DIFFERENTIAL RESPONSES OF GROEL AND METALLOTHIONEIN GENES TO DIVALENT METAL CATIONS AND THE OXYANIONS OF ARSENIC IN THE CYANOBACTERIUM *SYNECHOCOCCUS* SP. STRAIN PCC7942

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ABSTRACT

A better understanding of the resistance mechanisms used by the cyanobacterium *Synechococcus* sp. PCC 7942 could guide attempts to develop this organism for use in bioremediation. This bacterium exhibits extraordinary resistance to arsenate. Possible mechanisms include exclusion from the cell, intra- and extra-cellular binding, and potential interactions with proteins such as metallothioneins, which bind and detoxify divalent metal cations such as Cd2+, Cu2+, and Zn2+. *Synechococcus* sp. PCC 7942 expresses messenger RNA for metallothioneins in response to these divalent metal ions at various concentrations. Interestingly, a similar pattern of expression is induced in response to the oxyanions of arsenic, arsenate, and arsenite. The response to arsenate, however, occurs only when cells are cultured in phosphate-limited media. This suggests that under phosphate-sufficient conditions, arsenate is specifically excluded from the cell. Our hypothesis is that arsenate, upon entering phosphate-depleted cells, interacts with the sulfhydryl groups of the cysteine residues of the regulatory protein responsible for expression of the metallothionein gene. A thiol-alkylating reagent induced metallothionein synthesis, presumably by modifying the sulfhydryl residues of the regulatory protein, consistent with our proposed mechanism for arsenate induction of metallothionein expression. We further describe the effects of divalent metal cations and the oxyanions of arsenic on induction of the general stress protein GroEL. We have found that transcription of this gene begins within 15 minutes of exposure to the divalent metal cations Cd2+, Cu2+, and Zn2+ as well as to arsenate and arsenite. Thus, conditions that induce expression of the general stress protein GroEL and the metal-binding protein metallothionein are somewhat similar.

Key words: *metal binding, metallothioneins, GroEL, arsenate, cyanobacteria*

INTRODUCTION

The cyanobacterium *Synechococcus* sp. PCC 7942 is a single-celled photosynthetic prokaryote that is subject to a variety of physiological stresses in nature (Webb et al., 1994). This bacterium responds accordingly to variations in temperature, light intensity, nutrient deprivation, and heavy metal ion exposure.

The uptake of metal ions by cells depends upon the maintenance of a concentration gradient across bacterial cell membranes. Concentration gradients are established by the accumulation of ligands binding the metals on the cell surface, transfer across cell membranes by carrier ligands, and removal of metal ions inside the cell by other molecules. Cell surfaces are anionic due to the presence of ionized groups such as carboxylates, hydroxyl groups, and phosphate molecules embedded along the cell wall (Siegel, 1997). These molecules function as ligands for metal ion binding and transport into the cell. All organisms must possess mechanisms that regulate metal ion accumulation and therefore, avoid heavy metal toxicity while still procuring metals in trace amounts that are essential for normal cell growth. An efficient resistance system must exhibit specificity for a metal or a group of metals; otherwise, the resistance mechanism may deplete the cell of essential metals.

A number of mechanisms of metal resistance exist. This includes resistance to metals that are purely toxic to the organism and serve no biological function such as mercury and cadmium, and also extends to metals such as copper and zinc that are toxic at high concentrations but absolutely essential in trace amounts (Silver and Wauderhaug, 1992). The first mechanism involves extracellular binding. Cells may synthesize and release organic materials that chelate metals and reduce their bioavailability (Clarke et al., 1987) or the metal ions may be bound to the outer cell surface. These complexed forms are generally not readily transported into the cell because of structure and complexity. Secondly, cells can increase the excretion rate of certain metal ions using energy-driven efflux pumps (Siegel, 1997). Internal metal sequestration, a third resistance mechanism, is one of the most important mechanisms by which bacteria combat heavy metal exposure and subsequent accumulation. In the cyanobacteria, metal ion sequestration inside the cell is performed by the Class II metallothioneins.

Class II metallothioneins are thiol-containing, cysteine-rich, metal-binding proteins that sequester metal, thus preventing accumulation of potentially toxic-free metal ions within the cell (Zhou, 1994). Metal ion binding occurs through the interactions of the ions with the thiol groups of cysteine residues. The metallothionein genes are arranged as an operon called the smt locus, containing both smtA (metallothionein protein) and smtB (repressor, regulatory protein) genes. SmtB is a trans-acting repressor of expression from the smtA operator/promoter region. Metallothionein expression, from the gene to the functional protein, is induced by these metal ions and the regulation of transcription to messenger RNA is dependent upon the interaction between these metal ions and the repressor protein regulating transcription, again via interaction with thiol groups present on the repressor protein (Erbe et al., 1995). Loss of the smtB repressor gene and subsequent unregulated transcription of smtA may be advantageous to organisms constantly stressed with high levels of cadmium, copper, arsenic, and lead (Gupta et al., 1993) since cells devoid of functional repressor (smtB) show elevated concentrations of smtA messenger RNA transcripts even in the absence of an inducer.

Another important protective mechanism used by cells in response to a variety of stressors is the expression of heat shock genes. These proteins are present in highly conserved forms in all organisms studied including bacteria, plants, and animals. One of the most important of these heat shock proteins is GroEL. GroEL is a 58,000 amu protein which assembles into two stacked rings of seven subunits each with an additional ring of seven, 10,000 amu GroES subunits. Together, this complex has been shown to renature and make functional proteins that have been misfolded as a result of cellular stress (Weissman et al., 1996). In the bacteria, the genes for GroES and GroEL are arranged into an operon (groESL) and their transcription is coordinately expressed by the use of stress specific transcription factors (sigma factors). GroES and GroEL are essential proteins for cellular growth and are constantly expressed at low levels. However, the level of groESL transcrip-

tion increases dramatically with environmental changes such as temperature, light intensity, pH variations, and heavy metal exposure.

Cyanobacterial cells exhibit an extraordinarily high level of resistance to arsenate. Liquid cultures of these cells are capable of normal rates of growth in the presence of 1M arsenate. We believe that this high level of resistance is due primarily to the failure of arsenate to enter phosphate-sufficient cells. Arsenate may exert its toxic effects on cells by mimicking phosphate and by derivitizing essential cellular proteins. The phosphate-specific transporter (Pst) system is a slow, highly specific, inducible uptake mechanism in *E. coli* that discriminates between phosphate and arsenate under phosphate-sufficient conditions (Silver, 1995). The phosphate inorganic transporter (Pit) system is the major secondary phosphate transporter under phosphate-deficient conditions and is differentiated from the Pst system on the basis of its specificity for inorganic phosphate and the toxic anions of arsenic (Van Veen et al., 1994). We believe the phosphate-specific transport (Pst) system discriminates between phosphate and arsenate. This inducible system is the primary uptake mechanism used when phosphate is available in quantities sufficient for cyanobacterial growth. Under phosphate starvation, the phosphate inorganic transport (Pit) system becomes the major uptake mechanism for inorganic phosphate. However, this system appears not to differentiate between phosphate and arsenate. We believe that under conditions of phosphate limitation, arsenate is permitted to enter cyanobacterial cells resulting in cell toxicity.

The purpose of our investigation was to understand better the responses of the cyanobacterium *Synechococcus* sp. PCC7942 to divalent metal cations and the oxyanions of arsenic. The first aim was to compare the transcription of genes for metallothionein and GroEL in response to these stressors. We propose that these responses should be similar. Secondly, we aimed to explore the relationship between phosphate status and arsenate toxicity. Our hypothesis is that arsenate is excluded from phosphate-sufficient cells and that phosphate-limited cells allow the entry of arsenate where it is then able to exert its toxic effects. Lastly, we aimed to understand better the seemingly paradoxical finding that arsenate, a trivalent anion, could strongly induce metallothionein transcription, since this system responds more commonly to divalent metal cations. Our hypothesis is that arsenate covalently modifies sulfhydryl groups of the SmtB regulatory repressor protein, thus inducing metallothionein transcription. This was tested using the thiol modifying reagent N-Tosyl-L-Lysine Chloromethyl Ketone (TLCK) (Nobile, 1988). We expected this reagent to induce smtA expression in a manner analogous to arsenate induction, thus providing a working model for metallothionein induction by the oxyanions of arsenic.

PROCEDURES

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 (Allen, 1968) medium at room temperature, under a light intensity of approximately 100 microeinsteins per m^2 per second.

Phosphate-limited cyanobacterial cultures were prepared by growing cells in media containing 23 micromolar phosphate (as dibasic potassium phosphate) which is one-tenth of the concentration used for growing phosphate-sufficient cells.

Metal Ion, Arsenic, and Organic Stressors

Cells were subjected to the divalent cations Cu^{2+} (as copper sulfate), Cd^{2+} (as cadmium chloride), and Zn^{2+} (as zinc sulfate) at concentrations each of 1mM, 100 micromolar, 10 micromolar, and 1 micromolar. The oxyanions of arsenic, arsenate, and arsenite were also used to stress cells using the same concentrations listed above for the cations. The thiol alkylating reagent, N-Tosyl-L-Lysine Chloromethyl Ketone (TLCK), dissolved in ethanol, was added to cyanobacterial cultures to a final concentration of 1.6 mg per liter.

Oxygen Evolution Measurements

Oxygen evolution measurements of cyanobacterial cultures were performed at a light intensity of 100 microeinsteins per m^2 per second using a Clark-type oxygen electrode following the directions of the manufacturer, YSI Instruments Co. Inc., Yellow Springs, OH.

RNA Isolation, Electrophoresis, Northern Blotting, and Detection

Total cyanobacterial RNA was isolated after 0 (control), 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).

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, MA.

RESULTS AND DISCUSSION

This study focused on the effects of metal ion exposure and the oxyanions of arsenic on GroEL and metallothionein gene expression. The general stress protein GroEL is an important aspect of cellular responses to changing environmental conditions (Weissman et al., 1996). Metallothioneins are cysteine-rich proteins that bind metal ions and thus detoxify them by limiting their cellular availability (Zhou, 1994). Additional aims were to further explore the relationship between cellular responses to arsenic oxyanions and phosphate status and to propose a mechanism for the induction of metallothionein transcription, normally controlled by divalent metal cations, by these oxyanions.

The divalent cations of cadmium and zinc each elicit a stress response at concentrations of 10 micromolar, as evidenced by transcription of groEL and metallothionein genes (data not shown). Cadmium ions elicit these responses when present at concentrations as low as 1 micromolar. These findings suggest that groEL is not strictly a heat shock protein and that its transcription responds to potentially toxic metal ions. 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 in photosynthesis (Miccadei, 1993). Figure 1 shows the effects of cadmium ions on photosynthetic electron transport, measured here as oxygen evolution. Cellular exposure to concentrations of this ion as low as 1 micromolar have immediate effects on electron transport, decreasing oxygen evolution by nearly one-half. Similar effects are seen with copper and zinc ions only when their concentrations are 100-fold higher (100 micromolar) (data not shown).

We have found the divalent metal cations of copper, zinc, and cadmium elicit groEL and metallothionein transcription at different concentrations. Copper ions at a concentration of 10 micromolar do not induce transcription of groEL or metallothionein (data not shown). Concentrations of this ion in excess of 100 micromolar were required to induce transcription of these genes within 15 minutes (Figure 2 and Figure 3). Again, much lower concentrations of cadmium ions (1 micromolar) were sufficient to elicit a similar transcriptional response.

Figure 4 describes the generally accepted description of metallothionein induction by divalent cations. These ions enter the cell via transport proteins and bind sulfhydryl groups on the regulatory repressor protein. This protein then changes conformation and releases from the DNA. This allows RNA polymerase to begin transcription from smtA, resulting ultimately in metallothionein protein expression.

The second aspect of this work focused on gaining a better understanding of the extraordinarily high level of resistance to arsenate exhibited by all species of cyanobacteria examined to date. We believe that this resistance results from the failure of arsenate to enter phosphate-sufficient cells. The phosphate-specific transporter (Pst) system is a highly specific channel that selectively discriminates between arsenate and phosphate (Silver, 1995) and this is the major transporter used by normally grown cells. The phosphate inorganic transporter (Pit) system is the secondary phosphate transporter which becomes a major component of phosphate-limited cells. The Pit system is differentiated from the Pst system on the basis of its specificity for inorganic phosphate and the oxyanions of arsenic, arsenate, and arsenite (Van Veen et al., 1994). We do not observe any induction of either groEL (Figure 5) or metallothionein (data not shown) transcription in response to arsenate at concentrations in excess of 100 micromolar when cells are grown in phosphate-sufficient media. Similarly, these high concentrations of arsenate have no effect on photosynthetic oxygen evolution. When cells are grown under phosphate-limited conditions, we observe immediate effects

by arsenate ion on oxygen evolution at 10 and 100 micromolar concentrations (Figure 6). Further evidence supporting the entry of arsenate into phosphate-limited cells is shown in Figure 7 which shows metallothionein transcription by these cells in response to arsenate challenge. Interestingly, control cells (phosphate-limited, no arsenate challenge) express metallothionein transcripts. Upon arsenate challenge there is an initial decrease in the accumulation of this transcript; however, this transcript begins to accumulate again by 60 minutes.

We are designing experiments to test the notion that phosphate stress-specific transcription factors (sigma factors) may play a role in this observed response. This system may initially respond to arsenate as though it were structurally similar to phosphate by reducing metallothionein transcription. Later, as a consequence of either the toxic effects of arsenate or the continued lack of phosphate, metallothionein transcription is resumed.

The third aim of this work focused on developing a model to shed light on precisely how the trivalent anion arsenate induces metallothionein transcription. As described in Figure 4, smtA induction normally results from exposure to divalent metal cations such as copper, zinc, and cadmium interacting with sulfhydryl groups present in the repressor protein. We hypothesize that arsenate induces metallothionein transcription by covalently derivitizing these sulfhydryl residues. Support for this view comes from our experiments in which phosphate sufficient cells were exposed to N-Tosyl-L-Lysine Chloromethyl Ketone (TLCK). TLCK is an alkylating reagent that acts upon sulfhydryl groups on proteins (Nobile, 1988). Figure 8 shows that metallothionein transcription is rapidly induced to very high levels, within 15 minutes of exposure to 1.6 mg per liter TLCK.

Cyanobacteria are highly adaptable organisms. These organisms can respond to changing environmental conditions such as temperature, light, and metal ion exposure. They increase transcription of groEL in response to alterations in the environment to prevent protein aggregation and misfolding. In addition, these cells respond to a variety of metals ions and the oxyanions of arsenic by producing the metal-binding proteins called metallothioneins. It appears that this sequestration of these metals detoxifies them, thus decreasing their detrimental effects on the cell. Through genetic engineering, it will be possible to create cyanobacterial strains which express these proteins at very high levels, making such strains potentially valuable for attempts at bioremediation.

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REFERENCES

Allen, M.M., 1968. Simple conditions for growth of unicellular blue-green algae on plates. Journal of Phycology. 4:1-4.

Clarke, S.E., et al., 1987. Induction of siderophore activity in Anabaena species and its moderation of copper toxicity. Applied and Environmental Microbiology. 53(5):917-922.

Erbe, J.L., K.B. Taylor, and L.M. Hall, 1995. Metalloregulation of the cyanobacterial smt locus: Identification of the smtB-binding sites and direct interaction with metals. Nucleic Acid Research. 23(12):2472-2478.

Gupta, A., A.P. Morby, J.S. Turner, B.A. Whitton, and N.J. Robinson, 1993. Deletion within the metallothionein locus of Cd-tolerant Synechococcus PCC 6301 involving a highly iterated palindrome (HIP1). Molecular Microbiology. 7:189-195.

Miccadei, S., and A. Floridi, 1993. Sites of inhibition of mitochondrial electron transport by cadmium. Chemico-Biological Interactions. 89:159-167.

Nobile, S., and J. Deshusses, 1988. Evidence for a role of a vicinal dithiol in the transport of (-butyrobetaine in Agrobacterium sp. Biochimie. 70:1411-1416.

Reddy, K.J., R. Webb, and L.A. Sherman, 1990. Bacterial RNA isolation with one-hour centrifugation in a table-top centrifuge. Biotechniques. 8(3):250-251.

Silver, S., and M. Wauderhaug, 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiological Review. 56:195-264.

Silver, S., and M. Wauderhaug, 1995. Bacterial plasmid-mediated resistances to mercury, cadmium, and copper. Toxicology of Metals. Biochemical Aspects. 435-458.

Van Veen, H.W., et al., 1994. Phosphate Inorganic Transport (Pit) System in Escherichia coli and Acinetobacter johnsonii. Phosphate in Microorganisms: Cellular and Molecular Biology. Chapter 7:43-49.

Webb, R., and L.A. Sherman, 1994. The cyanobacterial heat-shock response and the molecular chaperones. The Molecular Biology of Cyanobacteria. Review Article. 751-767.

Weissman, J., et al., 1996. Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. Cell. 84:481-490.

Zhou, J., and P.B. Goldsborough, 1994. Functional homologs of fungal metallothionein genes in Arabidopsis. The Plant Cell. 6:875-884.

Figure 1. Response of cyanobacterial cells to cadmium. 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 the early stages of electron transfer (Miccadei and Floridi, 1993). Concentrations of ~1mM decreases oxygen evolution approximately 50%.

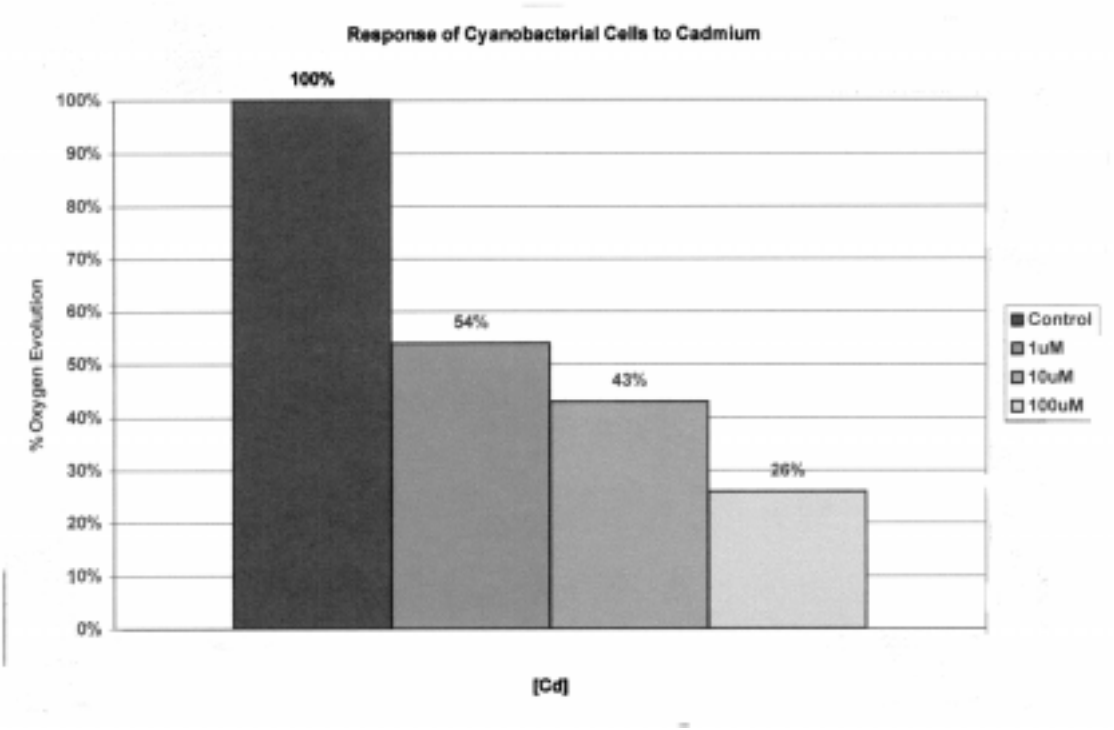

Figure 2. GroEL transcription is induced by Cu^{2+}. The transcriptional rate of the GroEL protein is constantly low. However, there is a definite increase in the transcriptional rate after 100µM $CuSO_4$ is introduced into the growth medium within 15 minutes. Levels decrease within 1 hour.

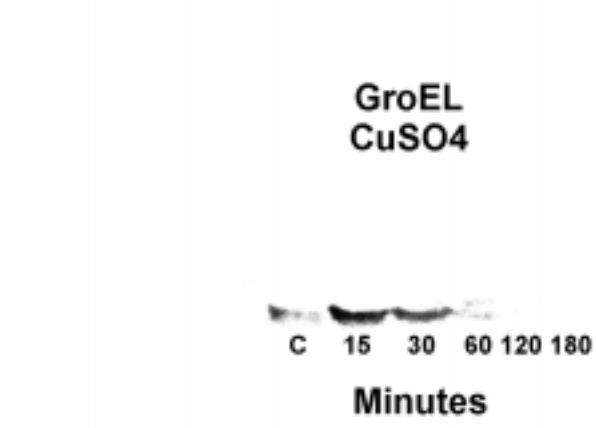

Figure 3. The transcriptional response of smtA with respect to divalent metal cations is similar to the stress protein operon GroEL. SmtA transcription is not detectable in the control, suggesting that smtA induction is metal-dependent. Metallothionein transcription is initiated within 15 minutes and levels of transcription remain constant up to 3 hours. Identical conditions as in Figure 2.

Figure 4. Proposed mechanism for metallothionein induction by metal cations and the oxyanions of arsenic. (1) Divalent metal cation challenge from the environment, (2) Sulfhydryl (-SH) modification by metal or arsenate binding, (3) SmtA expression producing metallothionein proteins, (4) Metal sequestration and detoxification by metallothionein proteins.

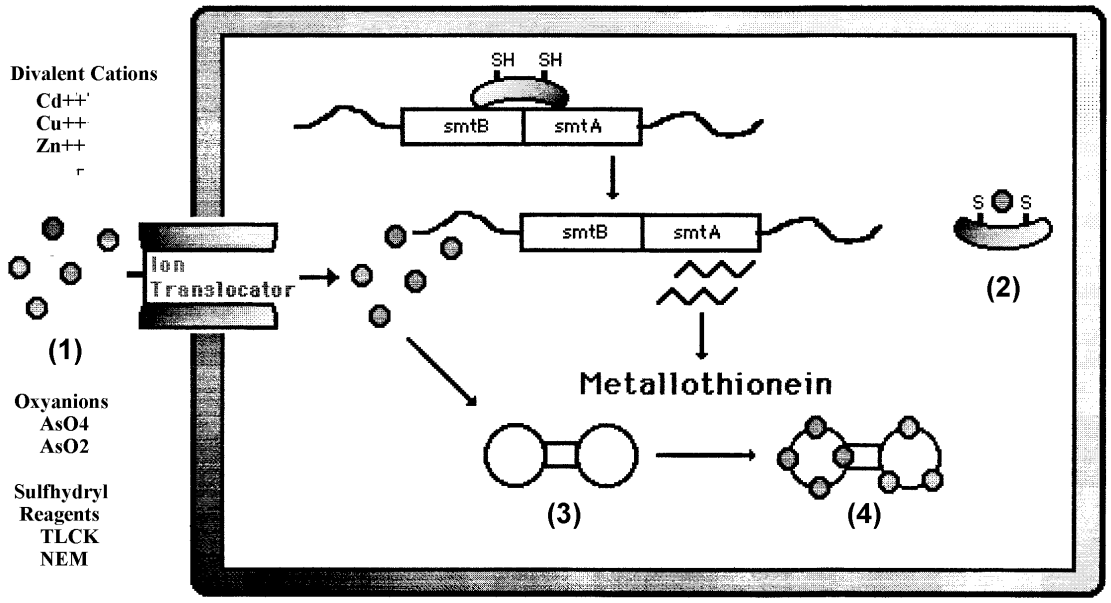

Figure 5. Cells competitively exclude arsenate when grown in phosphate-rich conditions. There is a higher affinity for phosphate; therefore, arsenate at concentrations of ≥100µM does not increase cellular stress (GroEL) or initiate the production of metal-binding proteins (data not shown).

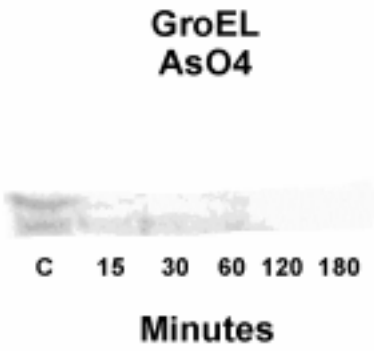

Figure 6. Response of phosphate-limited cells to arsenate. The extraordinarily high level of resistance to arsenate shown by cyanobacteria is due primarily to the failure of arsenate to enter phosphate-sufficient cells (data not shown). However, arsenate readily enters phosphate-depleted cells via the Pit translocator. Cells grown in minus phosphate media and then subjected to arsenate exposure (100µM) exhibit a decrease in oxygen evolution.

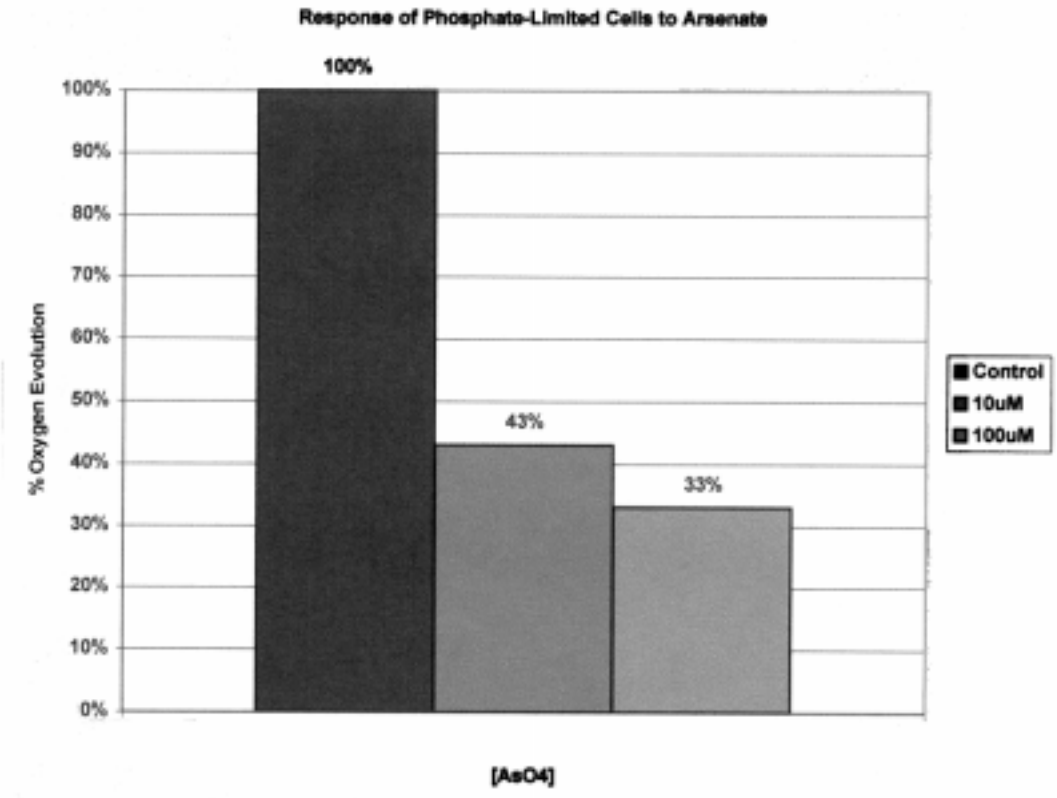

Figure 7. Metallothionein transcriptional response to arsenate in phosphate-limited cells. The control (no AsO4) exhibits some metallothionein synthesis that may be induced by a general stress sigma factor. The 15-minute time interval exhibits no metallothionein synthesis. Phosphate and arsenate are structurally similar and enter through the Pit pathway under phosphate starvation. The hypothesis is that the cell recognizes arsenate as phosphate and metallothionein production ceases. However, after a 15-minute exposure time, the cell recognizes it as arsenate and initiates metallothionein synthesis.

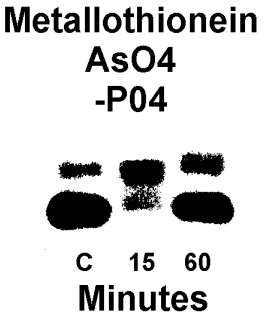

Figure 8. Modification of repressor sulfhydryl groups induces metallothionein transcription. N-Tosyl-L-Lysine Chloromethyl Ketone (TLCK) is an alkylating reagent that acts upon sulfhydryl groups on proteins. Initial concentration of TLCK: 1ml EtOH/5mg TLCK (1:6)-a final concentration of 1ml EtOH (5mg TLCK) per 3L of cyanobacterial cells grown in BG-11 liquid media. The control (no TLCK) did not induce metallothionein synthesis. Within 15 minutes following exposure to TLCK, metallothionein induction is evident. Metallothionein synthesis is continuous up to one hour.