Research Facility, University of Virginia. The facility utilizes a Finnigan-MAT TSQ7000 system with an electrospray ion source interfaced to a reverse phase capillary column. Amino acid analysis was performed by HPLC analysis of PTC derivatives after hydrolysis overnight at 100°C under vacuum.

RESULTS

Determination of inhibitory concentrations of $CuSO_4$.

Three distinct types of growth were seen when cultures of *Synechococcus* PCC 73109 were treated with varying copper levels. In the presence of 1.5-4.5 µM $CuSO_4$, cell densities were lower than the cell density of the control at every time point. However, the growth rate ($\approx$ 0.06/d) of these cultures was similar to that of the control.

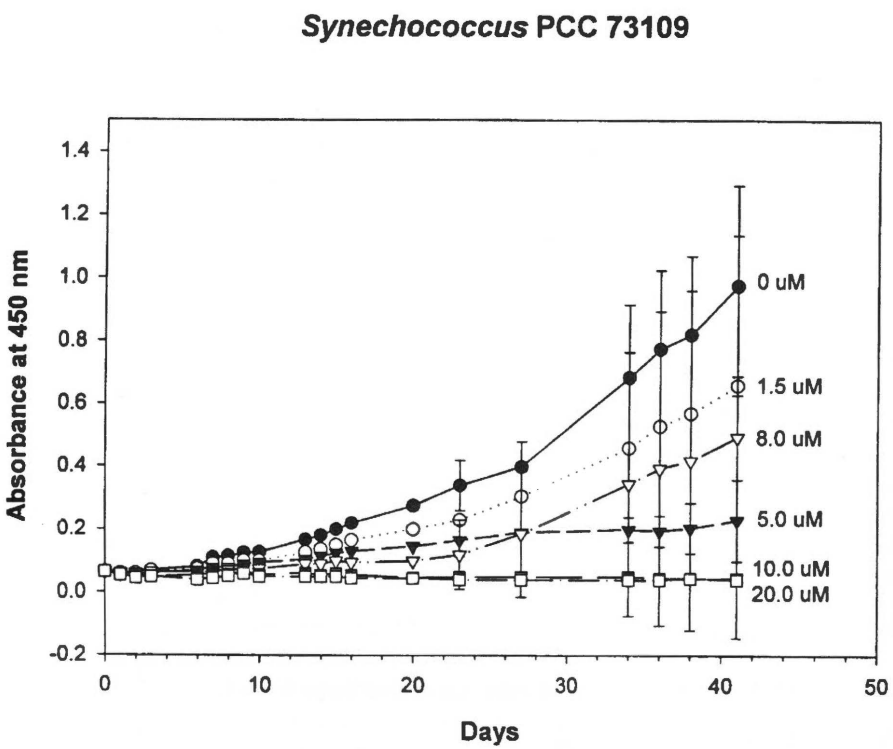


FIGURE 1. Growth of *Synechococcus* PCC 73109 in cultures containing varying concentrations of copper. $CuSO_4$ was added to cultures on day 0. 8 μM copper was selected as an inhibitory but non-lethal concentration for further studies of induction copper-complexing ligands. Error bars are standard deviations. For clarity not all copper concentrations tested are shown in the figure.

In the presence of 5.0-8.0 μM $CuSO_4$, cell densities were lower than the first group with the lowest cell densities in the presence of 8.0 μM $CuSO_4$ although the cultures remained green. After day 20, a shift to rapid growth was observed in 5.5, 6.0 and 8.0 μM $CuSO_4$ containing cultures (Figure 1). At higher concentrations of $CuSO_4$ (10 and 20 μM), the absorbance at later time points was lower than the absorbance at day 0 and the cultures were colorless. In later stages of the growth curve (after about 25 days), significant variation was observed in cell density between replicate cultures (Figure 1). The addition of 8.0 μM $CuSO_4$ to the culture resulted in significant growth inhibition during the first 20 days of incubation but cells were still alive (green color and recovery to rapid growth after 20 days). Thus growth was inhibited but cells were viable. 8.0 μM of $CuSO_4$ was chosen as the inhibitory copper concentration for further studies of the induction of copper-complexing compounds.

Southern blot of *smt* gene.

No detectable homologous (no hybridizing) band was found in *Synechococcus* PCC 73109 which hybridized to the freshwater *smt* gene probe from PCC 6301. However, hybridizing bands were readily observed in genomic DNA extracted from the freshwater strain from which the *smt* probe was derived. The intensity of a hybridizing band

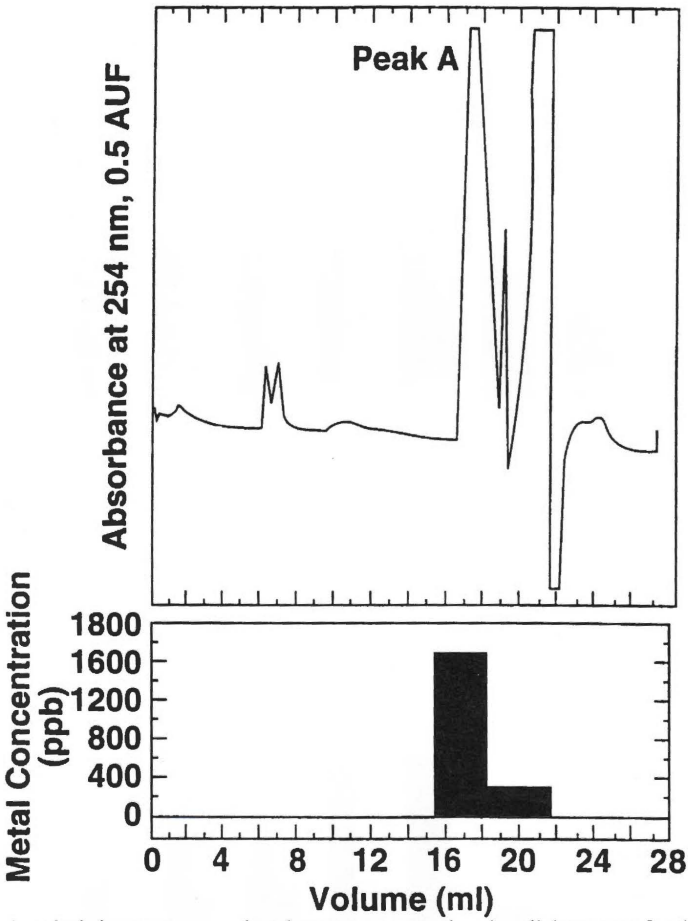


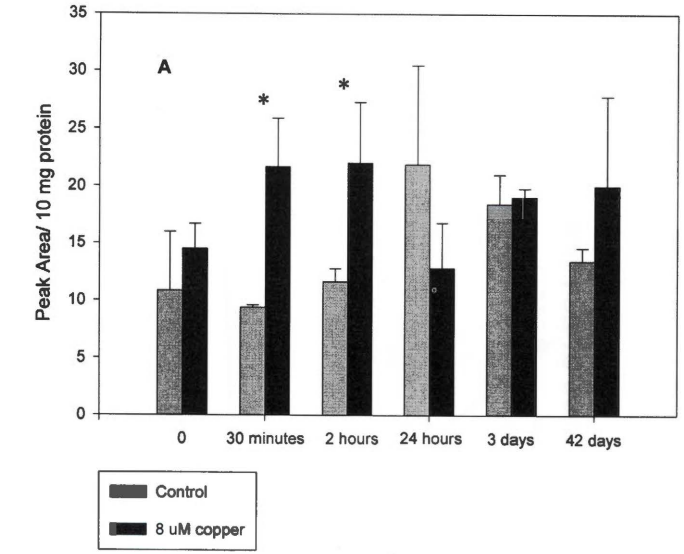
FIGURE 2. A typical chromatogram and total copper concentrations in cellular extract fractions. Extracts (200 μL) from a culture stressed with 50 μM copper were injected into a Superose size exclusion column connected to an FPLC system. The eluant buffer was 30 μM Tris Cl, pH 8.6 and 5 mM mercaptoethanol with a flow rate of 1 mL/min. Fractions of 3 mL were collected. Total copper concentration was determined

was higher when the freshwater culture was exposed to 2.2 μM $CdCl_2$ (as a positive control) possibly due to the amplification of *smt* gene induced by $CdCl_2$ (Gupta *et al.*, 1992).

Analyses of a copper-complexing component produced under copper stress.

Intracellular components isolated from cells subjected to three concentrations of copper (2.2 μM, 8μM and 50μM) were injected into a size exclusion Superose-FPLC column. Chromatograms of intracellular proteins isolated from every culture showed a distinct peak of MW <10,000 (retention volume 16-17 mL). This peak is hereafter referred to as peak A. The concentration of total copper was highest in the fraction containing this peak (Figure 2).

When cells were grown in 2.2 μM $CuSO_4$, the area of peak A was the same as peak A area from the control sample. However, when cells were exposed to 8 μM $CuSO_4$,



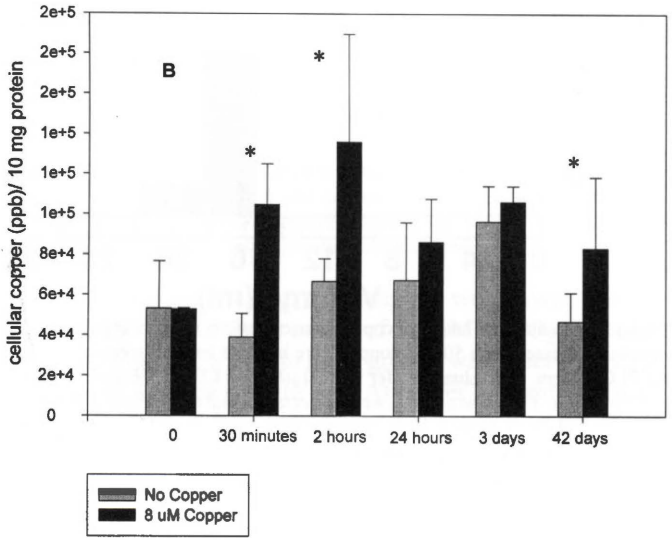


FIGURE 3a and b. Peak A area and copper concentration in cellular extracts from copper challenged (8 μM) and control cultures (a). Peak A area normalized to total protein in the cell extract. (b) Total cellular copper concentrations normalized to total protein. Error bars are standard deviations. * indicates significant ($p < 0.05$) difference from control (t-test).

peak A area from the copper amended cultures was generally larger than the control (Figure 3a) and was significantly larger at exposure times of 30 minutes and 2 hours. Total copper concentrations per mg protein in extracts of copper-treated cells were initially higher than the controls (30 min and 2 hours) but after 24 hours, intracellular copper concentrations were similar (Figure 3b).

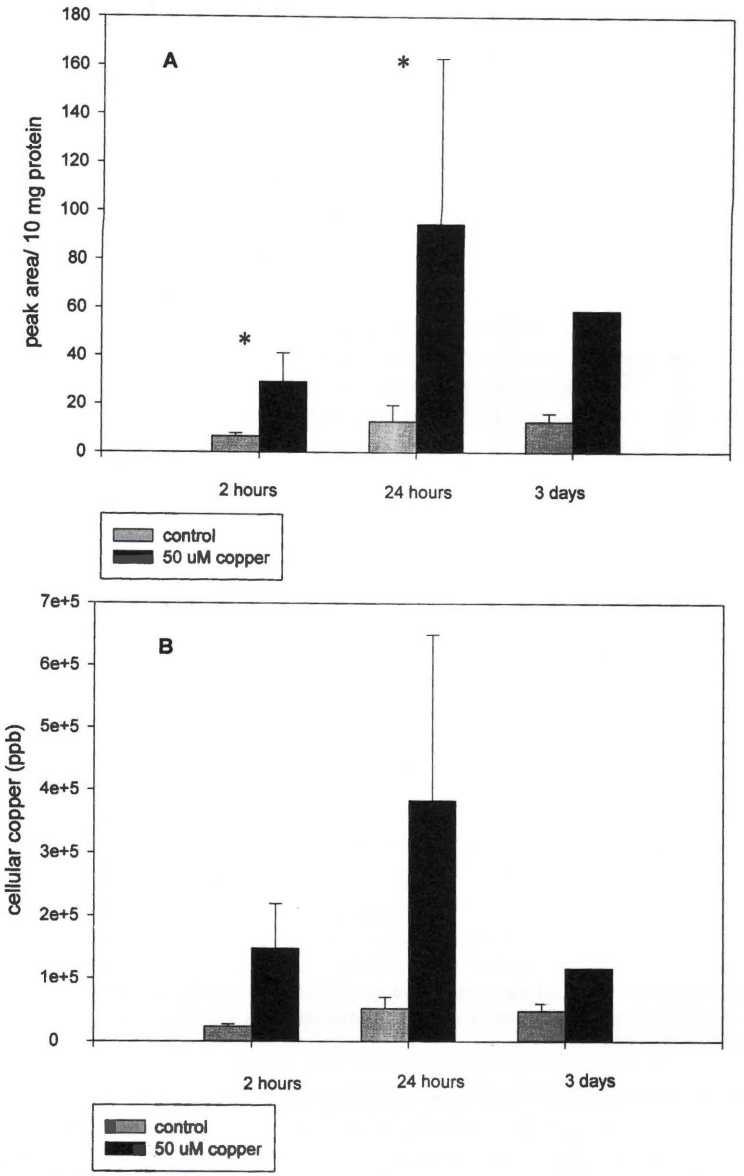


FIGURE 4a and b. Peak A area and copper concentration in cellular extracts from copper challenged (50 μM) and control cultures. (a) Peak A area normalized to total protein in the cell extract. (b) Total cellular copper concentrations normalized to total protein. Error bars are standard deviations. * indicates significant ($p < 0.05$) difference from control (t-test).

When 50 μM $CuSO_4$ was added, the culture changed color from green to yellowish green after 18 hr of exposure and then became clear and colorless after 10 d of exposure. The comparison of peak A area between the $CuSO_4$ treated sample (at 2 hr, 24 hr, and 3 d) and the control sample is shown in Figure 4a. At this higher copper concentration the area of peak A and intracellular copper concentration in copper challenged cultures

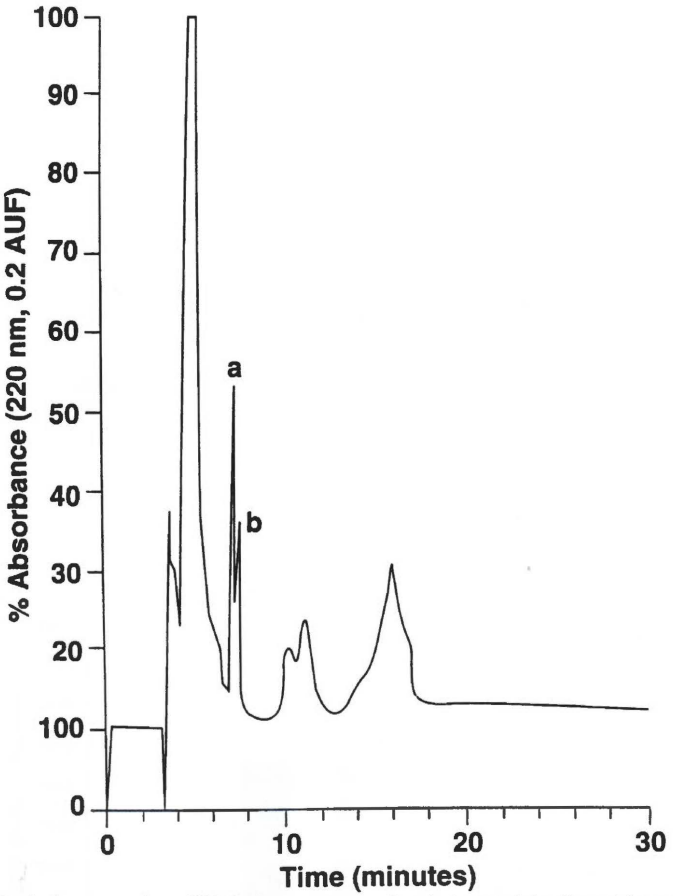


FIGURE 5. Typical reverse-phase HPLC chromatogram of peak A material collected from the Superose-FPLC separation of *Synechococcus* PCC 73109 cellular extract. Three fractions (3 mL) containing peak A eluted from a Superose-FPLC column were pooled, lyophilized, and resuspended in 150 µL of Milli-Q water. 100 µL (~78g protein) was injected into C_8-RPHPLC column. Peak a and b produced a single band (<6,500 MW) in SDS-PAGE gels.

exceeded that of the controls at each sampling time (Figure 4b). The difference was significant ($p < 0.05$) at 2 and 24 h time points. Large variation was observed in peak A area and cellular copper concentration in replicate copper-challenged cultures at the 24 hour sampling time.

Characterization of the intracellular components induced by the addition of $CuSO_4$.

The eluate fraction containing peak A was scanned using a UV-visible spectrophotometer (wavelength 200-900 nm) and a spectrofluorimeter. Maximum absorption was observed at 230 nm in all fractions containing peak A and the maximum emission was at 450 nm at excitation of 250 nm and 350 nm.

The RP-HPLC chromatogram of components contained within peak A is shown in Figure 5. The components that did not bind to the column (void volume) were eluted

at approximately 4 min. The peaks at retention time 6-7 min were absent in buffer blanks and their areas were consistently larger in copper-challenged cultures when normalized to total protein in the FPLC fraction. The material comprising peaks a&b eluted about 2 min. later from the RP-HPLC column when tetrabutylammonium bromide (TBA) was added to the mobile phase buffer as an ion pair reagent.

The -SH concentration in the fraction containing peak a and b from the RP-HPLC column was similar in copper-treated samples and in the control samples, i.e. 14.2 μm per mg total protein vs. 19.9μm per mg total protein, respectively.

The CAS assay for siderophores was negative in every fraction collected from the FPLC and in the crude extract. The sensitivity of the assay (less than 7.5 nmol of chelator with high affinity for iron) is sufficient to detect siderophores had they been present in these cellular extracts; thus, siderophores were not induced in the cultures.

Electrospray mass spectrometry indicated that the compound(s) in the fraction eluting at 6-7 min from RP-HPLC was not a peptide. A charging pattern characteristic of peptide mass spectra was not observed and no peptide ion was seen in the matrix assisted laser desorption time-of-flight mass spectrum (Michael Kinter, Ph.D., Director, W.M. Keck Biomedical Mass spectrometry Laboratory, personal communication). Amino acid analysis of this compound(s) demonstrated that lysine was present along with a large peak of unknown identity which absorbed at 254 nm. No cysteine was detected.

DISCUSSION

Freshwater *Synechococcus* sp. can produce MT in response to stress from elevated concentrations of copper, cadmium or zinc (Robinson *et al.*, 1990). The gene coding for MT was reportedly amplified when cells were repeatedly exposed to high concentrations of cadmium (Gupta *et al.*, 1992). To our knowledge, no marine strain of *Synechococcus* has been reported to produce MT in response to copper stress, but some strains produce it in response to cadmium and zinc (Olafson *et al.*, 1980). The role of Cu-MT in freshwater *Synechococcus* sp. is unclear because mutant strains lacking MT retain copper resistance but become sensitive to cadmium and zinc (Turner *et al.*, 1993).

Being the same genus as the freshwater strain that produces MT, marine *Synechococcus* PCC 73109 was hypothesized to also produce MT in response to copper stress. Since the unique structure of Cu-MT has been reported to result in a labile molecule in eukaryotes (Bremner *et al.*, 1978; Bremner *et al.*, 1986; Sato & Bremner, 1984), and since a previous study which tested for MT in a marine strain used a long incubation (Olafson *et al.*, 1980), we assayed cultures at a series of time intervals. Negative results from the Southern blot hybridization technique using a *smt* probe from a freshwater strain indicate that Cu-MT in the marine strain differs from that in the freshwater strain or that Cu-MT is absent in the marine strain. Absence of cysteine in ligands isolated from cultures exposed to copper indicates that MT is not produced by PCC 73109 in response to copper stress.

The ligand detected within thirty minutes of exposure to $CuSO_4$ in log phase *Synechococcus* PCC 73109 was neither phytochelatin, siderophore nor peptide, but contained lysine and an unidentified component that absorbed at 254 nm and fluoresced at 450 nm. These spectral characteristics suggest a compound with cyclic or aromatic structure. Toxic concentrations of copper induced increased production of this ligand

above the basal level. The observed copper-complexing capacity of the component, as indicated by its coelution with copper in FPLC, suggests a possible role in copper detoxification. Presence of the ligand in control cultures without an addition of $CuSO_4$ indicates a basal production at the low copper concentration present in the culture medium (0.32 μM is added to BG-11 medium). The production of the ligand rapidly increased when the cultures were exposed to high concentrations of copper, suggesting that the copper-complexing compound may be a metabolic defense at elevated copper concentrations.

Further study of the structure of the ligand isolated in this study is clearly needed. However structural characterization will require significantly more material than was obtained in the course of the present study. The influence of an ion-pairing agent on retention by RP-HPLC suggests the ligand is ionizable. Our data shows that the copper-complexing component is of low molecular weight (< 6,500), contains lysine, is probably a cyclic or aromatic component with ionizable functional groups. Lysine contains α-carboxylate, α-amino, and ε-amino group that can chelate free Cu^{2+} (Martin, 1979). This copper-complexing compound apparently functions to alleviate copper toxicity but is unlike copper binding ligands previously reported in *Synechococcus* spp.

ACKNOWLEDGEMENTS

This work was partially supported by ONR contract N00014-99-0093 to ASG. The U.Va. Biomedical Research Facility is supported by the U.Va. Pratt Committee and the Biomedical Mass Spectrometry Laboratory is funded by the W.M. Keck Foundation. We thank Dr. Nigel Robinson for providing the *Synechococcus* smt gene probe and Dr. Patricia Pleban, Department of Chemistry and Biochemistry, Old Dominion University, for assistance with atomic absorption spectrometry.

LITERATURE CITED

Ahner, B.A, S. Kong, & Morel, F.M.M. 1995. Phytochelatin production in marine algae: I. An interspecies comparison. Limnol. Oceanogr. 40: 649-57.

Ahner, B.A., Morel, F.M.M. & Moffett, J.W. 1997. Trace metal control of phytochelatin production in coastal waters. Limnol. Oceanogr. 42: 601-8.

Brand, L.E., Sunda, W.G. & Guillard, R.R.L. 1986. Reduction of marine phytoplankton reproduction rates by copper and cadmium. J. Exp. Mar. Biol. Ecol. 96:225-50.

Bremner, I., Mehra, R.K., Morrison, J.N. & Wood, A.M. 1986. Effects of dietary copper supplementation of rats on the occurence of metallothionein-I in liver and its secretion into blood, bile and urine. Biochem. J. 235:735-9.

Bremner, I., Hoekstra, W.G., Davies, N.T. & Young, B.W. 1978. Effect of zinc status of rats on the synthesis and degradation of copper-induced metallothioneins. Biochem. J. 174:883-92.

Bruland, K.W., Donat, J.R. & Hutchins, D.A. 1991. Interaction influences of bioactive trace metals on biological production in oceanic waters. Limnol. Oceanogr. 36:1555-77.

Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal. Biochem. 201:134-9.

Ellman, G.L. 1959. Tissues sulfhydryl groups. Arch. Biochem. Biophys. 82:70-7.

Emerson, S., Cranston, R.E. & Liss, P.S. 1979. Redox species in a reducing fjord: equilibrium and kinetic considerations. Deep-Sea Res. 26A:859.

Gledhill, M., M. Nimmo, SJ Hill & MT Brown. 1997. The toxicity of copper(II) to marine algae, with particular reference to macroalgae. J. Phycol. 33: 2-11.

Gordon, A.S., J.R. Donat, R.A. Kango, B.J. Dyer, &L.M. Stuart. 2000. Dissolved copper-complexing ligands in cultures of marine bacteria and estuarine water. Mar. Chem. 70: 149-160.

Grille, E., Winnacker, E.L. & Zenk, M.H. 1985. Phytochelatins: The principal heavy-metal complexing peptides of higher plants. Science 230: 674-6.

Gupta, A., Whitton, B.A., Morby, A.P., Huckle, J.W. & Robinson, N.J. 1992. Amplification and rearrangement of a prokaryotic metallothionein locus *smt* in *Synechococcus* PCC 6301 selected for tolerance to cadmium. Proc. R. Soc. Lond. B. 248:273-81.

Harwood-Sears V.H. & Gordon, A.S. 1990. Copper-induced production of copper-binding supernatant proteins by the marine bacterium *Vibrio alginolyticus.* Appl. Environ. Microbiol. 56: 1327-32.

Harwood, V.J. & Gordon, A.S. 1994. Regulation of extracellular copper-binding proteins in copper resistant and copper sensitive mutants of *Vibrio alginolyticus*. Appl. Environ. Microbiol. 60: 1749-53.

Klauser, S., Kagi, J.H.R. & Wilson, K.J. 1983. Characterization of isoprotein patterns in tissue extracts and isolated samples of metallothioneins by reverse-phase high-pressure liquid chromatograohy. Biochem. J. 209:71-80.

Martin, R.B. 1979. Complexes of a-amino acids with chelatable side chain donor atoms. *In* Sigel, H. (Ed.) *Metal ions in biological systems*. Marcel Dekker, Inc. New York Vol. 9. pp. 1-39.

McKnight, D.M. & Morel, F.M.M. 1979. Release of weak and strong copper-complexing agents by algae. Limnol. Oceanogr. 24:823-37.

McKnight, D.M. & Morel, F.M.M. 1980. Copper complexation by sideophores from filamentous blue-green algae. Limnol. Oceanogr. 25:62-71.

Moffett, J.W. & Brand, L.E. 1996. Production of strong, extracellular Cu chelators by marine cyanobacteria in response to Cu stress. Limnol. Oceanogr. 41:388-95.

Moffett, J.W. & Zika, R.G. 1983. Oxidation kinetics of Cu(I) in seawater: Implications for its existence in the marine environment. Mar. Chem. 13:239-51.

Olafson, R.W., Loya, S. & Sim, R.G. 1980. Physiological parameters of prokaryotic metallothionein induction. Biochem. Biophy. Res. Commun. 95:1495-503.

Porter, R.D. 1988. DNA transformation. Method. Enzymol. 167:703-12.

Rippka, R. 1988. Isolation and purification of cyanobacteria. Method. Enzymol. 167:3-27.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. & Stanier, R.Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111:1-61.

Robinson, N.J., Gupta, A., Fordham-Skelton, A.P., Croy, R.R.D., Whitton, B.A. & Huckle, J.W. 1990. Prokaryotic metallothionein gene characterization and expression: chromosome crawling by ligation-mediated PCR. Proc. R. Soc. Lond. B. 242:241-7.

Sato, M. & Bremner, I. 1984. Biliary excretion of metallothionein and a possible degradation product in rats injected with copper and zinc. Biochem. J. 223:475-9.

Schwyn,B. & Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160:47-56.

Tebo, B.M. 1995. Metal precipitation by marine bacteria: Potential for biotechnological applications. Genetic Engineering 17: 231-63.

Turner, J.S., Morby, A.P., Whitton, B.A., Gupta, A. & Robinson, N.J. 1993. Construction of Zn^{2+}/Cd^{2+} hypersensitive cyanobacterial mutants lacking a functional metallothionein locus. J. Biol. Chem. 268:4494-8.

Walsh, R.S., Cutter, G.A., Dunstan, W.M., Radford-Knoery, J. & Elder, J.T. 1994. The biogeochemistry of hydrogen sulfide: phytoplankton production in the surface ocean. Limnol. Oceanogr. 39:941-8.