GENETIC MANIPULATION OF A CYANOBACTERIUM FOR HEAVY METAL DETOXIFICATION

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INTRODUCTION

Cyanobacteria, sometimes referred to as blue-green algae, constitute the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes. There are unicellular and filamentous forms, fresh and salt water species, as well as numerous strains that have the capacity to undergo chromatic adaptation, to produce gas vacuoles, and/or to reduce atmospheric nitrogen by production of specialized cells known as heterocysts. Cyanobacterial existence is widespread, often abundant, and dependent upon only sunlight and carbon dioxide as basic energy and carbon sources, respectively. Their potential for use as a low cost means for aqueous decontamination is apparent.

Metallothioneins are a group of well-characterized metalloproteins that complex an array of metal ions, most notably copper, cadmium, and zinc. They display amazingly similar amino acid sequences in phylogenetically diverse organisms such as man, horse, crab, and fungi. However, the molecule has remained remarkably conserved in evolution. Low molecular weight, high metal content, and high cysteine content are characteristics of these polypeptides. The cysteines are coordinated to the metal ions through mercaptide bonds producing metal-thiolate clusters. Metallothionein proteins are subdivided into three classes. Classification into the first two of these is based upon the location of cysteine residues, either similarly related in position to equine renal metallothionein (Class I includes human, horse, and crab metallothionein) or only distantly related in position (Class II - sea urchin, nematode, yeast, and cyanobacteria). Class III metallothioneins are atypical, nontranslationally synthesized metal-thiolate polypeptides, encompassing plant metallothioneins (phytochelatins).

In *Saccharomyces cerevisiae*, copper resistance is mediated by the *cup* I gene which encodes the yeast copper metallothionein. The 53 amino acid polypeptide (the first 8 amino acids encoded in the gene are removed by post-translational modification of the mature protein) contains 12 cysteine residues capable of binding 8 copper ions. While inducible in the organism only by copper and silver ions, studies indicate *in vitro* ability of the protein to coordinate cadmium and zinc ions as well (Winge et al.

1985). A study by Fogel and Welch at the University of California at Berkeley showed that markedly enhanced copper resistance in yeast was a result of tandem gene amplification (Fogel and Welch 1982). Research has shown (Karin et al. 1984) that the copper chelatin gene alone (when present in sufficiently high copy number) seems to be sufficient for development of copper resistance in yeast. Given the structural similarity of the yeast and the cyanobacterial (Olafson et al. 1988) metalloprotein molecules (both are class II metallothioneins), the goal of this work will be to express the yeast copper metallothionein protein in a cyanobacterium. For this purpose, cloned sequences of the yeast *cup* I gene, both in single and multicopy constructs, have been obtained.

RESULTS AND DISCUSSION

Cell Strain. The cyanobacterium *Synechococcus* sp. strain PCC 7942, also very commonly known as *Anacystis nidulans* R2, was chosen for development and expression of the construct. It is unicellular, non-heterocystous, and naturally competent for DNA uptake. The ability to be easily transformed makes PCC 7942 (sometimes referred to as the *Escherichia coli* of experimental cyanobacteriology) an extremely attractive system for studies in genetic recombination.

Determination of Natural Metal Tolerance. Natural tolerance levels for various heavy metals was determined for the wild type PCC 7942. This initial assessment involved the metals cadmium, copper, and zinc. Tests were carried out in liquid culture and indicate the highest concentration for which appreciable growth could be detected by optical density determination (λ=550 nm) after seven days. The organism was most sensitive to copper and cadmium. Growth was inhibited at concentrations greater than 2 and 5µM, respectively, for these metals. Zinc was found to be somewhat less toxic, as growth was maintained at levels up to 25µM.

Plasmids and Cloning. Two plasmids have been used for cloning and transformation studies. The first is a cyanobacterial shuttle vector pUC303 (Kuhlemeier et al. 1983). This relatively large plasmid (10.9 kb) is a hybrid vector based on the small natural vector endogenous to this

species. It has been constructed with resistance markers for chloramphenicol and streptomycin and has a unique *Eco*RI restriction site within the chloramphenicol marker for insertion of foreign DNA.

The second plasmid, pAM1213, is a derivative of pBR322 and has been designed for integration by recombination into the PCC 7942 genome. (Plasmid pAM1213 was a gift from S. Golden.) Lacking a cyanobacterial origin of replication, it is unable to replicate in the cyanobacterial host. The plasmid contains a random segment of the *Synechococcus* chromosome which allows homologous recombination between the transforming plasmid DNA and the recipient cyanobacterial chromosome (Tsinoremas et al. 1994). A spectinomycin-resistance cassette to confer selection for transformants is located within and flanked by portions of the random segment. Also located within this area is the *E. coli conII* promoter, a unique *Sma*I site behind this promoter, and portions of the *lac* operon positioned to allow for IPTG induction.

The large *cup* I locus (1.95 kb) for the yeast copper metallothionein possesses two transcriptional units, the smaller of which contains the actual coding region (less than 200 bp) for the metal binding protein. While yeast may require multiple copies of this sequence for increased metal tolerance, the gene dosage effect has not been demonstrated in a cyanobacterium. A 309 bp fragment encompassing this metallothionein coding region was excised by digestion with *Rsa*I from the cloned sequence of the gene. (Cloned single and multiple copy sequences of the gene were a gift from Juliet Welch.) This fragment was inserted into the unique *Eco*RI site of the pUC303 vector, interrupting the chloramphenicol marker. Similarly, the fragment was inserted into the *Sma*I site of the pAM1213 vector. Molecular hybridization (using the 309 bp fragment as probe) confirmed the presence of inserts for both vectors.

Transformation. PCC 7942 was transformed (Golden and Sherman 1984) with the pUC303 construct, selected initially for streptomycin resistance, and subsequently examined against controls for increased metal tolerance. Since the coding sequence was cloned into the vector without concomitant insertion of a promoter, any expression of the metallothionein protein would probably be controlled by an endogenous promoter already present on the vector. With the foreign DNA inserted into the coding region for chloramphenicol acetyl transferase (CAT), expression via transcriptional read-through from the CAT promoter or creation of a fusion protein are possibilities. It is known that the CUP1 protein undergoes post-translational modification in yeast; however, absence of this process in the prokaryotic cyanobacterium would not be expected to greatly alter the metal-binding capability of this fairly simple protein (Berka et al. 1988; Sayers et al. 1993) Chelating activity depends primarily on metal coordination to the multiple cysteine residues, and even the unmodified protein is expected to be capable of metal sequestration.

Transformation of PCC 7942 with the pAM1213 construct has also been successfully accomplished. The cells were selected for spectinomycin resistance, transferred to a medium supplemented with IPTG, and examined against controls for increased metal tolerance. Recombination was confirmed by molecular hybridization of cyanobacterial genomic DNA using the 309 bp fragment of the *cup* I gene as probe.

Increased Heavy Metal Tolerance. Initial screening of the pUC303 cyanobacterial transformants was encouraging. Earliest results indicated a substantial increase in tolerance to copper relative to controls. Resistance to 10µM copper and possibly greater concentrations was observed with several transformants. However, with repeated culturing, these levels were unable to be maintained at a constant level of tolerance. Whether these results might be due to loss of the plasmid, recombination, or other events was unclear.

It was decided to concentrate efforts on the other plasmid construct which contains a promoter and provides for inducible expression. The *con* II synthetic promoter (Elledge et al. 1989) has been shown to function well as a heterologous promoter in a cyanobacterial system (Li and Golden 1993). Recombinants having increased tolerance to copper have been identified. Tests are currently underway to assess levels of protein expression from this construct and to determine physiological effects on the cyanobacterial cell. If the cells are successfully sequestering the metals and are not adversely affected by expression of the metallothionein protein, attempts will be made to increase expression by cloning of tandem repeats of the gene.

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