SEQUENCING OF GLUTATHIONE SYNTHETASE GENE FROM THE CHROMIUM RESISTANT SYNECHOCYSTIS SP. AHZ-HB-MK AND AHZ-HB-P2A

*Abdul Hameed and Shahida Hasnain
Department of Microbiology and Molecular Genetics University of the Punjab, Quid-e-Azam Campus, Lahore-54590, Pakistan.
*Email: ahz1963@yahoo.com

ABSTRACT: Various strains of cyanobacteria were previously isolated and characterized for chromium metal resistance, plant growth promotion and production of non protein thiols. Difference in polypeptide profiling under chromium stress in both strains and presence of Glutaredoxin gene in AHZ-HB-MK provoked for the amplification of glutathione synthetase in these organisms. Amplified products of around 800 base pairs size, gave satisfactory sequence of 475 and 604 base pairs with the sequence similarity of 99% and 98% from the Synechocystis sp. AHZ-HB-MK and AHZ-HB-P2A respectively with reference to the Synechocystis PCC 6803. These interpretations also lead us towards the finding that both these strains are different from each other in various aspects.

Key words: Synechocystis, Glutathione synthetase, Non protein thiol

INTRODUCTION
Glutathione (γ-glutamyl-l-cystinylglycine) is a non-protein thiol, widely distributed in animal tissues, plants and microorganisms. Glutathione is an abundant and ubiquitous low-molecular-weight thiol that may play a role in many cellular processes, including protection against the deleterious effects of reactive oxygen species [1]. It is synthesized in two ATP-dependent steps [2]. The dipeptide γ -glutamlycysteine is first synthesized from L-glutamic acid and L-cysteine by γ -glutamlycysteine synthetase (EC 6/3/22). In the second step, catalyzed by glutathione synthetase (EC 6/3/23), glycine is added to the C-terminal site of γ -glutamlycysteine to form glutathione [3]. Among prokaryotes it is specifically produced by Cyanobacteria and proteobacteria. Few gram positive bacteria are also involved [4]. These scavenging thiols protect organisms from oxygen toxicity and environmental hazardous encounters that effect the survival of cell by oxidizing protein, lipid and DNA content [5, 6]. These are produced not only during photosynthesis but also by the heavy metals such as chromium. In some studies it has been proved that these thiols are expressed only under such stress conditions [7, 8].

By growing cyanobacteria under chromium stress can help us to induce the expression of such thiols. [9] determined the nucleotide sequence of the entire genome of Synechocystis sp. PCC 6803 and found a gene encoding a polypeptide homologous to glutathione synthetase of E. coli. [10] cloned a homologue from Anabaena sp. PCC 7120. Although the cloned genes from these two organisms have been sequenced, functions have not been demonstrated. In another study a structural gene for glutathione synthetase, gshB, has been cloned from Synechococcus sp. PCC 7942 and its involvement in the biosynthesis of glutathione in that cyanobacteria had been demonstrated [11]. Presence of gshB gene is very much necessary for the proper functioning of glutathione in the scavenging of harmful reactive oxygen species. In one study deletion in glutathione synthetase gene decreased the level of reduced form of glutathione (GSH) in Cyanobacterial strain as well as its growth as compare to wild type under favorable conditions. In the presence of oxidative stress the growth reduced dramatically and even lead to the death [12]. Overexpression of glutathione synthetase offers a promising strategy for the production of plants with superior heavy-metal phytoremediation capacity [13]. Difference in polypeptide pattern by two different Synechocystis strains suggested that there are some proteins that are expressed under stress conditions. Sequencing for glutaredoxin confirmed it [8]. In the present study sequencing for glutathione synthetase was performed that worked with Glutaredoxin and helped to survive cyanobacteria in metal stress. Quest for the presence and metabolism of Glutathione in cyanobacteria is expected to improve our understanding of the functioning of this gene in higher organism as well [12]

Materials and methods
Isolation and characterization
Various filamentous and unicellular strains of cyanobacteria were previously isolated, characterized and molecularly identified[14, 15]. Protein profiling was also performed that has been reported previously and presence of glutaredoxin gene was also determined in Synechocystis sp. AHZ-HB-MK and AHZ-HB-P2A [8].

DNA Extraction, Amplification of Glutaredoxin synthetase gene and Sequencing
DNA isolation was performed according to previous study [8]. For amplification PCR was performed by using specific primers for glutathione synthetase gene. The programming was according to the primer used. The conditions of time durations and temperature were optimized and they were specified. Sequencing attempts for purified DNA fragments were made in Beckman Coulter® (CEQ 8000) Genetic Analysis System sequencer, which was fully automated and worked on Capillary Electrophoretic (CE) Genetic Analysis System (CEQ) and data was analyzed by the software, CEQ System (Ver.9.0.25). Out of total obtained data, the meaningful data was further processed.

BLAST (Basic Local Alignment Search Tool) Analysis
The sequences obtained were compared with known sequences of reference strains using BLAST [16]. The sequenced data then submitted to GenBank, in order to obtain the accession numbers for sequenced genes fragments.

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RESULTS
DNA Extraction, Amplification of Glutaredoxin synthetase gene and Sequencing
The primers were designed from this sequence for the amplification of this gene in *Synechocystis* isolated strains with following sequences 5'CTCGATCCGGGCCATGA'3 as forward primer and 5'CTCCCAGGCGGACTCCC'3 as reverse primer. The strains *Synechocystis* AHZ-HB-MK and *Synechocystis* AHZ-HB-P2A gave successful amplification of this gene, but amplification was not found in any other isolated cyanobacterial strains. Because the role of non-protein thiols was studied with *Synechocystis* strains, so the amplified genes in both strains were sequenced. The amplified gene was around 800 base pairs size. The satisfactory sequence was found up to 475 and 604 base pairs for *Synechocystis* AHZ-HB-MK and *Synechocystis* AHZ-HB-P2A respectively.

Sequence of Glutathione Synthetase gene in the reference strain *Synechocystis* sp. PCC 6803
The query asking glutathione synthetase gene from CyanoBase (The Genome Database for Cyanobacteria) for the reference strain *Synechocystis* sp. PCC 6803 gave result as follows:

“Gene Information (Chr) slr1238 Location: Init: 1051159 Term: 1052121 Length(aa):320Direction: direct Gene Products << Gene Name >> : glutathione synthetase << gshB >> The gene sequence was *Synechocystis* (Chr) *Synechocystis* PCC 6803 (Chr) 1051159-1052121”

Sequence of Glutathione Synthetase Gene in Strain *Synechocystis* “AHZ-HB-MK”
This strain gave sequence with forward primer 5'CTCGATCCGGGCCATGA'3:

```
GTGAAACTGCTGGTTTATTATCAGATCCCGCCGATGATCCACGTACG
ACCCTTGCCACATGGAAGCGGGCCCAAATAGTGGGACACGAAAGTTTTGTACG
GTGGGGGATTGGGCCGTATATACATCCAGGGCTGCTAAATAGCCTGGGCTACGT
CTCTCAGCGCTGTATTGCTGATGGGACAGTGGCAGATTTCCAAACCTTGCTCGG
ACTGTCGAACACCAGTGTACATCAATTCCTCACCACCTCTTTGACTTACTAGC
CCACCAACGCGGTCGGACCCGTGCAATCTATCTACGCCACCTCTTTGAGTTACTAGC
CCACACCCCGTACGGGCTACGCGGGAAGCCATGGAAGCCATGGAAGCG
AAATGTCACCCTCAGCTTGCTGCGGTATGCTGCTCCACGTTAGTGGGAC\nAGGGCCTGATCCCGCCAGTTTTGTTGGAAAGAGCACGGGGCGGCAGTGGAAAAACCTTTG
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The similarity with *Synechocystis* PCC 6803 was 99% (Table 1). The Accession Number is DQ381962. The Electropherogram of partial sequence of glutathione synthetase gene of this strain is shown as Figure 1.

**Sequence of Glutathione Synthetase Gene in Strain *Synechocystis* “AHZ-HB-P2A”**

This strain gave sequence with reverse primer 5'CTCCCAGGCGGACTCCC'3:

```plaintext
GTGACATTGACTTCTGTAGGTAGCCTCCGATAAACATCAATCCCAGCAAAAATATAAA
CCGTACAGCTGCAATTGTTTAGCAAAAGCGCAAATTCTATTTCCCTGGAGGTA
ATGGTAGTGAGGACCACTTGAACCAACACCCCATATTTCGCCCGGAATTCTGCTCCA
CTGGGAATGCGATTTACGACTCCAAATTGGGATCACCATCCACAGGATAATTGCTTA
TCACCCTCTTGGCTTGGCGAAGAAGACGCTGACATTACCGGCTTTTCCATGT
GGGTACTTAATCTCACCAGGAGGATGGAAGTTTCGATCGCAAGATCGAGAAACAAA
ATACCCCTCCGCCGCTTACCTCCTCAAGGTTTACACTGCGGCCCGTGCTTTTCCA
AAAACTGCGGATACGGCTTCTTGCACAACTAACCACCGTGAGAAGCATCACCAGCA
GCAAACGTGCAAGGTGTACA.
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The similarity with *Synechocystis* PCC 6803 was 98% (Table 2). The Accession Number is DQ398588. The Electropherogram of partial sequence of glutathione synthetase gene of this strain is shown as Figure 2.

**Table 1: The Homology/ Similarity Studies of *Synechocystis* “AHZ-HB-MK” Strain and *Synechocystis* PCC 6803 Glutathione synthetase Gene.**

The Retrieved Result with Alignment of Two Sequences are Given Below “gi|47118304|dbj|BA000022.2| Synechocystis sp. PCC 6803 DNA, complete genome
Length=3573470
Features in this part of subject sequence:
Solanesyl diphasophate synthase
Glutathione synthetase
Score = 934 bits (471), Expect = 0.0
Identities = 474/475 (99%), Gaps = 0/475 (0%)
Strand=Plus/Minus”
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**Query:** The Sequence of *Synechocystis* “AHZ-HB-MK” Submitted to Blast

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

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Figure 1: Electropherogram of Glutathione Synthetase Gene for Synechocystis "AHZ-HB-MK"
Table-2: The Homology/ Similarity Studies of *Synechocystis* “AHZ-HB-P2A” Strain and *Synechocystis* PCC 6803 Glutathione synthetase Gene.

The Retrieved Result with Alignment of Two Sequences are Given Below “gi|47118304|dbj|BA000022.2| Synechocystis* sp. PCC 6803 DNA, complete genome

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<th>Expect</th>
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<td>3573470</td>
<td>Solanesyl diphosphate synthase Glutathione synthetase</td>
<td>1126 bits (568)</td>
<td>0.0</td>
<td>589/596 (98%)</td>
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Query: The Sequence of *Synechocystis* “AHZ-HB-P2A” Submitted to Blast

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

May-june
Figure 2. Electropherogram of Glutathione Synthetase Gene for Synechocystis “AHZ-HB-P2A.”
DISCUSSION
Cyanobacterial strains evaluated in previous studies were able to resist chromium stress, promoted plant growth in chromium contaminated areas, produced non protein thiol. Alteration in polypeptide profile of chromium resistant cyanobacteria led us to the isolation, amplification and sequencing of genes involving in the formation of glutathione. Amplification of Glutaredoxin encouraged for the isolation, amplification and sequencing of glutathione synthetase gene.[7, 8, 14, 15, 17].

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. Glutathione synthetase (gshB) gene analog of Synechocystis PCC 6803 was sequenced in Synechocystis “AHZ-HB-MK” and Synechocystis “AHZ-HB-P2A”. Glutathione synthetase gshB gene in the cyanobacterium Synechococcus sp PCC 7942 encodes a functional glutathione synthetase [11], the gshB gene includes an open reading frame of 969 nucleotides, which encodes a polypeptide of 323 amino acid residues with an approximate molecular mass of 35 kDa. In both Synechocystis “AHZ-HB-MK” and “AHZ-HB-P2A” strains the amplified fragments were of around 800 base pairs length respectively. The direct sequencing of glutathione synthetase (gshB) fragment was achieved in this work while Okumura et al. (1997) [11] got its sequence only after cloning it.

Almost in all cyanobacterial strains a polypeptide of 35 KDa was found, which may be the product of gshB gene responsible for the synthesis of glutathione synthetase. A wild-type gene, designated gshB, encodes a polypeptide of 323 amino acids in Synechococcus sp. PCC 7942 with a molecular mass of 35 KDa was also reported by Okumura et al., 1997 [11]. But PCR amplification antagonized this theory. Sequencing of smaller fragment is quite difficult as compared to large fragment size; it may be due to less template availability for polymerase enzyme. The failure of gene fragment amplification in cyanobacterial stains analogue to glutathione synthetase (gshB), except Synechocystis “AHZ-HB-MK” and Synechocystis “AHZ-HB-P2A” strains, may have one explanation that the primers designed for glutathione synthetase gene probably was not form conserved region of this gene, in other cyanobacteria (Jeff Elhai, personal communication), so Synechocystis strains gave amplification, while other cyanobacterial strains did not give amplification for it. The differential behavior of same primers in one group of cyanobacteria also reported by [18]. The other possibility of this selective amplification was that the isolated cyanobacterial strains were from different sites and with different morphology, so the subject gene may entirely be absent in some of the strains. This may suggest presence of any other mechanism of chromium metal resistance/ detoxification other than non-protein thiols in the rest of the isolated cyanobacterial strains, as they failed to give amplification of gshB gene. The Synechocystis PCC 6803 has already been used for the study of some other heavy metal resistance mechanisms, but not for chromium [19]. Two genes glutathione synthetase and glutaredoxin 3 are involved in glutathione synthesis and 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 [3]. The 96% homology of Synechocystis “AHZ-HB-MK” glutaredoxin 3 gene with Synechocystis PCC 6803 was observed and reported recently [8]. In present study amplification and sequencing of glutathione synthetase gene in the same strains Synechocystis “AHZ-HB-MK” and Synechocystis “AHZ-HB-P2A” showed 99% and 98% homologies respectively with reference strain Synechocystis PCC 6803. This did not only illustrate the presence of these genes in closely related organisms, but also confirmed the role of glutathione in particular as one of the mechanism of heavy metal detoxification.

This study also shed some light on the differences between two strains of the same genus. Strain Synechocystis “AHZ-HB-MK” is not only different from Synechocystis “AHZ-HB-P2A” on the bases of glutathione synthetase gene homology which is greater in case of Synechocystis “AHZ-HB-MK”, but also from the other aspects. As indicated above gene product size is also larger for Synechocystis AHZ-HB-MK. This strain also gives high expression of 20 KDa polypeptide under 10μg mL⁻¹ and 100μg mL⁻¹ chromium. While Synechocystis sp.AHZ-HB-P2A lost/reduced these expression with passage of time which explains that this polypeptide may have involved in high chromium resistance of former strain over latter. This strain also showed the expression of 51.2 that was absent in others which explains that different cyanobacterial strains may have evolved different mechanisms for chromium resistance/ detoxification [8]. Both these strains also differ on the basis of their growth parameters, former strain gives improved and rapid growth than the other. Synechocystis sp. AHZ-HB-MK show more chromium resistance (250μg mL⁻¹), reduction (62.14%) [17] and production of non-protein thiols, when exposed to chromium stress, as compared to other strain [7].

REFERENCES


