

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

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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 -glutamylcysteine is first synthesized from L-glutamic acid and L-cysteine by γ -glutamylcysteine synthetase (EC 6.3.2.2). In the second step, catalyzed by glutathione synthetase (EC 6.3.2.3), glycine is added to the C-terminal site of γ -glutamylcysteine 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.

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):320 Direction: direct Gene Products << Gene Name >> : glutathione synthetase << *gshB* >> The gene sequence was *Synechocystis* (Chr) *Synechocystis* PCC 6803 (Chr) 1051159-1052121”

GTGAAACTGGCTTTTATTATCGATCCCCTAGAAAACTCGATCCGGGCCATGATTCC
 ACCGTTGCCATCATGGAAGCGGCCAAAAGTTGGGACACGAAGTTTTTGTACATCA
 GTGGGGGATTTGGCCGTTATCAATGGCCAGGCCTGGGCTAAATTAGCGGCGGTACG
 CCTTCAGCCTGTGATTTTGGTTCGATGGGCACTGGCAAGTTTCCCAACCTTGGTCCGA
 ACTGTCCAAATCCCAGTGGATGGCCCTGACGGAATGCCAAGCAGTCTTTATGCGTAA
 GGATCCGCCGGTGACCGTGCAATATCTCTACGCCACCTTCATTTTGGAGTTACTAGC
 CCCCACCAAGACCATGGTGATCAATTCCCCCAGGGGCTACGGGAAGCCAATGAAA
 AAATGTACACCCTGCAGTTTGCTGCGGTGATGCCTCCACGGTGGTTAGTTTGGACA
 AGGGCCTGATCCGCCAGTTTTTGGAAAGAGCACGGGGCGGCAGTGTTAAAACCTTG
 GGAGGTAAGGCCGGGGAGGGAATTTGTTTCTCGATCCTGGCGATCGAACTTCAAC
 TCCCTGGTGGAGATTAGTACCCAACATGGCAAAGAGCCGGTAATGGTACAGCGTTTT
 CTGCCAGAAGCGAAGGAAGGTGATAAGCGAATTATCCTGCTGGATGGTGATCCCAT
 TGGAGCCGTCAATCGCATTCCCAGTGGAGCAGAATTCCGGGGCAATATGGCGGTTG
 GTGGTCAAGTGGCTCCCACTACCATTACCTCCAGGGAAATAGAAATTTGCGCTTTGC
 TAAAACCCAAATTGCAGGCTGACGGTTTATATTTTGTTCGGTATTGATGTTATCGGAG
 GCTACCTAACAGAAGTCAATGTCACAAGTCCGACGGGGATCCGGGAGATTGATCGC
 CTTGAGGGAGTCCGCCTGGGAGAAAAAGTAATTTGCTGGTTGGAAAAACAATTTTA

G.

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

This strain gave sequence with forward primer 5'CTCGATCCGGGCCATGA'3:

GTGACATTGACTTCTGTTAGGTAGCCTCCGATAACATCAATACCGACAAAATATAAA
 CCGTCAGCCTGCAATTTGGGTTTTAGCAAAGCGCAAATTTCTATTTCCCTGGAGGTA
 ATGGTAGTGGGAGCCACTTGACCACCAACCGCCATATTGCCCGGAATTCTGCTCCA
 CTGGGAATGCGATTGACGGCTCCAATGGGATCACCATCCAGCAGGATAATTCGCTTA
 TCACCTTCCTTCGCTTCTGGCAGAAAACGCTGTACCATTACCGGCTCTTTGCCATGTT
 GGGTACTAATCTCCACCAGGGAGTTGAAGTTTCGATCGCCAGGATCGAGAAACAAA
 ATTCCTCCCCGGCCTTACCTCCAAGGGTTTTAACACTGCCGCCCGTGCTCTTCCA
 AAAACTGGCGGATCAGGCCCTTGTCAAACTAACCACCGTGGGAAGCATCACCGCA
 GCAAACCTGCAGGGTGTACA.

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'CTCCCAGCGGACTCCC'3:

GTGGGGACACGAAGTTTTGTACATCGGTGGGGGATTTGGCCGTTATCAATGGCCA
 GGCCTGGGCTACATTAGCGGCGGTACGCCTTCAGCCTGTGATTTTGGTCGATGGGCA
 CTGGCAAGTTTCCCAACCTTGGTCCGAACTGTCCAAATCCCAGTGGATGGCCCTGAC
 GGAATGCCAAGCAGTCTTTATGCGTAAGGATCCGCCGGTGACCGTGCAATATCTCTA
 CGCCACCTTCATTTTGGAGTTACTAGCCCCACCAAGACCATGGTGATCAATCCCC
 CCAGGGGCTACGGGAAGCCAATGCAAAAATGTACACCCTGCAGTTTGCTGCGGTGA
 TGCTCCACGGTGGTTGGTTTGGACAAGGGCCTGATCCGCCAGTTTTTGGAAAGAGC
 ACGGGGCGGCAGTGTTAAAACCCTTGGGAGGTAAGGCCGGGGAGGGAATTTTGT
 CTCGATCCTGGCGATCGAACTTCAACTCCCTGGTGGAGATTGATACCCAACATGGC
 AAAGAGCCGGTAATGGTACAGCGTTTTCTGCCAGAAGCGAAGGAAGGTCATAAGCG
 AATTATCCTGCTGGATGGTGATCCATTGGAGACCCG.

The similarity with *Synechocystis* PCC 6803 is 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 diphosphate synthase

Glutathione synthetase

Score = 934 bits (471), Expect = 0.0

Identities = 474/475 (99%), Gaps = 0/475 (0%)

Strand=Plus/Minus”

<u>Query 1</u>	GTGACATTGACTTCTGTTAGGTAGCCTCCGATAACATCAATACCGACAAAATATAAACCG	60
<u>Sbjct 1052033</u>	GTGACATTGACTTCTGTTAGGTAGCCTCCGATAACATCAATACCGACAAAATATAAACCG	1051974
<u>Query 61</u>	TCAGCCTGCAATTTGGGTTTTAGCAAAGCGCAAATTTCTATTTCCCTGGAGGTAATGGTA	120
<u>Sbjct 1051973</u>	TCAGCCTGCAATTTGGGTTTTAGCAAAGCGCAAATTTCTATTTCCCTGGAGGTAATGGTA	1051914
<u>Query 121</u>	GTGGGAGCCACTTGACCACCAACCGCCATATTGCCCCGGAATTCTGCTCCACTGGGAATG	180
<u>Sbjct 1051913</u>	GTGGGAGCCACTTGACCACCAACCGCCATATTGCCCCGGAATTCTGCTCCACTGGGAATG	1051854
<u>Query 181</u>	CGATTGACGGCTCCAATGGGATCACCATCCAGCAGGATAATTCGCTTATCACCTTCCTTC	240
<u>Sbjct 1051853</u>	CGATTGACGGCTCCAATGGGATCACCATCCAGCAGGATAATTCGCTTATCACCTTCCTTC	1051794
<u>Query 241</u>	GCTTCTGGCAGAAAACGCTGTACCATTACCGGCTCTTTGCCATGTTGGGTAATACTCC	300
<u>Sbjct 1051793</u>	GCTTCTGGCAGAAAACGCTGTACCATTACCGGCTCTTTGCCATGTTGGGTAATACTCC	1051734
<u>Query 301</u>	ACCAGGGAGTTGAAGTTTCGATCGCCAGGATCGAGAAACAAAATTCCTCCCCGGCCTTA	360
<u>Sbjct 1051733</u>	ACCAGGGAGTTGAAGTTTCGATCGCCAGGATCGAGAAACAAAATTCCTCCCCGGCCTTA	1051674
<u>Query 361</u>	CCTCCAAGGGTTTTAACACTGCCGCCCCGTGCTCTTCCAAAACCTGGCGGATCAGGCC	420
<u>Sbjct 1051673</u>	CCTCCAAGGGTTTTAACACTGCCGCCCCGTGCTCTTCCAAAACCTGGCGGATCAGGCC	1051614
<u>Query 421</u>	TTGTCCAACTAACCACCGTGGGAAGCATCACCGCAGCAAACCTGCAGGGTGTACA	475
<u>Sbjct 1051613</u>	TTGTCCAACTAACCACCGTGGGAGGCATCACCGCAGCAAACCTGCAGGGTGTACA	1051559

Query: The Sequence of *Synechocystis* "AHZ-HB-MK" Submitted to Blast
Subject: The Sequence of *Synechocystis* PCC 6803 Retrieved though Blast

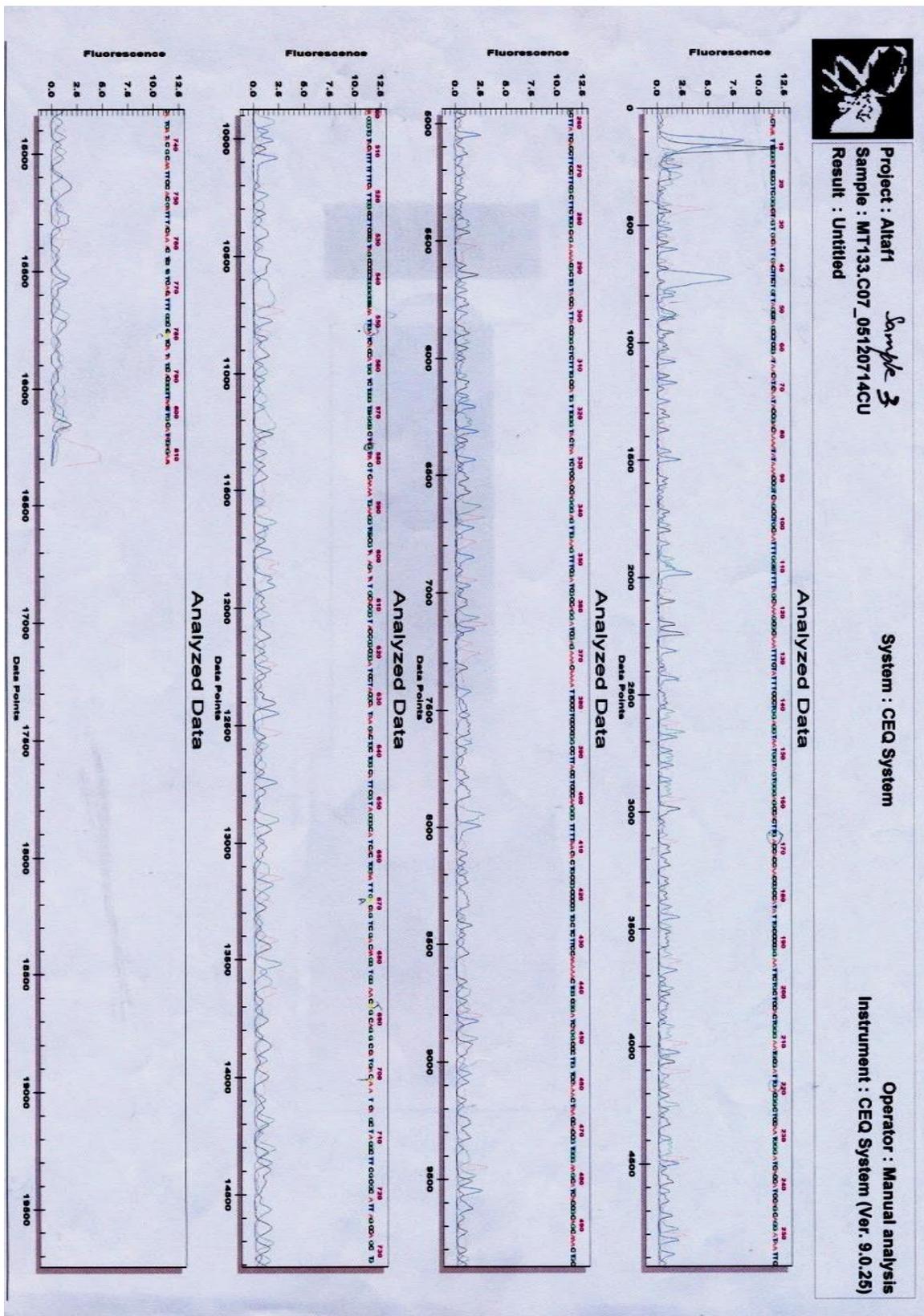


Figure-1 Electropherogram of Glutathione Synthetase Gene for *Synechocystis* "AHZ-HB-MK"

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

Length=3573470

Features in this part of subject sequence:

Solanesyl diphosphate synthase

Glutathione synthetase

Score = 1126 bits (568), Expect = 0.0

Identities = 589/596 (98%), Gaps = 0/596 (0%)

Strand=Plus/Plus”

Query_4	GGGACACGAAGTTT TGT CACATCGGTGGGGATT TGGCCGT TATCAATGGCCAGGCCTG	63
Sbjct_1051248	GGGACACGAAGTTT TGT CACATCAGTGGGGATT TGGCCGT TATCAATGGCCAGGCCTG	1051307
Query_64	GGCTACATTAGCGCGGTACGCCTTCAGCCTGTGATT TGGT CGATGGGCACTGGCAAGT	123
Sbjct_1051308	GGCTAAATTAGCGCGGTACGCCTTCAGCCTGTGATT TGGT CGATGGGCACTGGCAAGT	1051367
Query_124	TTCCCAACCTTGGTCGAACTGTCCAAATCCAGTGGATGGCCCTGACGGAATGCCAAGC	183
Sbjct_1051368	TTCCCAACCTTGGTCGAACTGTCCAAATCCAGTGGATGGCCCTGACGGAATGCCAAGC	1051427
Query_184	AGTCTTTATGCGTAAGGATCOGCCGGTGACCGT GCAATATCTCTACGCCACCTTCATTTT	243
Sbjct_1051428	AGTCTTTATGCGTAAGGATCOGCCGGTGACCGT GCAATATCTCTACGCCACCTTCATTTT	1051487
Query_244	GGAGTTACTAGCCCCACCAAGACCATGGTGATCAATTC CCCCAGGGGCTACGGGAAGC	303
Sbjct_1051488	GGAGTTACTAGCCCCACCAAGACCATGGTGATCAATTC CCCCAGGGGCTACGGGAAGC	1051547
Query_304	CAATGCAAAAATGTACACCTGCAGTTT GCTGCGGTGATGCCTCCACGGTGGTTGGTTT	363
Sbjct_1051548	CAATGAAAAAATGTACACCTGCAGTTT GCTGCGGTGATGCCTCCACGGTGGTTAGTTT	1051607
Query_364	GGACAAGGGCCTGATCOGCCAGTTT TGGAAAGACACGGGGCGGCAGTGT TAAAACCTT	423
Sbjct_1051608	GGACAAGGGCCTGATCOGCCAGTTT TGGAAAGACACGGGGCGGCAGTGT TAAAACCTT	1051667
Query_424	GGGAGGTAAGGCCGGGAGGGAATTT TGTCTTCTCGATCCTGGCGATCGAAACTTCAACTC	483
Sbjct_1051668	GGGAGGTAAGGCCGGGAGGGAATTT TGTCTTCTCGATCCTGGCGATCGAAACTTCAACTC	1051727
Query_484	CCTGGTGGAGATTGATACCCAACATGGCAAAGAGCCGGTAATGGTACAGCGTTTCTGCC	543
Sbjct_1051728	CCTGGTGGAGATTAGTACCCAACATGGCAAAGAGCCGGTAATGGTACAGCGTTTCTGCC	1051787
Query_544	AGAAGCGAAGGAAGGTGATAAGCGAATTATCCTGCTGGATGGTGATCCCATTGGAG	599
Sbjct_1051788	AGAAGCGAAGGAAGGTGATAAGCGAATTATCCTGCTGGATGGTGATCCCATTGGAG	1051843

Query: The Sequence of *Synechocystis* “AHZ-HB-P2A” Submitted to Blast

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



Project : Altair1
Sample : MT134.D07_05120714CU
Result : Untitled

System : CEQ System

Operator : Manual analysis
Instrument : CEQ System (Ver. 9.0.25)

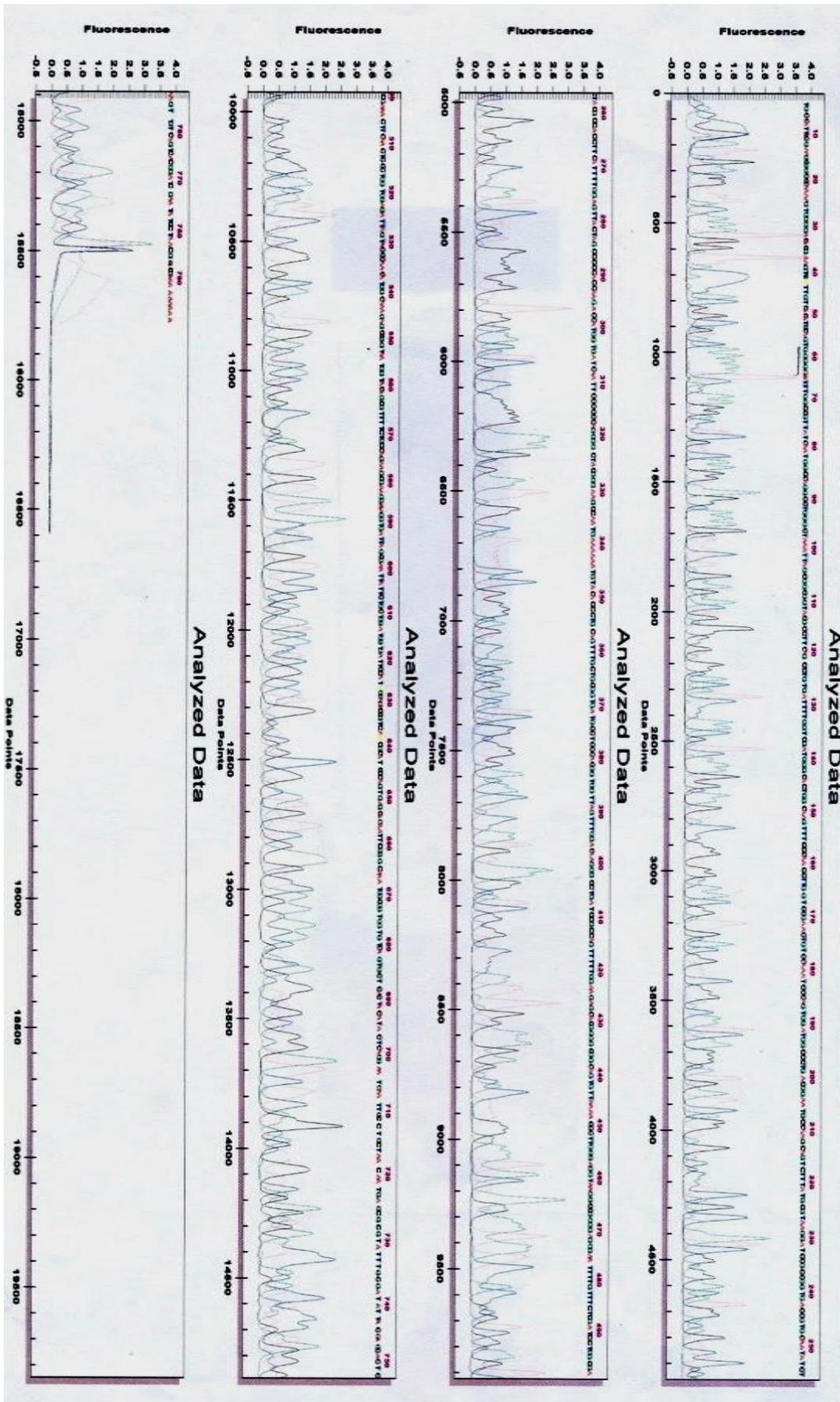


Figure-2 Electropherogram of Glutathione Synthetase Gene for *Synechocystis* "AHZ-HB-P2A"

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 compare 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 10 μ g mL⁻¹ and 100 μ g 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 (250 μ g mL⁻¹), reduction (62.14%) [17] and production of non-protein thiols, when exposed to chromium stress, as compared to other strain [7].

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