ROLE OF METAL DETOXIFICATION BY GLUTAREDOXIN GENE AND ITS ISOLATION FROM CHROMIUM RESISTANT CYANOBACTERIA

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ABSTRACT:Polypeptide profile of chromium resistant cyanobacteria strains Synechocystis sp. AHZ-HB-MK and S. sp. AHZ-HB-P2A was compared in the presence of K_2CrO_4 (0μg mL^{-1},50μg mL^{-1} and100μg mL^{-1}).Both strains showed difference in protein expression, at 50μg mL^{-1} conc. Polypeptides of 20KDa and 18KDa showed increased expression by S.AHZ-HB-MK and S. sp. AHZ-HB-P2A respectively. Metal concentrations and exposure time also effected the protein expressions. Isolation and sequencing of representative genes indicated Glutaredoxin is a protein that helps cyanobacteria to survive in oxidative stress caused by Chromium. Glutaredoxin gene was able to be sequenced only in Synechocystis sp. AHZ-HB-MK.

Key words: Glutaredoxin, oxidative stress, cyanobacteria, *Synechocystis*.

INTRODUCTION

With the advent of technologies and research cyanobacteria are now considered as potential candidates for bioremediation of metal contaminated sites, fields and promote plant growth even in the metal polluted cultivated areas. Cyanobacteria do so because of their metal tolerance and plant growth promoting ability. They can tolerate many metals by having different indigenous mechanisms. They have different detoxification system such as metal expulsion from the cell, their compartmentalization and synthesis of such proteins for example metallothioneins that can bind and make complex with metals [1].

Heavy metals such as Chromium can cause toxicity in all organisms including plant in which they induce the formation of reactive oxygen species mostly by autoxidation and Fenton reaction which requires divalent metals ion and a source of reducing agent to regenerate the metal [2]. In plants reactive oxygen species are also generated naturally during light independent photosynthesis [3].This oxidative stress caused by either way effects badly on the survival of microbes as well as plants. Reactive oxygen species targets biomolecules such as nucleic acid protein and lipid moieties [2]. Due to these harmful effects plants exhibit reduction in growth, phytomass, chlorosis, impaired photosynthesis, stunting and finally plant death [4] .

To protect themselves from the damage of these oxidants, cyanobacteria are supposed to produce some scavenger molecules as do all higher plant and eukaryotic organisms that can balance oxidative stress within the cell environment. These proteins can also be categorized as stress proteins that are not constitutively produced in normal circumstances rather they are produced in different situations (heat stress , metals stress , pH stress and salt stress) in order to nullify the effect of these stresses so that organism may survive. Non-protein thiols have already been investigated as probable mechanism of heavy metal detoxification in cyanobacteria [5]. Sometimes Reactive oxygen species by themselves induce the expression of these antioxidants [6]. Most important of these proteins are glutathione which is being produced by almost all organisms ranging from prokaryotes to eukaryotes including humans. But some phylum of archea and bacteria lack its gene [7].

Glutaredoxins have two active site cysteine residues in the sequence Cys-Pro-Tyr-Cys. The N-terminal cysteine is required for both protein disulfide reduction and reduction of mixed protein-glutathione disulfides, while the other cysteine is required only for reduction of intramolecular protein disulfide bonds. It gets reduced by the oxidation of Glutathione [8]. Many studies has been done to identify and isolate glutaredoxin genes from cyanobacteria specially genus *Synechocystis.* Its functionality and detoxification parameters have also been determined.

In present study cyanobacteria subjected to sudden shift in one or several parameters affecting their growth or survival due to which program of gene expression was initiated, which was manifested as an increased or decreased amount of a set of proteins synthesized in response to stress. In cyanobacteria chromium stress strongly stimulates the expression of a set of proteins that probably allow them to survive in the rapidly changing environment. Evaluation of difference in polypeptide profile of chromium resistant cyanobacteria was made that were growing in the presence (10 μg mL^{-}, 50μg mL^{-1} and 100 μg mL^{-1}) and absence of chromium. In addition to that the gene that expressed under stress condition was isolated, sequenced and identified.

MATERIALS AND METHOD

Isolation and characterization

Two strains of cyanobacteria genus *Synechocystis* sp. AHZ-HB-MK and *S*. sp. AHZ-HB-P2A were previously isolated, characterized and molecularly identified [9-11].

Protein profiling by Polyacrylamide gel electrophoresis (PAGE)

For extraction of cyanobacterial proteins, cyanobacteria were grown in BG 11 supplemented with 50μg $mL^{-1}$of K_2CrO_4 at 28±2ºC. After 15 days cells were harvested and total cell proteins were resolved on PAGE. The protein profile of *Synechocystis* strains was resolved after 1, 3 and 7 days at different concentrations of chromium K_2CrO_4, which were already used for the probable mechanisms of heavy metal detoxification through non-proteins thiols.

DNA Isolation, Amplification and Elution for Sequencing

DNA isolation was performed according to previous study [11].

Polymerase Chain Reaction (PCR) for Glutaredoxin gene amplification

PCR was performed in a Perkin-Elmer DNA Themai Cycler (Perkin-Elmer Co. Norwalk- CT, USA). PCR was performed in MASTERCYCLER® 5333 eppendorf, Version 2.30.33-99 under standard conditions. Glutaredoxin gene was amplified

in the genome of both *Synechocystis* sp. AHZ-HB-MK and *S.* sp. AHZ-HB-P2A. For all genes the template DNA used was 100 ng. After mixing Primers and other ingredients of PCR in amplification tube it was placed in thermoblock of thermocycler, which was fitted with heated lids. Amplified the nucleic acids during the Denaturation, annealing and extension cycles. The programming was according to the primer used. The conditions of time durations and temperature were optimized and they were specified.

Gel Extraction of Amplified DNA

After confirmation of amplified DNA by visualizing it through gel electrophoresis, this DNA was eluted and taken for sequencing. Two different kits were used for DNA elution. ***Fermentas*** *Kit # K 0513* And ***QIAquick*** *Gel Extraction Kit (Cat. # 28704, Qiagen, Inc.)*.

RESULTS

Protein profiling by PAGE

***Synechocystis* sp. AHZ-HB-MK**

In this strain, twenty proteins of 135, 132, 120, 110, 104, 62.5, 57.5, 52.4, 47.5, 44.9, 37.5, 35, 33.7, 30, 27, 25, 23.7, 20, 18 and 14.4 KDa were identified at 0µg mL^{-1} chromium. The expression of only one polypeptide of 20 KDa improved, however, twelve polypeptides of 110,104,62.5,57.5, 47.5, 35,33.7,30,27,23.7,18 and 14.4 KDa had reduced expression, while seven polypeptides of 135,132,120,52.4,47.5,44.9 and 27 KDa had completely vanished in the presence of 50µg mL^{-1} chromium (Table 1).

***Synechocystis* sp. AHZ-HB-P2A**

In this strain, twenty one proteins of 135, 132, 120, 110, 104, 62.5, 57.5, 52.4, 51.2, 47.5, 44.9, 37.5, 35, 33.7, 30, 27, 25, 23.7, 20, 18 and 14.4 KDa were identified at 0µg mL^{-1} chromium. The expression of only one polypeptide of 18 KDa remained same; however, fourteen polypeptides of 135, 132, 120, 110, 104, 62.5, 57.5, 52.4, 33.7, 30, 27, 25, 23.7 and 20 KDa had reduced expression, while six polypeptides of 51.2, 47.5, 44.9, 37.5, 35 and 14.4 KDa completely vanished in the presence of 50µg mL^{-1} chromium (Table 1).

To evaluate change in protein expression in more detail same procedure was also followed for different concentrations of metals such as 0µg $mL^{-1,}$ 10µg mL^{-1} and 100µg mL^{-1} at different time intervals (Table 2).

DNA Isolation, Amplification and Elution for Sequencing

DNA was obtained from the both strains of cyanobacteria and visualized by gel electrophoresis. Glutaredoxin gene amplification was done by PCR by adding appropriate primers and maintaining amplification temperature. 5'-TGAGAGCCCTGGCTTTATTG-3' was used as forward primer and 5'-CAACTTGCCTGCACCATCTA-3' as reverse primer. These were the primer that amplified the target gene. In order to confirm that whether the gene was present in both strains and amplification is done properly, gel electrophoresis was performed. After eluting DNA from the gel it was subjected to sequencing. Results of sequencing were manipulated as below:

Sequence of Glutaredoxin Gene for *Synechocystis* sp. AHZ-HB-MK

This strain gave sequence with forward primer 5' TGAGAGCCCTGGCTTTATTG 3' during first attempt. The partial sequence of this gene, the blast [12] gave 96% similarity to *Synechocystis* PCC 6803 genome with Glutaredoxin 3 gene. The data was submitted to GenBank and was issued an accession number as DQ398587. The Electropherogram of partial sequence of Glutaredoxin gene of this strain is shown as Figure 1.

BLAST (Basic Local Alignment Search Tool) Analysis

Sequencing was difficult because fragment lengths were very short that is why only *Synechocystis* sp. AHZ-HB-MK could be sequenced and analyzed further. The sequence obtained was compared with known sequences using BLAST [12]. The sequenced data then submitted to GenBank, in order to obtain the accession numbers for sequenced gene fragments. The query asking glutathione reductase gene from CyanoBase (The Genome Database for Cyanobacteria) for the reference strain *Synechocystis* sp. PCC 6803 gave result as follows:

Gene Information (Chr) ssr2061 Location: Init: 1050812 Term: 1051078 Length (aa): 88

Gene Products: Glutaredoxin 3.

The Gene Sequence was *Synechocystis* PCC 6803 (Chr) 1050812-1051078

ATGGCTGTCTCGGCAAAAATTGAAATTTATACATGG
AGCACTTGCCCTTTTTGCATGAGAGCCCTGGCTTTAT
TGAAACGTAAAGGAGTAGAGTTCCAAGAATATTGC
ATTGACGGCGACAACGAAGCAAGGGAAGCCATGGC
GGCAAGGGCCAACGGCAAAAGGAGCTTGCCCCAAA
TTTTTATTGACGACCAACACATTGGTGGCTGTGATG
ACATCTATGCCCTAGATGGTGCAGGCAAGTTGGACC
CCCTGCTCCATAGTT

The Homology/ Similarity Studies of *Synechocystis* sp. AHZ-HB-MK

Strain and *Synechocystis* PCC 6803 Glutaredoxin 3 Gene. The Retrieved Result with Alignment of Two Sequences is Given Below.

The blast gave 96% similarity to *Synechocystis* PCC 6803 "gi|47118304|dbj|BA000022.2| *Synechocystis* sp. PCC 6803 DNA, complete genome
Length=3573470
Features in this part of subject sequence:
Solanesyl diphosphate synthase
Glutaredoxin 3
Score = 254 bits (128), Expect = 7e-65
Identities = 146/152 (96%), Gaps = 0/152 (0%)
Strand=Plus/Plus"

```
Query                                                    1
TTCCAAGAATATAGCATTGATGGCGATTACGAAGCAAGGGA
GGCCATGGCGGCAAGGGCC  60
                 ||||||||||||       |||||||
|||||  |||||||||||| ||||||||||||||||||
Sbjct                                 1050905
TTCCAAGAATATTGCATTGACGGCGACAACGAAGCAAGGGA
AGCCATGGCGGCAAGGGCC  1050964

Query                                                   61
AACGGCAGAAGGAGCTTGCCCCAAATTTTTATTGACGACCA
ACACATTGGTGGCTGTGAT  120
                 |||||||
||||||||||||||||||||||||||||||||||||||||||
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Sbjct                                          1050965  Sbjct1051025
AACGGCAAAAGGAGCTTGCCCCAAATTTTTATTGACGACCA  GACATCTATGCCCTAGATGGTGCAGGCAAGTT  105105
ACACATTGGTGGCTGTGAT  1051024

Query                                              121
GACATCTATGCCCTAGATGGTGCAGGCAAGTT  152

||||||||||||||||||||||||||||||||
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Query: The Sequence of *Synechocystis* sp. AHZ-HB-MK Submitted to Blast

Subject: The Sequence of *Synechocystis* PCC 6803 Retrieved though Blast

Table 1: Polypeptide profile of chromium resistant cyanobacterial strains under 0µg mL^{-1} and 50µg mL^{-1} chromium

r=reduced expression of polypeptide,**d**=polypeptide inhibited,**m**=more expression

		135	132	120	110	104	62.5	57.5	52.4	47.5	44.9	37.5	35	33.7	30	27	25	23.7	20	18	14.4
Synechocystis sp. AHZ-HB-MK	**0µg mL^{-1}**																				
	50µg mL^{-1}	d	d	d	r	r	r	r	d	r	D	d	r	R	r	r	d	R	m	r	r
Synechocystis sp. AHZ-HB-P2A	**0µg mL^{-1}**	135	132	120	110	104	62.5	57.5	52.4	47.5	44.9	37.5	35	33.7	30	27	25	23.7	20	18	14.4
	50µg mL^{-1}	d	d	d	r	r	r	r	d	r	d	d	r	R	r	r	d	r	m	r	r

Table 2: Polypeptide profile of chromium resistant *Synechocystis* strains under 0µg mL^{-1}, 10µg mL^{-1} and 100µg mL^{-1} chromium.

CYANOBACTERIAL STRAINS													
Synechocystis sp. AHZ-HB-MK							*Synechocystis* sp. AHZ-HB-P2A						
0µg mL^{-1}	**10µg mL^{-1}**			**100µg mL^{-1}**			**0µg mL^{-1}**	**10µg mL^{-1}**			**100µg mL^{-1}**		
Cont.	**1day**	**3day**	**7day**	**1day**	**3day**	**7day**	**Cont.**	**1day**	**3day**	**7day**	**1day**	**3day**	**7day**
135	m	m	r	r	r	d	135	m	M	r	r	m	r
132	m	m	d	m	m	d	132	m	M	r	r	m	r
120	m	m	d	m	m	d	120	m	M	r	r	m	r
110	m	m	r	m	m	r	110	m	R	r	m	m	r
104	m	m	r	m	m	r	104	m	R	r	r	m	r
62.5	m	m	r	r	m	r	62.5	m	M	r	m	m	r
57.5	m	m	m	m	m	r	57.5	m	R	r	r	r	r
52.4	r	r	r	r	m	d	52.4	m	M	r	m	m	r
47.5	r	m	d	r	r	r	51.2	r	D	r	r	r	d
44.9	r	m	d	r	d	d	47.5	m	R	m	m	r	d
37.5	r	r	d	r	r	d	44.9	r	D	r	m	r	d
35	m	r	d	r	r	r	37.5	r	R	r	r	d	d
33.7	r	r	d	r	r	r	35	m	R	m	m	r	d
30	r	m	r	r	r	r	33.7	m	R	d	r	r	r
27	r	r	d	r	r	r	30	r	R	d	r	r	r
25	m	r	d	r	d	d	27	r	R	r	r	r	r
23.7	r	m	r	r	m	r	25	r	R	d	d	r	r
20	m*	m*	m	m*	m*	m	23.7	m	M	m	m	m	r
18	m*	m*	r	r	r	r	20	m*	M	d	m	m*	r
14.4	same	r	r	r	r	r	18	same	R	same	same	r	same
							14.4	r	R	r	r	r	d

r=reduced expression of polypeptide,**d**=polypeptide inhibited,**m**=more expression,**m***=many fold more expression

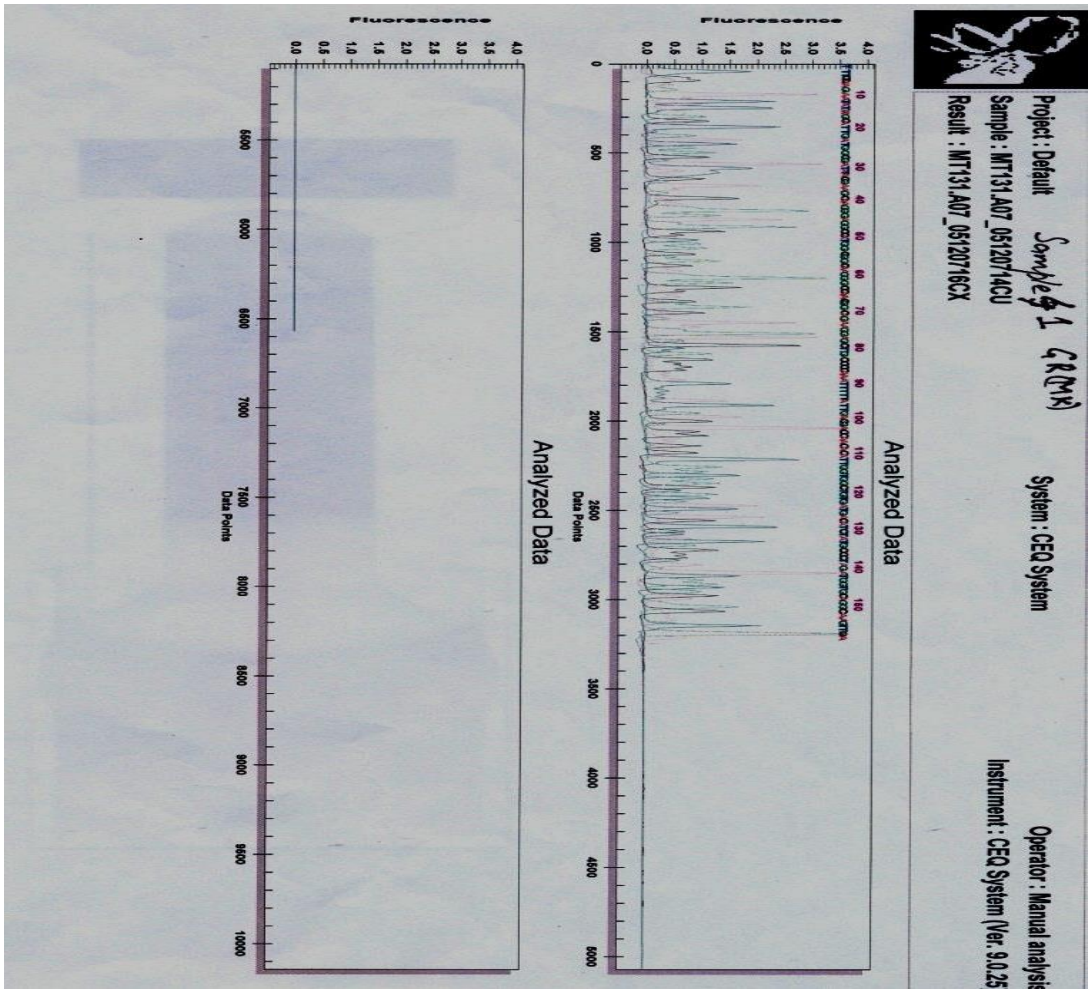

Figure 1: Electropherogram of Glutaredoxin Gene for *Synechocystis* sp. AHZ-HB-MK

DISCUSSION

Cyanobacteria are well known and potential candidate for bioremediation and metal detoxification. *Synechocystis* sp. AHZ-HB-MK and *Synechocystis* sp.AHZ-HB-P2A were already studied for their role in detoxification through non-protein thiols. In present study they were also tested for the presence and consequently sequencing of Glutaredoxin gene. Presence of Glutaredoxin gene was observed when polypeptide profiling was done in order to determine the effect of chromium on the synthesis of stress proteins. The *Synechocystis* PCC 6803 has already been used for the study of some other heavy metal resistance mechanisms, but not for chromium [13-15]. The reference strain *Synechocystis* PCC 6803 was obtained and subsequently used for comparative study of probable mechanism of heavy metal detoxification through non-protein thiols. Chromium stress resulted in alteration in polypeptide profile of chromium resistant cyanobacteria. Polypeptide pattern revealed that three different types of changes in these strains in response to chromium stress were observed i.e., (i) loss of certain proteins (ii) reduction in expression of some proteins and (iii) improvement in expression of some proteins. The interesting comparisons in between *Synechocystis* sp. AHZ-HB-MK and *Synechocystis* sp.AHZ-HB-P2A strains for a polypeptide of 20 KDa was that its expression in *Synechocystis* sp. AHZ-HB-MK was high in both chromium treatments of 10µg mL^{-1} chromium and 100µg mL^{-1} chromium with passage of time than *Synechocystis* sp. AHZ-HB-P2A, where its expression was reduced/ lost at 7 days of chromium treatment, this polypeptide may have

involved in high chromium resistance of former strain over latter. In strain *Synechocystis* sp. AHZ-HB-P2A a polypeptide of 51.2 KDa was found, however, it was absent in *Synechocystis* sp. AHZ-HB-MK. This clearly differentiates between both strains. There is another finding form the above study that different cyanobacteria strains may have evolved different mechanisms for chromium resistance/ detoxification.

These findings urged to identify their respective genes. That is why the amplified genes in both strains were isolated and sequenced. All sequencing attempts were made in Beckman Coulter® (CEQ 8000) sequencer and data was analyzed by the software, CEQ System (Ver.9.0.25). The sequencing was only achieved after obtaining high purity of amplified fragment through spin column QIAquick Gel Extraction Kit (Cat. # 28704) purification, rather than silica beads treatment (Fermentas # K 0513). Same result has also been reported by [16-17]. Hence proved that QIAquick Gel

Extraction Kit (Cat. # 28704)is more efficient.The amplified gene being less than 250 base pairs was very difficult to get sequenced, after many attempts this gene in strain *Synechocystis* sp. AHZ-HB-MK was sequenced, but all attempts were in vein for *Synechocystis* sp. AHZ-HB-P2A. The smaller fragment sequencing is quite difficult as compare to large fragment; it may be due to less template availability for polymerase enzyme.

In this study an attempt has been made to target and sequence gene analogue to glutaredoxin3 of*Synechocystis* PCC 6803 in *Synechocystis* sp. AHZ-HB-MK and *Synechocystis* sp. AHZ-HB-P2A strains isolated and characterized previously. The Glutaredoxin 3 (*grxC*) probable function of Glutaredoxinssr2061 has already been studied in *Synechocystis* PCC6803 that it has a glutathione-disulfide oxidoreductase activity in the presence of NADPH, reduces low molecular weight disulfides and proteins[18]. The glutaredoxin was amplified in both cyanobacterial strains in this present study, some scientists [19] were also successful to amplify this gene in all strains reported by him, it is also strengthening a possibility that the designed primers of this gene may be from conserved region of different cyanobacteria or cyanobacterial strains isolated in present study. The amplified fragment in *Synechocystis* strains were of 230 base pairs size approximately, which correspond to 250 base pairs fragment isolated by some scientists [19].Literature review suggests that glutaredoxin 3 works along with another gene product glutathione synthetase, that form glutathione. It causes reduction of oxidized form of glutathione (GSSG in to GSH) to reduced form for its further use to overcome oxidative stress by binding with heavy metals. Finding of the 96% homology of *Synechocystis* "MK (S)" glutaredoxin 3 gene did not only illustrate the presence of this gene in closely related organisms, but also confirmed the role of glutathione in particular as one of the mechanism of heavy metal detoxification. Moreover increased expression of other polypeptide after chromium stress can lead us to the identification of glutathione synthetase genes that also provide additional help to the organism like cyanobacteria to combat with metal stress in the environment. To date much less is known about the roles of glutathione in cyanobacteria that are the evolutionary precursor of the chloroplast. An understanding of glutathione in cyanobacteria is expected to provide novel insight into the evolution of the elaborate and extensive pathways that utilize glutathione in photosynthetic organisms [14].

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