

Differential protein expression in a marine-derived *Staphylococcus* sp. NIOSBK35 in response to Arsenic (III)

Shruti Shah and Samir R. Damare*[§]

Biological Oceanography Division,
CSIR-National Institute of Oceanography,
Dona Paula, Goa - 403004.

*corresponding author: samir@nio.org

Ph. +91 832 2450447

Fax. +91 832 2450602

Abstract

Peptide mass fingerprinting of Gram-positive marine-derived *Staphylococcus cohnii* #NIOSBK35 gave us an insight into the proteins involved in conferring arsenic resistance as well as the probable metabolic pathways affected under metal stress. Analysis of the protein profiles obtained from LC/MS QToF (Liquid Chromatography-Mass Spectrometry-Quadrupole Time of Flight) resulted in the identification of 689 proteins. Further grouping of these proteins based on the arsenic concentration (0, 250, 500 and 850 ppm) and the time points (6, 9, 12, 18, 24 and 36 h) in growth phase showed that a total of 13 proteins were up-regulated while 178 proteins were down-regulated across all the concentrations and time points. Arsenic specific proteins like arsenical pump driving ATPase, ArsR family transcriptional regulator and Arsenic operon resistance repressor were found to be highly up-regulated throughout all the conditions indicating their possible involvement in the tolerance to arsenic. MBL fold metallo-hydrolase, a known stress protein, was the only protein that was up-regulated at all time points across all arsenic concentrations. Metabolic pathways like translation, carbohydrate metabolism, amino acid metabolism, membrane transport, metabolism of cofactors and vitamins, replication and repair, nucleotide metabolism along with stress proteins and hypothetical proteins were found to be significantly expressed. Our results also suggest that arsenic stress at higher levels is negatively affecting the expression of many normal functional proteins required for cell survival.

Keywords: Arsenic, *Staphylococcus cohnii*, LC/ MS QToF, Protein expression.

1. Introduction

Arsenic is a ubiquitous, 20th most occurring trace element in the earth's crust, 14th in seawater and 12th in the human body and is highly toxic to all life forms (Mandal and Suzuki 2002). It is released in the environment mainly by the volcanic activity (Smedley and Kinniburgh 2002). Arsenic exists primarily in four oxidation states— arsenate (+5), arsenite (+3) (most toxic state), arsenic (0) and arsine gas (-3) (Zhao et al. 2010; Sharma and Sohn 2009; Botes et al. 2007). Arsenic and its compounds enter the environment through surface run-offs from agricultural activities and effluent discharge (Mishra et al. 2010; Pacyna and Pacyna 2001). Arsenic is persistent in nature (Kaur et al. 2011) and toxic to living organisms and hence its removal and transformation are necessary.

The capability of bacteria for arsenic removal is beneficial in bioremediation studies. Bacteria have developed different mechanisms to transform arsenic which include arsenite oxidation, cytoplasmic arsenate reduction, respiratory arsenate reduction and arsenite methylation (Srivastava et al. 2012; Silver and Phung 2005; Mukhopadhyay et al. 2002). Therefore, understanding the role of microorganisms in the cycling of metals may lead to improved processes that are employed to detoxify contaminated sites.

Efforts have been made in understanding the underlying mechanisms with which the cells survive in the presence of high concentrations of heavy metals and its responses for the same. One of the approaches is the use of DNA microarray as a high throughput method for global analysis of gene expression to explore the cellular response to heavy metal toxicity. However, it does not correlate with the relative protein abundance in the cell and also the post-translational modifications of the proteins are not considered (Krizek et al. 2003). Proteomics in comparison to genomics is a better tool to identify differentially expressed proteins as they reflect the activity with respect to metabolic reactions and provide more information about microbial activity than functional genes or corresponding mRNAs (Bernhardt et al. 2013; Lankadurai et al. 2013; Wohlbrand et al. 2013). For a better understanding of the differentially expressed proteins, proteomic analysis such as Two-dimensional electrophoresis (2DE), protein microarray, micro-fluidics could be used. However, these techniques have their drawbacks like improper resolution of hydrophobic proteins and failure to achieve the data of the entire proteome in 2DE; Protein microarray though is a gel-free technique, it has limited affinity reagents (recombinant proteins and monoclonal antibodies) and hence, cannot be used for samples from variable sources, self-assembling of the protein microarray platform, need for improved surface chemistry for immobilization and capturing of affinity reagents and in quantitative analysis of proteins. However, these drawbacks are nullified in Liquid Chromatography coupled with Mass Spectrometry (LC-MS-MS), where the peptides and proteins are separated by the LC, and then the analytes are split based on their mass to charge ratio (m/z), then fragmented, detected and identified in the MS. Also, LC-MS/MS is widely used workbench

for quantitative expression proteomics (Tuli and Resson 2009). With this technique, it has become possible to identify and decipher the role of every protein that a microorganism expresses.

Proteomics approach has been widely used to study the response of arsenic in *Pseudomonas* (Patel et al. 2007), *Chromobacterium* (Ciprandi et al. 2012), *Staphylococcus* sp. (Srivastava et al. 2012), and *Thiomonas* (Bryan et al. 2009). There are no detailed cellular reports of *Staphylococcus* at proteomics level using modern soft ionization techniques. This study focuses on characterizing bacteria from different sites and screening for potential strains capable of biotransformation of arsenite, and the regulation of differentially expressed proteins under the influence of arsenite by peptide mass fingerprinting using mass spectrometry (LC/MS QToF) to elucidate the proteome of the organism under arsenic stress.

2. Materials and Methods

2.1 Growth characteristics of #NIOBKB35

Staphylococcus cohnii #NIOBKB35 (NCBI accession no- MH220297) isolated from the sediments of the Karwar mangroves was tolerant to 1000 ppm arsenite supplemented in Luria Bertini (LB) broth. A synchronous culture was inoculated in the experimental flasks containing 200 mL of LB broth (HiMedia, India) in seawater supplemented with arsenic concentrations 250, 500 and 850 ppm respectively. Control without arsenic was also inoculated similarly. The flasks were incubated at 28°C at 120 rpm for about 48 h (approx. till culture reached its stationary phase). Absorbance at 600 nm was measured using UV Visible Spectrophotometer (Gensys 20, Thermo Scientific, USA) every 3 h and cell pellets harvested for protein extraction as mentioned below.

2.2 Whole cell protein extraction

Based on the growth pattern, cell pellets were harvested from 15 mL of the culture broth at different time points considering the three bacterial growth phases (lag, exponential and stationary), i.e., 6, 9, 12, 18, 24 and 36 h respectively. The cell pellets were washed with sterile 1 X phosphate buffer followed by centrifugation at 14000 rpm for 10 min. The cell pellets were then re-suspended in 1 mL of urea thiourea buffer [7 M urea, 2 M thiourea, 20 mM Tris (pH 8), 4% CHAPS, 20 mM DTT (Sigma Aldrich, USA), 0.8% IPG buffer (pH 3 to 10) (GE Healthcare, Sweden), 1 X protease inhibitor cocktail (Serva, Germany)] and transferred to bead bashing tubes (zirconia beads). This cell suspension was homogenized twice at 6.5 m s⁻¹ for 45 s (FastPrep, MP Biomedicals, USA) with a gap of 5 min on ice between two homogenizations. The cell debris was separated from the supernatant (protein extract) by centrifugation at 14000 rpm at 4°C for 10 min. The protein content of the extract was quantified using Folin- Lowry method (Lowry et al. 1951). The protein extracts were stored at -20°C until further use.

2.3 In solution digestion

Protein extract (100 µg) was precipitated by adding nine times volume of methanol and incubated at room temperature for 60 min. This was followed by 20 min of centrifugation at 14000 rpm at 4°C. The protein pellet was washed with 90 % methanol and centrifuged again. Pellets were dried in a vacuum concentrator (Eppendorf, Germany) and stored at room temperature until further use. These protein pellets were subjected to in-solution trypsin digestion (Lotlikar and Damare, *In Press*). The tryptic digests were transferred to MS vials for LC-MS/MS analysis.

2.4 LC-MS/MS-based protein identification

Protein identification was carried out using in-house LC-MS QTOF facility (6538 UHD Accurate Mass QTOF LC/MS, Agilent Technologies, USA). The trypsin-digested peptides were injected (8 µL) through the auto-sampler onto the Prot ID chip 150 II 300A C18 150 mm column. Samples were run as four technical replicates. Peptides were separated using a 3 to 97 % gradient (non-linear) for 100 min (Water and 90% acetonitrile) at a flow rate of 0.4 µL min⁻¹. Formic acid (0.1 %) was used as adduct in both water and acetonitrile. The data was acquired in positive ion mode using Mass-Hunter Data Acquisition software B.06.00 (Agilent Technologies, USA). MS was performed from 200-2000 *m/z* and MS/MS from 50-2000 *m/z*. A maximum of 14 precursors were selected for each cycle with intensity over 1000. The spectral data were analyzed further using the Spectrum Mill MS Proteomics Workbench ver. B.04.01.141 (Agilent Technologies, USA). The MS-MS data was searched against species-specific protein database in NCBI (*Staphylococcus cohnii*, Txid- 29382 enlisting 39480 proteins accessed on 5th Jan, 2017) to identify the proteins expressed. Precursor and product mass tolerance cut-off given was 50 and 100 respectively while a maximum of two missed cleavages by trypsin was allowed for identification. Auto-validation was performed at 1.2% False Discovery Rate (FDR).

2.5 Proteomic change

The identified proteins were further analyzed using Mass Profiler Professional ver. 13 (GeneSpring GX, Agilent Technologies, USA) for their regulation. Primarily, the samples were grouped based on the arsenic concentration they were subjected to (0, 250, 500 and 850 ppm). Secondly, the samples were grouped based on the time points at which cell pellets were harvested for protein extraction. For analyzing the regulation of proteins, a cut-off of 2.0 for fold change was applied at a p-value of 0.2. Venn diagram was created by comparing up-regulation of proteins expressed at 250 ppm v/s 0 ppm, 500 ppm v/s 0 ppm and 850 ppm v/s 0 ppm (same was done for comparing down-regulation of proteins). Similarly, Venn diagrams were also created for the time point comparison of the protein regulation, i.e., 250 ppm v/s 0 ppm, 500 ppm v/s 0 ppm and 850 ppm v/s 0 ppm at 6, 9, 12, 18, 24 and 36 h respectively.

The Venn diagram revealed the list of proteins that were commonly up-regulated and down-regulated and also the list of proteins that were unique to a particular condition. The heat maps for the proteins expressed at different arsenic concentrations (250, 500 and 850 ppm) as compared to the proteins expressed in the absence of arsenic were constructed. The relative intensities in the heat map correspond to the respective protein regulation, i.e., higher the colour intensity, higher was the fold change value for up or down-regulation of proteins (fold change cut-off used was 2). All the up and down-regulated proteins were categorized based the metabolic pathway that the protein is involved in as per the KEGG (Kyoto Encyclopedia for Genes and Genomes) pathway ver. 37 database.

3. Results

3.1 Protein identification

A total of 689 proteins (1.7% out of the total proteins listed in NCBI database under *Staphylococcus cohnii*) were identified across all arsenic concentrations (0, 250, 500 and 850 ppm) across all time points, i.e., 6, 9, 12, 18, 24 and 36 h.

3.2 Proteomic change

3.2.1 Effect of concentration

All the 689 proteins identified across all the conditions in the Spectrum Mill software were analyzed further in the MPP software. However, a fold change cut-off of 2.0 resulted in filtering out of 40 proteins. Hence, only 649 proteins were analyzed for their regulation pattern and the metabolic pathways affected. On comparing the proteins expressed at 250 ppm with those in the absence of arsenic, a total of 147 proteins were found to be up-regulated. Similarly, 114 and 42 proteins were found to be up-regulated at 500 and 850 ppm arsenic respectively as compared to proteins expressed in the absence of arsenic. Correspondingly, 236, 285 and 406 proteins were found to be down-regulated at 250, 500 and 850 ppm when compared with the proteins expressed in the absence of arsenic. Of the total proteins up-regulated at 250, 500 and 850 ppm, 13 proteins were up-regulated across all the above three conditions, while 118, 80 and 22 proteins were unique to 250, 500 ppm and 850 ppm respectively (Fig.1). Also, out of the total down-regulated proteins, 178 proteins were found to be common across all three conditions, i.e., 250, 500 and 850 ppm arsenic as compared to proteins expressed in the absence of arsenic. Amongst the down-regulated proteins, 11, 14 and 100 were unique to 250, 500 and 850 ppm arsenic as compared to proteins expressed in the absence of arsenic.

Fig. 2a and 2b show heat maps as constructed in MPP software giving a clear picture of the regulation of proteins across all the arsenic concentrations.

The 13 up-regulated proteins (Table 1) across the three different arsenic concentrations include 50S ribosomal protein L27, glyoxal reductase, pyridine nucleotide-disulfide oxidoreductase, MBL metallo-hydrolase, dihydroneopterin aldolase, 3-oxoacyl-ACP synthase, excinuclease ABC subunit B, MarR family transcriptional regulator, phenylalanine-tRNA ligase subunit beta, hypothetical protein BSF33_00540 and arsenic specific proteins.

Proteins like DNA starvation protein, membrane protein, lipoprotein, metal ion transporting ATPase (Lead, cadmium, zinc and mercury transporting ATPase), zinc-dependent dehydrogenase, UMP-kinase, organic hyperoxide resistance protein, iron citrate ABC transporter substrate binding protein, intracellular adhesion protein, ABC transporter ATPase, monooxygenase, outer surface protein, autolysin Atl were found to be up-regulated only at 250 ppm arsenic. 2,5-diketo-D-glucuronic acid, 3-hydroxyl-3-methylglutaryl-CoA reductase, ComE operon protein 1, manganese ABC transporter substrate-binding protein, mercuric reductase, metallophosphoesterase, pyridoxal biosynthesis protein, redox-regulated ATPase YchF zinc ABC transporter substrate binding protein were among the 80 proteins that were up-regulated only at 500 ppm arsenic. Similarly, MerR family transcriptional regulator, Mn²⁺/Zn²⁺ ABC transporter ATPase, glycine/betaine ABC transporter substrate-binding protein, cell cycle regulation protein HIT, cold shock protein and multidrug transporter ATPase are among the 22 proteins that were uniquely up-regulated only at 850 ppm arsenic concentration.

The number of commonly down-regulated proteins is quite high as compared to the number of up-regulated proteins (Table 2 and Supplementary Data). These 178 proteins consisted mainly of the ribosomal proteins (30S ribosomal protein S7, 50S ribosomal protein L23) and other proteins like homoserine dehydrogenase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, DNA-binding protein, ATP synthase F0F1 subunit alpha, alkaline shock protein 23, catalase, cysteine synthase, dihydrolipoyl dehydrogenase, endoribonuclease L-PSP, ferritin, formate C-acetyltransferase, general stress protein, GNAT family N-acetyltransferase, lipid hyperoxide peroxidase, malate: quinone oxidoreductase, nitric oxide dioxygenase, peroxiredoxin, phosphopyruvate hydratase, serine hydroxymethyl transferase, serine-tRNA ligase, superoxide dismutase, thioredoxin, transketolase, universal stress protein UspA and translational proteins such as translation elongation factor Ts, translation initiation factor IF-3 and were found to be highly down-regulated.

Proteins like 50S ribosomal protein L6, phosphoenolpyruvate carboxykinase, dihydrolipoamide succinyltransferase, nucleoside-diphosphate kinase, glycine-tRNA ligase were among the 11 proteins that were found to be down-regulated only at 250 ppm arsenic (Supplementary Data). Similarly, malate dehydrogenase (acceptor), cell-cycle regulation protein HIT, PTS glucose transporter subunit IIA, aldehyde dehydrogenase, NADH dehydrogenase, transglycosylase, 30S ribosomal protein S18 were among the 14 unique proteins down-regulated at 500 ppm arsenic. About 15 ribosomal proteins along

with DNA polymerase III subunit beta, DNA- directed RNA polymerase subunit alpha and beta, homoserine kinase, N-acetylmuramoyl-L-alanine amidase, UDP-glucose 4-epimerase, acetate kinase, glutamate-1-semialdehyde-2,1-aminomutase, glutathione-dependent formaldehyde dehydrogenase, glycine dehydrogenase, phosphoglucosamine mutase, succinate CoA ligase sub-unit beta were among the 100 uniquely down-regulated proteins at 850 ppm arsenic concentration (Supplementary Data).

3.2.2 Temporal expression of proteins in conjecture with different concentrations of arsenite

There was only one protein up-regulated at 6, 9 and 12 h respectively across 250, 500 and 850 ppm arsenite when compared to proteins expressed in the absence of arsenic, while there were 4, 6 and 2 proteins up-regulated at 18, 24 and 36 h respectively across 250, 500 and 850 ppm. Also, it was observed that 72, 119, 70, 42, 85 and 92 proteins were down-regulated at 6, 9, 12, 18, 24 and 36h respectively across 250, 500 and 850 ppm when compared to proteins expressed in the absence of arsenic (Supplementary Data).

MBL-fold metallo-hydrolase was up-regulated across all the three different concentrations at all the time points, i.e., 6, 9, 12, 18, 24 and 36 h respectively. While, arsenical pump driving ATPase was found to be up-regulated at 18, 24 and 36 h respectively. In addition to that, at 18 h, two more proteins viz., arsenic resistance operon repressor and ferritin were found to be highly up-regulated. At 24 h, along with arsenic operon repressor proteins, pyridine nucleotide-disulfide oxidoreductase, dihydroepterinaldolase and phosphopyruvate hydratase were also found to be up-regulated.

The number of commonly down-regulated proteins across the different arsenic concentrations at different time points was more as compared to the up-regulated proteins. At 6 h, six ribosomal proteins along with proteins such as ATP-dependent DNA helicase, DNA polymerase III subunit beta, UDP-N-acetylglucosamine 1-carboxyvinlytransferase, dihydrolipoyl dehydrogenase, PTS beta-glucoside transporter subunit IIBC, iditol 2-dehydrogenase, nucleoside-diphosphate kinase, prolyl-tRNA synthetase were down-regulated across the three arsenic concentrations. Similarly, at 9 h, the 50S ribosomal proteins (L15, L18, L23, L3, L31 and L6) were down-regulated along with ATP synthase F0F1 subunit alpha and gamma, cell division protein FtsA, cysteine synthase, elongation factor P, fructose 1,6- bisphosphatase. Lactate dehydrogenase, peptide deformylase, tetrahydrodipicolinateacetyltransferase, 6-phosphogluconolactonase, aminopeptidase T, chorismate synthase, prolyl-tRNA synthetase were commonly down-regulated proteins at 12 h. Similarly, at 18 h, proteins like dihydroxyacetone kinase, non-canonical purine NTP pyrophosphatase, peptidase M28, 6-phosphogluconolactonase, glutamate ligase, thioredoxin reductase, heat shock protein 60 family chaperone GroEL were found to be down-regulated. Likewise, at 24 h, 1,4-dihydroxy-2-naphthoyl-CoA synthase, RNA- binding protein, beta-ketoacyl- ACP reductase, delta-aminolevulinic acid dehydratase, pyruvate carboxylase, GNAT family N-acetyltransferase, Translation elongation factor Ts, beta-

ketoacyl-ACP reductase, lipid hydroperoxide peroxidase, signal transduction protein TRAP were found to be down-regulated. Also, at 36 h, proteins like NAD-dependent dehydratase, phosphopentomutase, butanediol dehydrogenase, threonine synthase, ATP synthase F0F1 subunit delta, ATP-dependent DNA helicase RecG, D-alanine aminotransferase, cytochrome ubiquinol oxidase subunit I, mannitol-1-phosphate 5-dehydrogenase, molecular chaperone GrpE, teichoic acid ABC transporter ATP-binding protein, thioredoxin were found to be down-regulated.

3.3 Arsenic specific proteins

Arsenic specific proteins also called as arsenical resistance proteins such as ArsR family transcriptional regulator was found to be 17.10, 17.90, 17.46 times up-regulated at 250, 500 and 850 ppm arsenic respectively. Similarly, arsenical pump driving ATPase was 19.49, 17.99, 17.40 times and arsenic resistance operon repressor was 19.97, 19.87, 18.99 times up-regulated at 250, 500 and 850 ppm arsenic (Table 1). However, arsenical pump membrane protein was 17.10 times up-regulated only at 500 ppm arsenic at 18 h. Also, arsenate reductase was up-regulated with less than 2.0 fold change values at 6, 12, and 24 h at 500 ppm As and 36 h at 850 ppm arsenic concentration (Supplementary data).

3.4 Metabolic pathways

All the 649 proteins identified were classified as per the metabolic pathways they were involved (Fig. 3). The maximum proteins (88) were grouped as hypothetical proteins (proteins whose functions are hypothesized), carbohydrate metabolism (68), stress proteins (63) and amino acid metabolism (63) (Fig. 3).

4. Discussion

Arsenic is a widely studied heavy metal due to its toxic nature and persistence in the environment. There are many reports with respect to the accumulation of arsenic compounds in fungi, yeasts, and plants. Microorganisms play an essential role in the geochemical cycle of arsenic. Many free-living organisms possess metabolic mechanisms to resist arsenic. The activity of microbes strongly influences the bioavailability of arsenic in the environment. Hence, understanding of microbial reactions to arsenic is fundamental to develop improved bioremediation of arsenic-contaminated environments. Arsenic operon in bacteria is also well elucidated, and the corresponding proteins are known too. However, not much is known about the bacterial detoxification strategies and the biotransformation potential.

In this study, we focused on the regulation of differentially expressed proteins that expressed under arsenite stress and the metabolic pathways involved in bacterial resistance to arsenic. Arsenite is water soluble and most toxic form of arsenic, to which *Staphylococcus* sp. #NIOBK35 was tolerant at very high concentrations (1000 ppm). Arsenic tolerance in bacteria is usually mediated by the gene products of *ars* operon. *ars* genes can be present on either the genome (gram negative as well as gram-positive

prokaryotes) or the plasmid (mostly gram-positive prokaryotes) (Carlin et al. 1995; Rosen 2002). The simplest form of ars operon that confers arsenic tolerance in prokaryotes consists of a set of three co-transcribed genes *arsR*, *arsB* and *arsC* (Silver and Phung 2005). These genes encode transcriptional repressor protein, arsenite efflux pump protein and arsenate reductase in *Staphylococcus aureus* (Ji and Silver 1992; Silver and Phung 1996; Mateos et al. 2006; Páez-Espino et al. 2009). Similar results were obtained in our study where the arsenic specific proteins such as ArsR family transcriptional regulator, arsenical pump-driving ATPase, arsenic resistance operon repressor were highly up-regulated in the presence of arsenic which indicates up-regulation of the corresponding genes. Different organisms have different gene clusters which confer arsenic resistance or may be responsible for arsenic metabolism or both resistance and metabolism of arsenic (Andres and Bertin 2016). In some organisms, along with *arsRBC*, some supplementary genes may also be present, viz., *arsA* and *arsD* which code for anion stimulated ATPase and As (III) chaperone (Silver and Phung 2005a; Páez-Espino et al. 2009, Rosen 1999). *ArsRDABC* operon is found to confer arsenic resistance in *E.coli* R733 and was present on the plasmid (Mateos et al. 2006, Páez-Espino et al. 2009). Some other arsenic operons include *aioBA* operon in *Alcaligenes faecalis* which consists of 21 genes which confer resistance as well metabolize arsenic (Silver and Phung 2005b). Arsenic operon in prokaryotes consists of different arsenic gene clusters responsible for arsenic resistance. For example, *Campylobacter jejuni* RM1221 has *arsP*, *arsR*, *arsC*, and *acr3*, *Thiomonas* sp. 3As has *arsR*, *glo*, *arsC* and *arsB* genes (Li et al. 2014; Andreas and Bertin 2016).

The proteome results obtained in our study were compared with the transcriptome data in other organisms as no studies have reported proteome or transcriptome change with respect to tolerance to arsenic in *Staphylococcus cohnii*. The transcriptomic analysis in *Herminiimonas arsenicoxydans* showed that induction and expression of the genes involved in protein synthesis, central intermediary metabolism, energy metabolism, transport and cellular processes (Cleiss-Arnold et al. 2010). Zhang et al. (2016) have reported adaptive response of Enterobacteriaceae strain LSJC7 to arsenate exposure by transcriptomic analysis where the amino acid metabolism, protein folding, protein metabolic process, homeostatic process, carbon metabolism and nitrogen metabolism were the pathways which were found to be overexpressed.

Proteomic analyses of various bacteria under arsenic stress have shown the presence of unique and arsenic specific proteins (Sacheti et al. 2014). Arsenic even though is a non-redox active metal, is known to generate oxidative stress and lipid peroxidation in organisms (Ahsan et al. 2009) which was also observed in our study. Proteins like monooxygenase were up-regulated at 250 ppm, while down-regulated at 500 ppm arsenic, mercuric reductase was up-regulated only at 500 ppm, and NAD(P)-

dependent oxidoreductase was up-regulated only at 850 ppm arsenic, NAD(P)H- dependent oxidoreductase was up-regulated only at 250 ppm arsenic; oxidoreductase was up-regulated only at 250 ppm, while down-regulated at 500 and 850 ppm arsenic. Superoxide dismutase, peroxiredoxin and alkyl hydroperoxide reductase subunit F were down-regulated across all three arsenic concentrations. This is similar to the findings reported by Jones et al. (2007) where they have shown the generation of superoxide under normal conditions in root hair development process in *Arabidopsis* roots. Under chromate stress, *S. aureus* has been reported to show mild up-regulation of RNA polymerase sigma factors to cope with oxidative stress (Teitzel et al. 2006). Proteins like heat shock proteins (HSPs) are known to be up-regulated under stress conditions (Visioli et al. 2010; Chen et al. 2009). However, it is quite interesting to note that in current study heat shock protein 60 family chaperone was commonly down-regulated across all the concentrations indicating that they may not be playing any role in arsenic tolerance. But thioredoxin-disulfide reductase which is involved in the metabolism of other amino acids was highly up-regulated under the metal stress.

Metal-related proteins like MBL fold metallo-hydrolase were highly up-regulated which could be the probable mechanism for the survival of the organism. The maximum proteins that were affected due to arsenic were involved in translation, amino acid metabolism and carbohydrate metabolism followed by stress proteins, membrane transport proteins, metabolism of cofactors and vitamins, energy metabolism. Majority of the membrane transport proteins such as phosphate transporters were found to be down-regulated which could be due to the interference of arsenic with the phosphate transport system (Mehrag and Macnair 1992; Meharg and Harley-Whitaker 2002).

Zhao et al. (2010) reported that the arsenite binds to the sulfhydryl groups of proteins affecting their structure or activity. This could be the possible reason for the down-regulation of most of the sulfur-containing proteins like cysteine synthase, methionine sulfoxide reductase B which are involved in normal cell functioning. This indicates that the arsenic has a toxic effect on the normal cell functioning and to survive the metal stress, there are either up or down-regulation of proteins.

Proteins involved in lipid metabolism like 3- hydroxy acyl- CoA dehydrogenase, 3- oxoacyl- ACP synthase, beta-ketoacyl- [acyl-carrier-protein] synthase II were found to be highly up-regulated across all three concentrations (250, 500 and 850 ppm) of arsenic when compared to proteins expressed in the absence of arsenic which indicates that the acylated proteins are affected by the metal stress. Similarly, dihydroneopterinaldolase (metabolism of cofactors and vitamins), excinuclease ABC subunit B (replication and repair), glyoxalreductase (carbohydrate metabolism), MarR family transcriptional regulator (transcription), aspartate aminotransferase (translation) were found to be up-regulated across all three concentrations. Proteins like type I glyceraldehyde- 3- phosphate dehydrogenase (carbohydrate

metabolism), succinate CoA ligase subunit alpha (energy metabolism) and most of the ribosomal proteins (translation), uracil phosphoribosyltransferase (nucleotide metabolism), PTS glucose transporter subunit IIA (membrane transport) were found to be highly down-regulated which shows that the primary metabolic functions of the cell were affected by the presence of metal in their environment.

Most of the proteins involved in normal cell functioning like amino acid metabolism, lipid metabolism, membrane transport, metabolism of cofactors and vitamins, nucleotide metabolism, replication, and repair were found to be up-regulated at 250 ppm arsenic while down-regulated at 500 and 850 ppm of arsenic. This could be possible because the higher concentration of arsenic interfered more with the protein functions than the bacterial repair mechanisms could reverse the damage. However, up-regulation of arsenic specific proteins indicate that these proteins help the organism for tolerance and survival in the environments containing high arsenic concentrations.

5. Conclusion

In this decade, a significant amount of information has been published on arsenic metabolism in bacteria and the genes involved in these processes and their regulation. In the present study, most of the proteins involved in normal metabolic functions of the cell were found to be down-regulated indicating that the cell metabolism is hampered in the presence of arsenic. Increase in the concentrations of arsenic, however, showed no significant difference in the expression of the proteins. The proteins involved in tolerance mechanisms and arsenic-related proteins, i.e., arsenical resistance proteins were found to be strongly up-regulated. The identification of differentially expressed proteins under arsenic stress improves our understanding of the cellular response of the arsenic-tolerant *Staphylococcus* sp.

Acknowledgement

The authors wish to thank the Director of CSIR-National Institute of Oceanography and Head, Biological Oceanography Division for their support and providing all facilities to carry out this study. The authors gratefully acknowledge the funding support received from the project BSC0111 funded by CSIR, India. We are also thankful to the anonymous reviewers whose suggestions and constructive comments helped us to improve the manuscript. This manuscript is NIO contribution number #####.

Conflict of interest

Both the authors state that there is no conflict of interest.

References

1. Ahsan N, Renaut J, Komatsu A (2009) Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. *Proteomics* 9, 2602–2621. doi:10.1002/pmic.200800935.
2. Andreas J, Bertin PN (2016) The microbial genomics of arsenic. *FEMS Microbiol Rev* 40, 299-322.
3. Bernhardt J, Michalik S, Wollscheid B, Volker U, Schmidt F (2013) Proteomics approaches for the analysis of enriched microbial sub-populations and visualization of complex functional information. *Curr Opin Biotechnol* 24, 112-119.
4. Botes E, Heerden EV, Litthauer D (2007) Hyper-resistance to arsenic in bacteria isolated from an antimony mine in South Africa. *S Afr J Sci* 103, 279-281.
5. Bryan CG, Marchal M, Battaglia-Brunet F, Kugler V, Lemaitre-Guillier C, Licvremont D, Bertin PN, Arsene-Ploetze F (2009) Carbon and arsenic metabolism in *Thiomonas* strains: differences revealed diverse adaptation processes. *BMC Microbiol* 9, 1471-2180.
6. Carlin A, Shi WP, Dey S, Rosen BP (1995) The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. *J Bacteriol* 177, 981-986.
7. Chen DS, Chan KM (2009) Changes in the protein expression profiles of the Hepa- T1 cell line when exposed to Cu²⁺. *Aquat Toxicol* 94 (3), 163- 176. doi:.10.1016/j.aquatox.2009.06.014.
8. Ciprandi A, Baradna RA, Santos AV, Goncalves EC, Carepo MSP, Schneider MPC, Silva A (2012) Proteomic response to arsenic stress in *Chromobacterium violaceum*. *J Integr OMICS* 2, 69-73.
9. Cleiss-Arnold J, Koechler S, Proux C, Fardeau ML, Dillies MA, Coppee JY, Arsène-Ploetze F, Bertin PN (2010) Temporal transcriptomic response during arsenic stress in *Herminiimonas arsenicoxydans*. *BMC Genomics* 11, 709.
10. Ji, G, Silver S (1992) Regulation and Expression of the Arsenic Resistance Operon from *Staphylococcus aureus* Plasmid pI258. *J Bacteriol* 174 (11), 3684-3694. doi:10.1128/jb.174.11.3684-3694.1992.
11. Kaur S, Kamli MR, Ali A (2011) Role of arsenic and its resistance in nature. *Can J Microbiol* 57, 769-774. doi: 10.1139/w11-062.
12. Krizek BA, Prost V, Joshi RM, Stoming T, Glenn TC (2003) Developing transgenic Arabidopsis plants to be metal-specific indicators. *Environ Toxicol Chem* 22, 175-181. doi: 10.1002/etc.5620220123.
13. Lankadurai BP, Nagato EG, Simpson MJ (2013) Environmental metabolomics: an emerging approach to study organism responses to environmental stressors. *Environ Rev* 21, 180-205
14. Li X, Zhang L, Wang G (2014) Genomic evidence reveals the extreme diversity and wide distribution of the arsenic-related genes in Burkholderiales. *PLoS ONE* 9(3): e92236. doi:10.1371/journal.pone.0092236.
15. Lotlikar NP, Damare SR (2018) Variability in protein expression in a marine-derived *Purpureocillium lilacinum* subjected to salt and chromium stresses. *Ind J Microbiol. In Press* doi: 10.1007/s12088-018-0733-1
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193 (1), 219- 275. PMID: 14907713.
17. Mandal BK, Suzuki KT (2002) Arsenic round the world: a review. *Talanta*. 58, 201–235. doi: 10.1016/S0039-9140(02)00268-0.
18. Mateos LM, Ordóñez E, Letek M, Gil JA (2006) *Corynebacterium glutamicum* as a model bacterium for the bioremediation of arsenic. *Int Microbiol* 9 (3), 207- 215.
19. Meharg A.A, Macnair MR (1992) Suppression of the high- affinity phosphate uptake system- a mechanism of arsenate tolerance in *Holcuslanatus* L. *J Exp Bot* 43, 519–524. doi:10.1093/jxb/43.4.519.

20. Meharg AA, Hartley-Whitaker J (2002) Arsenic uptake and metabolism in arsenic resistant and non-resistant plant species. *New Phytol* 154, 29–43. doi:10.1046/j.1469-8137.2002.00363.x.
21. Mishra SP, Das M, Dash UN (2010) Review on adverse effects of water contaminants like arsenic, fluoride and phosphate and their remediation. *J Sci Ind Res* 69, 249-253.
22. Mukhopadhyay R, Rosen BP, Phung LT, Silver S (2002) Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol Rev* 26, 311-325. doi:10.1111/j.1574-6976.2002.tb00617.x.
23. Pacyna JM, Pacyna EG (2001) An assessment of global and regional emissions of trace metals to the atmosphere from anthropogenic sources worldwide. *Environ Rev* 9, 269-298. doi: 10.1139/a01-012.
24. Páez- Espino D, Tamames J, de Lorenzo V, Cánovas D (2009) Microbial responses to environmental arsenic. *Biometals* 22, 117-130. doi:10.1007/s10534-008-9195-y
25. Patel P, Goulhen F, Boothman C, Gault A, Charnock J, Kalia K, Lloyd J (2007) Arsenate detoxification in a *Pseudomonad* hypertolerant to arsenic. *Arc Microbiol* 187, 171-183. PMID: 17061211.
26. Sacheti P, Bhonsle H, Patil R, Kulkarni MJ, Srikanth R, Gade W (2013) Arsenomics of *Exiguobacterium* sp. PS (NCIM 5463). *RSC Adv* 3, 9705-9713.
27. Sharma VK, Sohn M (2009) Aquatic arsenic: toxicity, speciation, transformations, and remediation. *Environ Int* 35, 743–759.
28. Silver S, Phung LT (1996) Bacterial Heavy Metal Resistance: New Surprises. *Ann Rev Microbiol* 50, 753-789. doi:10.1146/annurev.micro.50.1.753.
29. Silver S, Phung LT, (2005a) A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. *J Ind Microbiol Biotechnol* 32, 587-605. doi: 10.1007/s10295-005-0019-6.
30. Silver S, Phung LT, (2005b) Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. *Appl Environ Microbiol* 71, 599-608. doi:10.1128/AEM.71.2.599-608.2005
31. Smedley PL, Kinniburgh DG (2002) A review of the source, behaviour and distribution of arsenic in natural waters. *Appl Geochem* 17, 517-568.
32. Srivastava S, Verma PC, Singh A, Mishra M, Singh N, Sharma N (2012) Isolation and characterization of *Staphylococcus* sp. strain NBRIEAG-8 from arsenic contaminated site of West Bengal. *Appl Microbiol Biotechnol* 95, 1275-1291. doi: 10.1007/s00253-012-3976-5.
33. Teitzel GM, Geddie A, Susan K, Kirisits MJ, Whitley M, Parsek MR (2006) Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J Bacteriol* 188, 7242-7256.
34. Tuli L, Ransom HW (2009) LC-MS Based Detection of Differential Protein Expression. *Journal of Proteomics and Bioinformatics* 2, 416-438. doi: 10.4172/jpb.1000102.
35. Visioli G, Marmiroli M, Marmiroli N (2010) Two- dimensional Liquid Chromatography Technique Coupled with Mass Spectrometry Analysis to Compare the Proteomic Response to Cadmium Stress in Plants. *J Biomed Biotechnol* 2010: 567510. doi:10.1155/2010/567510.
36. Wohlbrand L, Trautwein K, Rabus R (2013) Proteomic tools for environmental microbiology – a road-map from sample preparation to protein identification and quantification. *Proteomics* 13, 2700-2730.
37. Zhang Y, Chen S, Hao X, Su JQ, X X, Yan Y, Zhu YG, Ye J (2016) Transcriptomic analysis reveals adaptive responses of an Enterobacteriaceae strain LSJC7 to arsenic exposure. *7*, 636 doi: 10.3389/fmicb.2016.00636.
38. Zhao FJ, McGrath SP, Meharg AA (2010) Arsenic as a food chain contaminant: mechanism of plant uptake and metabolism and mitigation strategies. *Ann Rev Plant Biol* 61, 535–559. doi: 10.1146/annurev-arplant-042809-112152.

Legend to figures

Figure 1. (i) Venn diagram showing the up-regulation of proteins. (ii) Venn diagram showing the down-regulation of proteins. (A- List of proteins from 250 ppm against 0 ppm As, B- List of proteins from 500 ppm against 0 ppm As, C- List of proteins from 850 ppm against 0 ppm As).

Figure 2. (a) Heat map showing the up-regulation of proteins at 250, 500 and 850 ppm As against 0 ppm (b) Heat map showing the down-regulation of proteins at 250, 500 and 850 ppm As against 0 ppm.

The color range from 0 to 20 was used where 0 indicated lowest intensity and 20 indicated highest intensity in the protein expression. Gray color in the heat map indicated absence of the particular protein at that respective concentration. The boxed proteins were expressed at all the As concentrations.

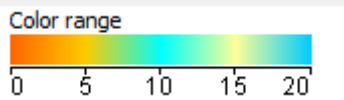


Figure 3. Pie chart depicting the metabolic classification of the identified proteins. The numbers in the bracket denote the total proteins identified for each category

Fig.1

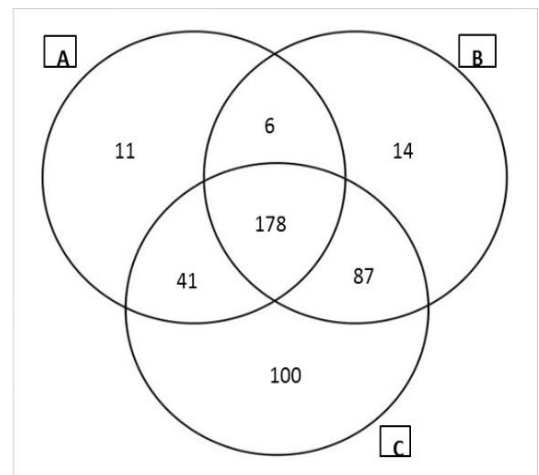
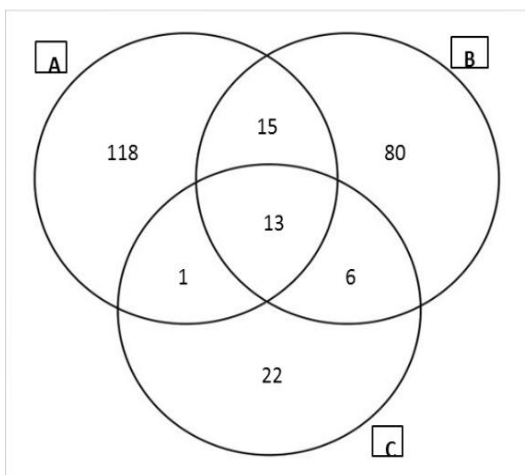




Fig2a_k35_up regulated

Fig. 3

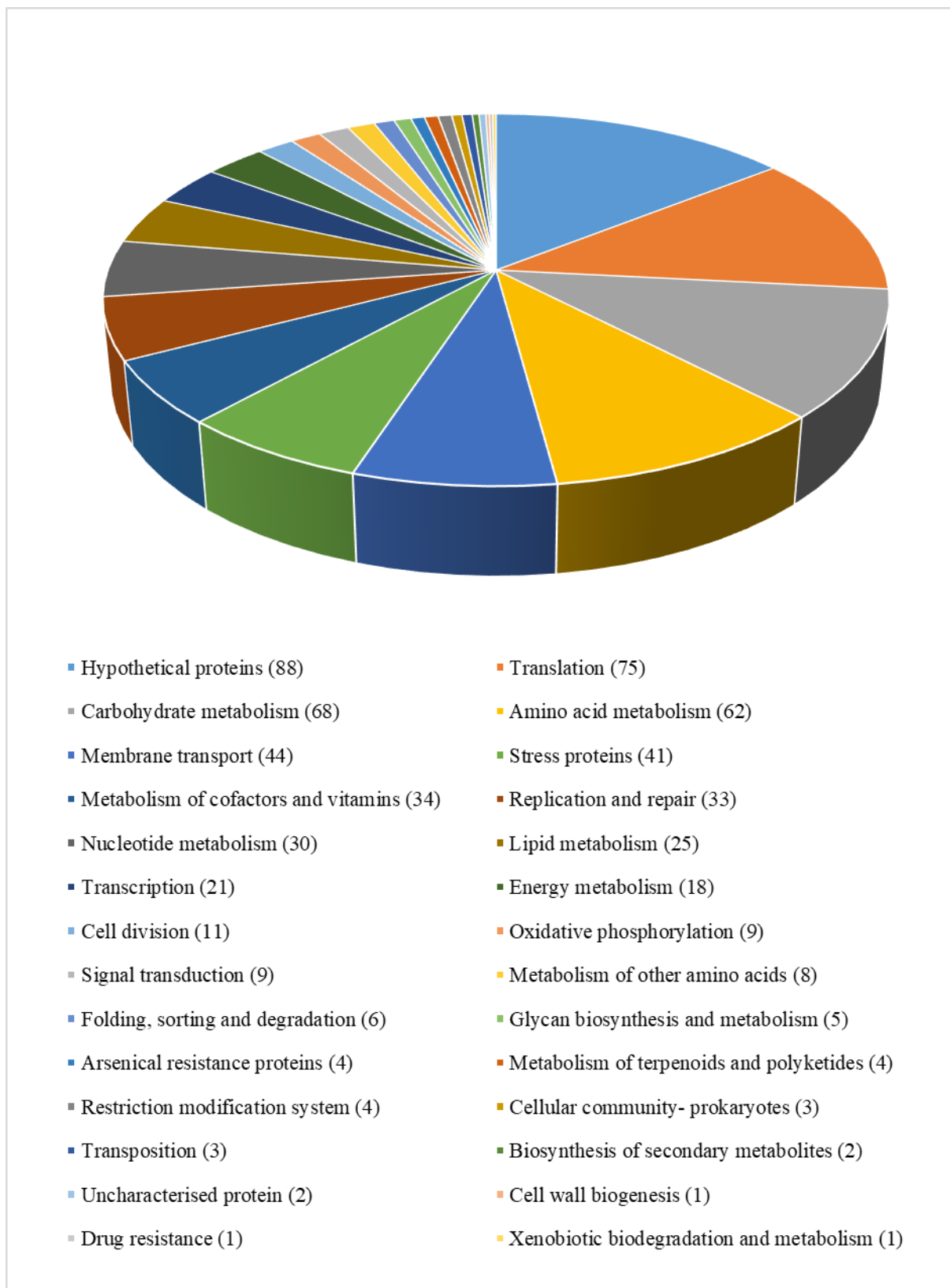


Table 1: List of all the proteins that were commonly up-regulated in *Staphylococcus* sp. NIOSBK35 across varying arsenic concentrations and their fold change values

Protein	Fold change at the arsenic concentration (ppm) as compared to proteins expressed in the absence of arsenic			Pathway involved (KEGG)	Function	Accession number (NCBI)
	250	500	850			
3-oxoacyl-ACP synthase	15.656	15.566	16.422	Lipid metabolism	Fatty acid biosynthesis	1032137338
50S ribosomal protein L27	2.791	2.538	1.344	Translation	Ribosomal proteins	1032137058
Arsenic resistance operon repressor	19.967	19.865	18.990	Arsenical resistance protein	Arsenic resistance	1101914326
Arsenical pump-driving ATPase	19.491	17.996	17.394	Arsenical resistance protein	Arsenic resistance	815782762
ArsR family transcriptional regulator	17.097	17.898	17.459	Arsenical resistance protein	Arsenic resistance	1101914329
Dihydroneopterin aldolase	18.176	17.961	17.812	Metabolism of cofactors and vitamins	Folate biosynthesis	1101916492
Excinuclease ABC subunit B	17.513	17.015	17.582	Replication and repair	Nucleotide excision repair	1032203995
Glyoxal reductase	1.217	3.637	4.507	Carbohydrate metabolism	Pentose and glucouronate interconversions	1032139056
MarR family transcriptional regulator	16.430	16.640	15.589	Transcription	Transcriptional factors-prokaryotic type	1101906693
MBL fold metallo-hydrolase	18.026	18.704	17.868	Stress proteins	Stress protein	1113037530
Phenylalanine--tRNA ligase subunit beta	18.151	17.862	18.478	Translation	Aminoacyl tRNA biosynthesis	748779103
Pyridine nucleotide-disulfide oxidoreductase	18.507	19.043	17.894	Energy metabolism	Oxidative phosphorylation	815782763
Hypothetical protein BSF33_00540	3.812	3.851	3.116	Hypothetical proteins	Hypothetical proteins	1113040740

Table 2: List of a select proteins that were commonly down-regulated in *Staphylococcus* sp. NIOSBK35 across varying arsenic concentrations and their fold change values

Protein	Fold change at the arsenic concentration (ppm) as compared to proteins expressed in the absence of arsenic			Pathway involved (KEGG)	Function	Accession number (NCBI)
	250	500	850			
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-1.878	-3.308	ND	Amino acid metabolism	Glycine, serine and threonine metabolism	748778761
30S ribosomal protein S7	-1.008	-1.400	-2.504	Translation	Ribosomal proteins	1032139017
50S ribosomal protein L23	-1.067	-1.263	ND	Translation	Ribosomal proteins	1032204812
Alkaline shock protein 23	-1.084	-2.382	-1.990	Stress proteins	Stress proteins	1101916547
ATP synthase FOF1 subunit alpha	-1.348	-1.199	-3.156	Energy metabolism	Oxidative phosphorylation	1072350166
Catalase	-1.068	-1.640	-2.857	Amino acid metabolism	Tryptophan metabolism	1113041128
Cysteine synthase	-1.197	-1.415	-2.622	Amino acid metabolism	Cysteine and methionine metabolism	1032202317
Dihydrolipoyl dehydrogenase	-1.212	-2.310	-3.588	Carbohydrate metabolism	Citrate cycle	1113041335
DNA-binding protein	-1.944	-2.350	-1.80	Replication and repair	DNA replication	1032139654
Elongation factor Ts	-1.562	-1.854	-2.512	Translation	Ribosomal proteins	1032139251
Endoribonuclease L-PSP	-1.793	-2.273	ND	Nucleotide metabolism	Pyrimidine metabolism	1101907053
Ferritin	-5.500	-1.290	-2.208	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism	1032138405
Formate C-acetyltransferase	-3.906	ND	ND	Carbohydrate metabolism	pyruvate metabolism	1113040684

General stress protein	-1.211	-1.255	-3.008	Stress proteins	Stress protein	1032203216
GNAT family N-acetyltransferase	-1.458	-1.375	ND	Amino acid metabolism	Arginine and proline metabolism	1113041377
Homoserine dehydrogenase	-1.227	ND	ND	Amino acid metabolism	Glycine, serine and threonine metabolism	815785866
Lipid hydroperoxide peroxidase	-1.561	-1.395	-2.366	Stress proteins	Stress proteins	1032140073
Malate:quinone oxidoreductase	-1.743	-2.533	-3.699	Carbohydrate metabolism	Citrate cycle	1032202422
Nitric oxide dioxygenase	-1.261	-2.127	ND	Energy metabolism	Nitrogen metabolism	1113039696
Peroxiredoxin	-1.169	-2.214	-4.119	Metabolism of other amino acids	Glutathione metabolism	1032139694
Phosphopyruvate hydratase	-3.282	-5.130	-3.241	Carbohydrate metabolism	Glycolysis	1032137863
Serine hydroxymethyl transferase	-2.030	-2.040	-1.293	Amino acid metabolism	Glycine, serine and threonine metabolism	1032202817
Serine-tRNA ligase	-1.054	-3.111	ND	Translation	Aminoacyl tRNA biosynthesis	1102013349
Superoxide dismutase	-1.090	-1.251	-1.175	Stress proteins	Stress proteins	1032205332
Thioredoxin	-2.820	-2.443	-2.905	Metabolism of other amino acids	Selenocompound metabolism	1101915066
Transketolase	-1.192	-1.213	-3.109	Carbohydrate metabolism	Pentose phosphate pathway	1072351365
Translation initiation factor IF-3	-1.705	-1.511	ND	Translation	Translational factors-prokaryotic type	1032140041
Universal stress protein UspA	-1.279	-2.169	-2.170	Stress proteins	Stress proteins	1032140069

*Negative values indicate down regulation of proteins; ND-proteins were not detected at that arsenic concentration.