EFFECTS OF DIVALENT METAL CATIONS AND RESISTANCE MECHANISMS OF THE CYANOBACTERIUM *SYNECHOCOCCUS* SP. STRAIN PCC 7942

G.R. Ybarra and R. Webb

Department of Biological Sciences, The University of Texas at El Paso, El Paso, TX 79968; Phone: (915)747-6889; Fax: (915)747-5808.

ABSTRACT

Cyanobacteria exhibit an extraordinary resistance to many environmental factors including nutrient limitation, changes in hydrogen ion concentration, temperature, and light extremes. A better understanding of the biological effects and response mechanisms of cyanobacteria to heavy metal exposure could be used to develop these bacteria for use in bioremediation. *Synechococcus* sp. strain PCC 7942 expresses messenger RNA for the stress protein GroEL and for the metal-binding protein metallothionein in response to a wide range of divalent metal ion concentrations. Although *groEL* is expressed at low levels regardless of environmental conditions, a high rate of transcription is initiated within 15 minutes following exposure to divalent metal cations at concentrations ranging from 10 μM to 100 μM for copper and zinc, and concentrations as low as 1 μM for cadmium. Transcript levels return to normal within one hour following exposure to each metal. Induction of the metallothionein operon also occurs within 15 minutes of these exposures. We speculate that these resistance mechanisms are working together to protect the cell from damage.

Key words: *metallothionein, GroEL, cyanobacteria, resistance mechanisms*

INTRODUCTION

The cyanobacterium *Synechococcus* sp. PCC 7942 is a single-celled photosynthetic prokaryote that is subject to a variety of environmental stressors in nature (Webb et al., 1994). This bacterium responds accordingly to variations in temperature, light intensities, and heavy metal exposure by the induction of the stress protein GroEL and the metal-binding protein metallothionein. These proteins act together to diminish or eliminate cellular damage.

All organisms must possess mechanisms that regulate metal ion accumulation and thus, avoid heavy metal toxicity. Several resistance mechanisms exist to lessen or prevent metal toxicity. These include resistance to metals that are always toxic to the cell and serve no beneficial role, such as cadmium and mercury, and also include resistance to metals such as copper, iron, and zinc, which are toxic at high concentrations but are absolutely essential in trace amounts (Silver and Wauderhaug, 1992). A first-resistance mechanism involves extracellular binding whereby cells synthesize and release organic materials that chelate metals to reduce their bioavailability (Clarke et al., 1987), or the metal ions may be bound to the outer cell surface. These complex forms are generally more difficult to transport into the cell. Secondly, cells can increase the rate of metal ion excretion using energy-driven efflux pumps (Siegel, 1997). A third method of resistance is through internal metal sequestration. This is one of the most important mechanisms by which bacteria combat heavy metal exposure and subsequent accumulation. In the prokaryotic cyanobacteria, metal ion sequestration within the cell is performed by the class II metallothioneins.

Class II metallothioneins are sulfhydryl-containing, cysteine-rich, metal-binding proteins that sequester metal, thus preventing accumulation of potentially toxic forms of metal ions within the cell (Zhou et al., 1994). Metal ion binding occurs through the interactions of the ions with the sulfhydryl groups of cysteine residues (Erbe et al., 1995). The s*mt* locus of *Synechococcus* PCC 7942 contains a metal-regulated gene, s*mtA* (Morby et al., 1993). This operon encodes a class II metallothionein and a divergently transcribed repressor of *smtA* transcription, *smtB* (Morby et al., 1993). SmtB is a trans-acting repressor of expression from the *smtA* operator-promoter region. Metallothionein protein expression is dependent upon the interaction between these metal ions and the repressor protein which regulates the expression of metallothionein mRNA (Morby et al., 1993). Loss of the repressor gene, *smtB*, and subsequent unregulated transcription of *smtA*, has been shown to be advantageous to organisms constantly stressed with changing levels of cadmium, copper, lead, nickel, zinc, or arsenate (Gupta et al., 1993). These mutant strains, devoid of the functional repressor, show elevated levels of *smtA* messenger RNA even in the absence of a metal inducer.

Another important resistance mechanism used by cells in response to a variety of environmental stressors is the expression of heat shock genes. These proteins are present in highly conserved forms in bacteria, plants, and animals. One of the most important of the heat shock proteins is GroEL. GroEL is a 58-kDa protein that assembles into two stacked rings of seven subunits each with an additional ring of seven 10-kDa GroES subunits. This complex has been shown to renature proteins, making them again functional (Weissman et al., 1996). Since their major role is in assisting protein folding with the consumption of ATP, GroEL and GroES are termed chaperonins. Chaperonins provide kinetic assistance to the process of folding of newly translated proteins or proteins disrupted as a result of cellular stress (Xu et al., 1997). In the bacteria, the genes for GroES and GroEL proteins are arranged into an operon (*groESL*) and transcription is coordinately expressed by the use of specific stress sigma factors. GroEL has been shown to be an essential component for maintaining viability with changes in temperature (Webb et al., 1990). GroEL and GroES are essential proteins for cellular growth and are always transcribed at baseline levels; only under conditions of stress does the transcription rate increase.

The purpose of our investigation was to better understand the response of the cyanobacterium *Synechococcus* sp. strain PCC 7942 to divalent metal ion exposure. The first aim was to compare the transcription of genes for GroEL and metallothionein proteins in response to these stressors. We propose that these resistance mechanisms act together during metal ion exposure, and thus are expressed in a similar fashion. We also examined the effects of heavy metal exposure on the photosynthetic rate of cyanobacteria by measuring oxygen evolution. This was used as an indicator of cellular stress, and our aim was to correlate this data with the induction of *groEL* and metallothionein gene expression.

MATERIALS AND METHODS

Cyanobacterial strain and growth conditions

Synechococcus species strain PCC 7942 (wild type) was used in all experiments. Cyanobacterial cultures were grown with continuous aeration in liquid BG-11 medium (Allen, 1968) at room temperature, under a light intensity of approximately 100 $\mu E/m^2/s$. Culture density for all studies was approximately 0.8 A680.

Metal ions

Cells were subjected to the divalent cations by adding a single dose of Cu^{2+} (as copper sulfate), Cd^{2+} (as cadmium chloride), and Zn^{2+} (as zinc sulfate) to give final concentrations each of 1 mM, 100 μM, 10 μM, and 1 μM.

Oxygen evolution measurements

Oxygen evolution measurements of cyanobacterial cultures were performed at a light intensity of 200 $\mu E/m^2/s$ using a Clark-type oxygen electrode following the directions of the manufacturer, YSI Instruments Co. Inc., Yellow Springs, OH.

Oligonucleotides

Polymerase chain reaction on isolated *Synechococcus* sp. strain PCC 7942 DNA was used to generate DNA probes for both *groEL* and *smtA*. The primers used in generating the PCR products for *groEL* were 5′ ATG GCT AAA CGG ATC ATT TAC A 3′ for the forward primer and 5′ GTA GTC GAA GTC GCC CAT GCC A 3′ for the reverse primer. A second set of primers was used to generate the PCR product for *smtA*. The sequences of these primers were 5′ GGC GTC GAC CTG AAT CAA GAT TCA GAT GTT AGG 3′ for the forward primer and 5′ GGC GTC GAC ATG TTA GGC TTA AAC ACA T 3′ for the reverse primer.

RNA isolation, electrophoresis, northern blotting, and detection

Total cyanobacterial RNA was isolated after 0 (control-no metal added), 15, 30, 60, and 180 minutes of exposure to each concentration of each stressor independently. The RNA was isolated and subjected to agarose gel electrophoresis using the procedures described in Reddy, Webb, and Sherman (1990). Twenty micrograms of total RNA were loaded to each lane.

Northern blotting, probe labeling, and detection were performed as described in the instructions for the Phototope Star Labeling and Detection kits manufactured by New England Biolabs, Beverly, Mass. Experiments were performed on stripped, reprobed membranes, and repeated in triplicate to ensure consistency. The membranes were prehybridized and hybridized with *groEL* probe, and then stripped and reprobed with *smtA* and vice versa.

RESULTS AND DISCUSSION

This study focused on the cellular effects of exposure to divalent metal cations and the roles of

the specific resistance mechanism of metallothionein expression, and the expression of the stress protein GroEL. GroEL has been shown to be an integral component that combats heavy metal toxicity (Weissman et al., 1996). Metallothioneins are cysteine-rich proteins that bind metal ions and thus detoxify these metals by limiting their cellular availability (Zhou, 1994).

Metal ions may enter cells via transport proteins that bind metal and transport the complexed form into the cell. These metals can then bind sulfhydryl groups on the regulatory repressor protein *smtB*. This bound metal then alters the conformation of the repressor protein and releases it from the DNA. This allows RNA polymerase to begin transcription from *smtA*, resulting in metallothionein expression.

The divalent metal cations of copper, zinc, and cadmium each elicit a stress response at concentrations of ^{3}1 μM, as evidenced by the transcriptional rate of GroEL and metallothionein (Table 1). Cadmium ions induce metallothionein and GroEL expression within 15 minutes at concentrations of 100 μM (Figures 1 and 2) and at concentrations as low as 1 μM (Table 1). Cadmium is a highly toxic metal that quickly assimilates into photosynthetic structures and is a potent uncoupler of oxidative phosphorylation and a potent inhibitor of electron transfer in the electron transport system of photosynthesis (Miccadei, 1993). Our data also indicate that zinc ions at concentrations of ^{3} 10μM induce *groEL* gene expression (Figure 3) as well as metallothionein transcription (Figure 4) within 15 minutes of exposure. These findings suggest that GroEL is not strictly a heat shock protein (hsp 60), and that its transcription responds to potentially toxic metal ions.

The transcription kinetics for *groEL* and *smtA* differ from each other and vary with type of metal ion. Zinc ions induce *groEL* maximally by 30 minutes, and the levels of these transcripts return to near baseline levels within one hour. Zinc ions induce metallothionein transcripts more quickly (15 minutes), and the levels of these transcripts remain high beyond one hour. Cadmium ions rapidly (15 minutes) induce high levels of transcription of both *groEL* and *smtA* genes. While *groEL* transcription has returned to basal levels by 30 minutes, metallothionein gene transcription remains high beyond two hours. These results suggest that *groEL* responds to immediate, acute metal ion stress, while metallothioneins are important at all times during continued metal ion exposure.

Another aspect of this work focused on illustrating a possible relationship between heavy metal exposure and photosynthetic rate as an indicator of cellular stress. We subjected cyanobacterial cells to 100 μM, 10 μM, and 1 μM concentrations of copper, zinc, and cadmium. Upon exposure to a metal cation, these cells were subjected to darkness for five minutes, followed by a period of light exposure (200 $\mu E/m^2/s$ over10 minutes) during which oxygen evolution was measured. The values for each metal treatment were calculated and compared to a control with no metal ion exposure (100%). Table 2 illustrates the percentage of decrease compared to control cells. Cop-

per and zinc ions each produce a slight decrease in oxygen evolution at the concentrations tested. The greatest effect on oxygen evolution is produced by cadmium ions. Concentrations as low as 1 μM decrease oxygen evolution by approximately 50% (Table 2). These results are consistent with our RNA transcription measurements. Cadmium has a far greater effect than other metals, even at lower concentrations.

Cyanobacteria are highly adaptable organisms that can respond to changing environmental conditions such as temperature, light, and metal ion exposure. They increase the transcription of *groEL* as a resistance mechanism in response to the many types of cellular alterations resulting from environmental contamination. This resistance mechanism works to prevent protein aggregation that results from protein denaturation (Llorca et al., 1996) and also works to promote proper protein folding. In addition, these cells respond to a variety of metal ions by producing metal-binding proteins called metallothioneins. Our findings suggest that *groEL* and *smtA* gene expression and the rate of oxygen evolution in response to divalent metal ions can be correlated. This is especially true for cadmium ions whose effects are not only to cause protein denaturation but also to affect electron transport during photosynthesis. It appears that the sequestration of these metals by metallothioneins detoxifies them, thus decreasing their detrimental cellular effects. Through genetic engineering, it may be possible to create strains of cyanobacteria that could prove to be valuable in bioremediation.

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Table 1. Summary of the induction of *groEL* and *smtA* with respect to metal ion type and concentration.

	1mM	100μM	10μM	1μM
Cu^{2+}	+	+	-	-
Zn^{2+}	+	+	+	-
Cd^{2+}	+	+	+	+

Table 2. Effects of metal ion type and concentration on rate of photosynthetic oxygen evolution. All experiments conducted in duplicate.

	Control No Metal Ion Exposure	100μM	10μM	1μM
Cu^{2+}	100%	83%	78%	75%
Zn^{2+}	100%	84%	80%	80%
Cd^{2+}	100%	54%	43%	25%

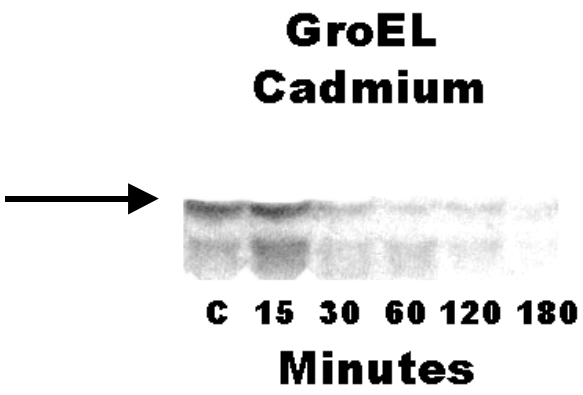

Figure 1. Northern blot analysis for *groEL* mRNA expression following exposure to 100µM cadmium.

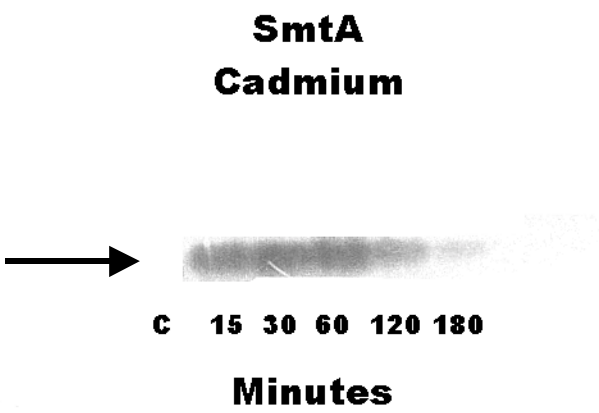

Figure 2. Northern blot analysis of *smtA* expression following exposure to 100µM cadmium. Nylon membrane from Figure 1 was stripped and reprobed with *smtA*.

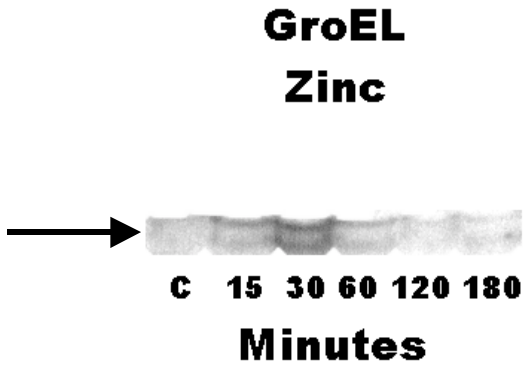

Figure 3. Northern blot analysis for *groEL* mRNA expression following exposure to 100µM zinc sulfate.

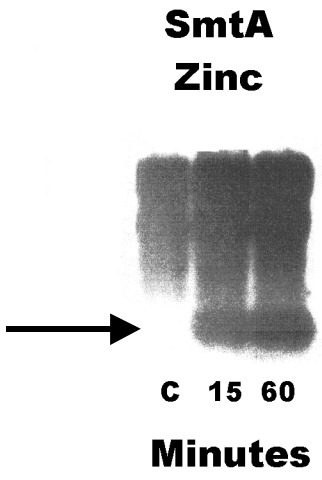

Figure 4. Northern blot analysis for *StmA* following exposure to 10 µM zinc sulfate.