

## Differential Protein Analysis of Hexavalent Chromium Stress Response in Marine *Staphylococcus xylosus*

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### Abstract:

#### Background

Several bacteria tolerate chromate with this attribute important in bacteria intended for biotechnological applications on chromate clean-up. Marine bacteria are beginning to show promise in this field due to their range and novelty. However, the general mechanisms of chromate tolerance employed by marine bacteria remain unknown.

#### Objective

To understand differential protein expression of a marine *Staphylococcus* sp. in terms of response to hexavalent chromium stress

#### Method

In this study, protein extracted from *Staphylococcus xylosus* grown in varying chromate (Cr (VI)) conditions (50, 100, 150 and 200  $\mu\text{g mL}^{-1}$  Cr (VI)) was subject to spectral counting strategy where unlabelled protein samples were analyzed separately using the same protocol as each other, and the relative protein quantification was established by comparing the number of MS/MS spectra identified for each protein

#### Results

A total of 613 proteins detected in *S. xylosus* were differentially expressed when grown in varying Cr (VI) conditions and were grouped functionally. Response in medium with 50  $\mu\text{g mL}^{-1}$  Cr (VI) was not very different from that of the control (no Cr). Whereas, in medium with 200  $\mu\text{g mL}^{-1}$  Cr (VI), the ability of *S. xylosus* to cope with Cr stress seemed to be reduced as the number of upregulated stress tolerant proteins (4) were less than those observed in medium with 150  $\mu\text{g mL}^{-1}$  (15) or 100  $\mu\text{g mL}^{-1}$  Cr (VI) (16). Up-regulation patterns showed that superoxide removal, directed DNA repair and proteins involved in peptide mis-folding were mechanisms employed by *S. xylosus* for surviving Cr induced stress in medium with 100 and 150  $\mu\text{g mL}^{-1}$  Cr (VI).

#### Conclusions

These findings have important implications for understanding mechanisms of chromate tolerance, which provides new information of how a marine *Staphylococcus* deals with chromate toxicity.

**Keywords:** *Staphylococcus xylosus*, chromate, protein profiling

## 1. INTRODUCTION

Chromium (Cr), the 17<sup>th</sup> most abundant element in the earth's crust, is used extensively in industrial processes [1] and has been a public and regulatory concern because of its toxicity to ecosystems. Its indiscriminate disposal or dumping causes ground water contamination through dissolution and sub-surface-percolation. Although its oxidation states range from -2 to +6, only the +3 and +6 states are stable under normal environmental conditions [2] exerting toxic effects to a variety of life forms.

The mobility of soluble hexavalent chromium renders it toxic, mutagenic and carcinogenic. Among living organisms, it causes allergies, eczema and other negative effects because of its strong oxidizing nature [3]. In comparison, relative immobility of trivalent chromium [Cr(III)] makes it considerably less toxic [4].

Due to various socio-economic elements and developments on a local and global scale, as well as modifications of river flows carrying pollutants from the land to coasts and seas, the level of pollution in marine environments is steadily on the rise [5].

As Silver [6] proposed, the emergence of metal tolerant determinants seems to have been a very early event in evolution. Bioremediation technology, realizing the metabolic potential of microorganisms, aims to clean contaminated environments. Since bioremediation is an attempt to accelerate naturally occurring degradation by optimizing the growth conditions, the metabolic ability of microorganisms to mineralize or transform contaminants into less harmful substances can be used and integrated into natural biogeochemical cycles [7]. However, the sole obstacle in bioremediation *in situ* is the unfavorable or extreme conditions (temperature, pH, high salt concentration or pressure) in contaminated environments/sites.

Marine bacteria exposed to such unfavorable conditions naturally would prove useful [8]. Hence, marine bacteria capable of tolerating chromate [9], may provide an understanding of molecular mechanisms of marine bacteria involved in tolerating chromate and its detoxification, providing clues on overall effectiveness of microbial bioremediation and may help with the engineering of more effective microbes for *in situ* bioremediation.

Proteomics is a powerful tool for identifying genome-level defense mechanisms by microbes [10]. Many studies primarily focused on Cr (VI), Cd (II), and radionuclide stress by environmentally-relevant bacteria are available. For example proteome descriptions on *Shewanella* sp. [11, 12], *Caulobacter* sp. [13], *Acidiphilium* sp. [14], and *Pseudomonas* sp. [15, 16] subjected to heavy metal stress are available.

When chromate enters a bacterial cell, it leads to generation of reactive oxygen species (ROS) and may damage many cellular components, such as DNA and proteins [17 and 18]. The thioredoxin-

dependent reduction systems found to be responsible for maintaining the reducing environment of the *Escherichia coli* and *Saccharomyces cerevisiae* cytosol acts as a defense against oxidative stress. Carmel-Harel and Storz [19] proposed that, DNA repair genes also take part in the chromate resistance process [10, 20].

Most proteomic studies on Cr stress have examined bacteria from metal contaminated sites such as industrial effluents. While Cr detoxification by dissimilatory reduction is known [21], apparently the proteomic response in salt-tolerant or marine bacteria is yet to be understood. The protein profiling of a salt-tolerant *S. xylosus* under Cr (VI) exposure was carried out using a LC-MS-QToF peptide fingerprinting (PMF) approach. We identified many proteins that were differentially expressed under chromate stress in this salt-tolerant bacterium. To our knowledge, this is the first comprehensive proteomic description of *S. xylosus* subjected to Cr (VI) metal stress.

## **2. MATERIALS AND METHODS**

### **2.1. Bacterial strains, media, and materials**

A bacterial strain was isolated (identified as *Staphylococcus xylosus* using 16s rDNA gene sequencing submitted to NCBI with Acc. No. KY672997) from water sample collected at the Betim fishing jetty point (Goa, India) was used in this study. The culture was grown and maintained on seawater nutrient agar (SWNA with composition [l<sup>-1</sup>]: peptic digest of animal tissue 5.0 g, sodium chloride 5.0 g, beef extract 1.5 g, yeast extract 1.5 g, agar 15 g, distilled water 1000 mL, pH 7.4 ± 0.2) amended with 50 µg mL<sup>-1</sup> of dichromate (prepared by mixing appropriate amounts of filter sterilized potassium dichromate [K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, A.R grade from Merck] stock solution and **NA** media) with 50% seawater. Liquid cultures were grown at 30°C in nutrient broth (NB, containing [l<sup>-1</sup>] 5.0 g peptone and 3.0 g yeast extract) at pH 7.0.

### **2.2 Growth conditions and heavy metal induction**

For proteomic analysis, a single colony of the culture was inoculated in 10 mL of **NB** and grown to saturation overnight. Fresh broth was inoculated with one mL of overnight culture and monitored for growth. The cells were grown until the OD<sub>600</sub> reached 0.4 (mid-exponential phase), at which point a fixed volume (0.1%) of grown culture was used to inoculate fresh broth with (50, 100, 150 or 200 µg mL<sup>-1</sup> Cr) and without Cr (control). After a fixed period of 7h incubation, a time point during mid-log phase growth condition, cells in each (with optical densities 0.854, 0.802, 0.651, 0.422, 0.193 and 0.04 respectively for 0, 50, 100, 150 and 200 µg mL<sup>-1</sup> Cr at 600 nm) were then harvested by centrifugation at 14,000 rpm for 10 min at 4°C, washed twice with 1X **PBS**, and the resulting pellet subjected to cell lysis and protein extraction.

### ***2.3 Cell lysis and protein extraction***

The cell pellets were dissolved in 500  $\mu$ L urea thiourea buffer in bead-bashing tubes (zirconia beads) and the cell slurry homogenized at 4.5 m/s for 40 sec (FastPrep, MP Biomedicals, USA). The bacterial cells and debris were then centrifuged at 14,000 rpm for 20 min at 4°C, and supernatant was transferred to a fresh microcentrifuge tube. For extraction of residual proteins, if any, another 500  $\mu$ L urea thiourea buffer was added to the cell debris in the bead bashing tube, followed by vortexing the tube on the TurboMix<sup>TM</sup> (Scientific Industries Inc.). The supernatants were pooled to which 1:9 (v/v) 100% methanol (Fluka MS grade) was added followed by methanol precipitation [22]. The protein samples were vacuum dried for 20 min at 30°C. The dried samples were resuspended in 1 mL of TE buffer (pH 8.3) containing 10  $\mu$ L of 1X protease inhibitor (Serva, Germany) and the protein concentrations were estimated using Folin-Lowry's method [23]. The protein extract was stored at -20°C until they were analyzed.

### ***2.4 LC/MS-QToF analysis***

In-solution trypsin digestion (modified from Kinter et al. [24]) was performed to prepare the samples for **LC-MS-QToF** analysis. 50  $\mu$ L aliquots of digests were transferred to MS vials and sealed. The tryptic digested peptides were analyzed using the in-house **LC-MS-QToF** facility (6538 UHD Accurate Mass QTOF LC/MS, Agilent Technologies, USA). Sub-volumes of 8  $\mu$ L tryptic digest were injected into the auto-sampler. The LC separations were carried out using water and acetonitrile (90 % in water) on a Prot ID Chip 150 II 300A C18 150 mm column. Formic acid (0.1%) was used as adduct in both the aqueous and organic phases. The peptides were eluted using a standardized 71 min protocol. The MS/MS for the separated ions was carried over a range of 50 to 2000 m/z in positive mode at 2 ppm accuracy with nitrogen gas inside the collision cell. The spectral data was acquired using Mass Hunter Software ver. 5.0 (Agilent Technologies, USA).

### ***2.5 Protein identification***

The spectra collected through Mass Hunter were analyzed as per manufacturer's instructions using Spectrum Mill MS Proteomics Workbench ver. B.04.01.141 (Agilent Technologies, USA). The MS/MS search was done against specific sub-set database created using SwissProt database at an accuracy 50 ppm (for MS) and 100 ppm (for MS/MS). Auto validation was done on all MS searches using a false discovery rate of 1.2%, followed by the generation of peptide and protein summary files. The hits above a cutoff score of more than 1, and % **SPI** more than 10 were considered for further analysis.

### ***2.6 Statistical analyses***

Mass Profiler Professional ver. 13 (MPP, Agilent Technologies, USA) was used to analyze the data generated in Spectrum Mill. The samples were grouped as technical replicates with respect to the chromate concentrations (0, 50, 100, 150 and 200  $\mu\text{g mL}^{-1}$ ). Every entity in the sample was filtered based on the frequency of occurrence among the samples in at least one condition. The differential significance of the samples was assessed with a p-value cut-off of 0.2 and a fold-change cut-off of 2.0 when control samples (no chromate) and chromate amended samples were compared. The proteins which were satisfying the fold-change cut-off were used to create Venn diagrams to check proteins that were up or down-regulated at different chromate concentrations and those proteins common to all conditions. The proteins thus identified were used to create a functional based classification where the gene's corresponding functions were grouped, based on the Kyoto Encyclopedia of Genes and Genomes, ver. 37 (KEGG) pathways and Clusters of Orthologous Groups (COGs) of the proteins and cross-referenced with their UniProtK Accession numbers.

### 3. RESULTS

#### 3.1 Protein identification

In addition to a control (no chromate), four conditions of increasing chromate concentrations *viz.*, 50, 100, 150 and 200  $\mu\text{g mL}^{-1}$  were examined to observe dose-dependent variations in protein expression. As many as 613 proteins were identified by mass spectroscopic measurements across all samples with 83, 83, 366, 304 and 94 proteins identified respectively from this isolate grown in 0, 50, 100, 150 and 200  $\mu\text{g Cr mL}^{-1}$ . More than 50 proteins were differentially expressed under each condition including both up-regulated and down-regulated proteins. Of the 613 identified proteins, nearly 45 % (293 proteins) were unidentified and not comparable to the ones in UniProt database ([www.uniprot.org](http://www.uniprot.org)).

#### 3.2 Comparison of protein profiles between control and Cr stress treatments

Quantitative analysis of the proteomic data revealed significant changes in protein expression due to metal exposure. As many as 59, 364, 300 and 86 proteins were uniquely up-regulated in *S. xylosus* grown in medium respectively with 50, 100, 150 and 200  $\mu\text{g mL}^{-1}$  Cr. Among these, 10 proteins (~ 2%) were common in all concentrations of Cr (**Fig. 1a**).

As many as 364 proteins were up-regulated in medium with 100  $\mu\text{g mL}^{-1}$  Cr. The numbers of up-regulated proteins common in medium with 50, 150 and 200  $\mu\text{g mL}^{-1}$  were 23, 149 and 37 respectively. In the medium with 150  $\mu\text{g mL}^{-1}$  Cr, 300 proteins were upregulated with 27, 149 and 40 proteins common in medium with 50, 100 and 200  $\mu\text{g mL}^{-1}$  Cr, respectively. In medium with 200  $\mu\text{g mL}^{-1}$  Cr, 86 proteins were up-regulated with 14, 37 and 40 proteins common in medium with 50, 100 and 150  $\mu\text{g mL}^{-1}$  Cr. Of the 59 proteins up-regulated in medium with 50  $\mu\text{g Cr mL}^{-1}$ , 23 were also up-regulated in medium with

100  $\mu\text{g mL}^{-1}$  Cr. Similarly 27 and 14 proteins were up-regulated in medium with 150 and 200  $\mu\text{g mL}^{-1}$  Cr respectively (**Fig. 1a**). From **Fig. 1a**, most number of up-regulated proteins was observed in medium with 100 and 150  $\mu\text{g mL}^{-1}$  Cr. Additionally, there was a substantial down-regulation of expressed proteins under Cr stress. As many as 554, 294, 313 and 527 proteins were uniquely down-regulated in *S. xylosus* grown in medium with 50, 100, 150 and 200  $\mu\text{g mL}^{-1}$  Cr, respectively. Among these, 36 proteins (~ 6%) were commonly suppressed in all media containing Cr (**Fig. 1b**).

Of the 554 proteins down-regulated in medium with 50  $\mu\text{g mL}^{-1}$  Cr, as many as 213, 281 and 442 were common when *S. xylosus* was grown in medium with 100, 150 and 200  $\mu\text{g mL}^{-1}$  Cr. Similarly, of the 249 proteins down-regulated in 100  $\mu\text{g mL}^{-1}$  Cr, as many as 213, 98 and 200 were common for *S. xylosus* grown in medium with 50, 150 and 200  $\mu\text{g mL}^{-1}$  Cr, respectively. In medium containing 150  $\mu\text{g mL}^{-1}$  Cr, 313 proteins were down-regulated with 281, 98 and 267 of these proteins also observed in medium with 50, 100 and 200  $\mu\text{g mL}^{-1}$  Cr, respectively. In medium containing 200  $\mu\text{g mL}^{-1}$  Cr, 527 proteins were down-regulated. Of these 482, 200 and 267 proteins were also observed in medium with 50, 100 and 150  $\mu\text{g mL}^{-1}$  Cr. As can be seen from **Fig. 1b**, the most number of down-regulated proteins were in the medium with 50 and 200  $\mu\text{g mL}^{-1}$  Cr. Some of these commonly up-regulated proteins and their fold change in each condition relative to the proteins expressed in medium without Cr are listed in **Table 1**.

### 3.3 Cr (VI)- induced proteins

Under varying concentrations of Cr (VI), proteins were expressed differentially. For instance, in medium with the 50  $\mu\text{g mL}^{-1}$  Cr (**Table 2**), one stress protein, OsmC-like protein (C273\_01260) was up-regulated. In addition, UvrABC system protein A (UvrABC) (*uvrA* to catalyze recognition and processing of DNA lesions as part of the nucleotide-excision repair system and SOS response) was also up-regulated.

In medium with 100  $\mu\text{g mL}^{-1}$  Cr (VI) (**Table 3**), six stress related proteins were up-regulated. Thioredoxin reductase (SXYLSMQ121\_1932), superoxide dismutase (*sodA*) and oxidoreductase (SXYLSMQ121\_0647) known to remove superoxide radicals, alkyl hydroperoxide reductase subunit C (*ahpC*) with peroxidase activity, ATP-dependent Clp protease ATP-binding subunit ClpC (*clpC* active in protein metabolism), and universal stress family protein (HMPREF0793\_2441). In addition to **UvrABC** (*uvrA*), four other repair proteins were up-regulated; DNA repair protein radA (SAGN\_06134), DNA repair protein RecN (*recN*), DNA-3-methyladenine glycosylase (HMPREF0798\_00143) enabling base-excision repair and ImpB/MucB/SamB family protein (IMPB) (SEVCU012\_1547) all associated with DNA damage and repair. Five proteins related to peptide folding and transport were up-regulated including bifunctional preprotein translocase subunit SecD/SecE (SCHR\_04972) and oligopeptide transport ATP-binding protein OppD (SPSINT\_0670).

In medium with 150  $\mu\text{g mL}^{-1}$  Cr (VI) (**Table 4**), four stress related proteins were up-regulated. In addition to **SOD** (*sodA*), **CLPC** (*clpC*) and **AHPC** (*ahpC*), another subunit of the alkyl hydroperoxide reductase, alkyl hydroperoxide reductase subunit F (SARL\_04221), an oxidoreductase was up-regulated. Six DNA repair related proteins were up-regulated. In addition to **IMPB** (SEVCU012\_1547) and **UvrABC** (*uvrA*), another SOS response protein, UvrABC system protein B (*uvrB*); DNA repair protein (C273\_00955); ATP-dependent helicase/nuclease subunit A (*addA*) and ATP-dependent helicase/deoxyribonuclease subunit B (*addB*); both concerned with double-stranded DNA break repair were up-regulated. There was no increase in the number of peptide folding and transport related proteins with oligopeptide/dipeptide ABC transporter, ATP-binding protein (SAGN\_10870); and oligopeptide transport system permease protein oppB (SS7213T\_13162) specifically up-regulated in medium with 150  $\mu\text{g mL}^{-1}$  Cr (VI). ABC transporter, ATP-binding protein (HMPREF0793\_1590) and two chaperones, chaperone protein DnaK (*dnaK*) and 60 kDa chaperonin (*groL*) were up-regulated in both media containing 100 and 150  $\mu\text{g mL}^{-1}$  Cr. Chaperones are known to prevent mis-folding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions.

In medium with 200  $\mu\text{g mL}^{-1}$  Cr (VI) (**Table 5**), the response was comparatively suppressed with a decrease in the number of DNA repair related proteins up-regulated followed by a complete absence of stress related proteins. Competence protein comEA (*comEA* known to have a function in DNA repair), and a chaperone (ribosome maturation factor RimM, *rimM*) were specifically up-regulated in medium with 200  $\mu\text{g mL}^{-1}$  Cr.

Additionally chaperone protein DnaK and 60 kDa chaperonin were also up-regulated, first observed in medium with 100 and 150  $\mu\text{g mL}^{-1}$  Cr, but not in medium with 50  $\mu\text{g mL}^{-1}$  Cr. From the latter's fold changes there was no tendency discernible albeit at 200  $\mu\text{g mL}^{-1}$  Cr (VI), it was decreased in comparison to media containing 100 and 150  $\mu\text{g mL}^{-1}$  Cr.

### **3.4 Functional characterization of differentially expressed proteins**

To gain insight into the expression differences in response to metal stress conditions, all the differentially expressed proteins were segregated based on Clusters of Orthologous groups (COG) categories (**Fig. 2**). The percentage of proteins in each of the **COG** categories was different under different levels of Cr stress. Majority of the differentially expressed proteins (293) did not have matches in Uniprot database ("uncharacterized category"). The largest groups of annotated differentially expressed proteins were involved in enzymatic functions (11%), translation (10%) followed by membrane transport (5%). Other metabolic processes like metabolism of cofactors and vitamins, terpenoids and polyketides, carbon, peptidoglycan, of lipids etc. were grouped under the umbrella of "Other metabolic processes" accounting for 6% of the 613 proteins.

### 3.5 Common proteins under various Cr concentrations

The ten commonly up-regulated proteins among all four Cr (VI) treatments (**Table 1**) had a variety of predicted constitutive functions in translation, replication and energy metabolism, of which one protein was uncharacterized. 10 kDa chaperonin (*groS*), YSIRK family Gram-positive signal peptide (ERYG\_03274), 50S ribosomal protein L23 (*rplW*) and Carboxyl-terminal protease (C273\_07362) were commonly up-regulated in all Cr conditions but were not observed in medium without Cr (**Table 1**).

The other proteins in **Table 1**, two of which functioned as elongation factors, (Translation elongation factor Tu, *tuf* and Translation elongation factor Ts, SXYLSMQ121\_1549), one a ribosomal constituent (30S ribosomal protein S1, *rpsA*), one acting as a DNA binding protein involved in chromosome condensation, (DNA-binding protein HU, HMPREF1208\_00854) and one in energy metabolism (Small GTP-binding protein) all showed appreciable fold-changes. With the exception of DNA-binding protein HU, they follow an identical pattern of being highly up-regulated, many folds over in both media with 100 and 150  $\mu\text{g mL}^{-1}$  Cr in comparison to media with only 50 or 200  $\mu\text{g mL}^{-1}$  Cr.

As many as 36 proteins were expressed specifically in the control, but not in media containing Cr (**Table 1**). Ten of the 36 were part of several metabolic processes, with proteins such as aconitate hydratase A (*acnA*) part of organic acid metabolism and isoprenyl transferase (V070\_00285), known to be active in the metabolism of terpenoids, for instance. Seven down-regulated proteins were enzymatic in function ranging from oxidoreductases to hydrolases. Additionally, one protein involved in transcription (sucrose operon repressor (STP1\_0485)); and two proteins involved in replication, one in DNA unwinding, DNA Helicase (*pcrA*) and the other, Ribonucleotide reductase of class III (Anaerobic), (SXYLSMQ121\_0155) were also down-regulated.

## 4. DISCUSSION

In this study the objective was to examine expressional proteomic differences in response to varying Cr. Although *S. xylosus* can tolerate concentrations up to 350  $\mu\text{g mL}^{-1}$ , a decrease in growth rate and biomass yield is observed at 50  $\mu\text{g mL}^{-1}$  and greater. This is evident from the significantly reduced OD<sub>600</sub> at higher Cr concentrations. 50  $\mu\text{g mL}^{-1}$  Cr was selected for challenging mid-log phase *S. xylosus* as this was the lowest concentration at which cell toxicity was observed. Cr concentration was sequentially increased up to 200  $\mu\text{g mL}^{-1}$  (above which obtaining sufficient cell pellet was a problem), for delineating protein expression responses when grown in different Cr concentrations.

Cr toxicity is primarily attributable to oxidative stress via reactive intermediates formed during intracellular reduction of Cr (VI) to Cr (III). This reduction brought about by cytoplasmic proteins, cytochrome P-450 and/or mitochondria generates short-lived intermediates Cr (V) and/or Cr (IV). The



reduction of Cr (V) to Cr (IV) is accompanied by the reduction of molecular oxygen to peroxide, which in turn forms hydrogen peroxide. This process produces reactive oxygen species (ROS) including single oxygen (O) and superoxide ( $O_2^-$ ), hydroxyl (OH) and hydrogen peroxide ( $H_2O_2$ ) radicals which combine readily with DNA-protein complexes. Additionally, intracellular cationic Cr (III) also interacts with phosphate groups of DNA. This binding to cellular material hampers normal physiological function and in the case of DNA, affects replication, transcription and cause mutagenesis. [17, 25, 26, 27, 28],

Comparative analysis showed that the number of proteins up-regulated were least when *S. xylosum* was grown in medium containing  $50 \mu\text{g mL}^{-1}$  Cr with one DNA repair and one stress protein up-regulated as a response to Cr stress. Evidently, a challenge of  $50 \mu\text{g mL}^{-1}$  Cr invoked a minor response defense-wise and posed no significant threat to the survival of *S. xylosum*. This is corroborated by the limited effect  $50 \mu\text{g mL}^{-1}$  Cr has on growth in medium, as observed in the growth curve (**Fig. 3**) as well as the  $OD_{600}$  at time of harvest.

In comparison,  $100 \mu\text{g mL}^{-1}$  Cr elicited a much more pronounced response both in total number of proteins up-regulated as well proteins possibly predicted to protect *S. xylosum* from Cr stress. Medium containing  $150 \mu\text{g mL}^{-1}$  Cr induced a similar response to medium with  $100 \mu\text{g mL}^{-1}$  Cr both in terms of number of proteins up-regulated and as well proteins conferring protection from Cr stress. This is further highlighted by how their growth curves and proteomic expression resemble each other. *S. xylosum* reacted differently to medium with  $200 \mu\text{g mL}^{-1}$  Cr, in comparison to the other Cr concentrations with one unique (Competence protein comEA) DNA repair protein and three proteins in protein folding and transport up-regulated, one of them specific to medium with  $200 \mu\text{g mL}^{-1}$  Cr. Here the the cells are negatively responding to the challenge, showing first signs of inability to cope with chromate induced stress supported by poor growth in medium (with  $200. \mu\text{g mL}^{-1}$  Cr) in comparison to growth in medium with either  $100$  or  $150 \mu\text{g mL}^{-1}$  Cr.

Of the many superoxide removal proteins up-regulated in medium containing  $100$  and  $150 \mu\text{g mL}^{-1}$  Cr, Mn/ Fe-binding **SOD** (*sodA*) was (known to catalyze decomposition of superoxide anion ( $O_2^{\cdot-}$ ) to hydrogen peroxide and molecular oxygen) up-regulated in both, further demonstrating the similarity between the two conditions. Thompson et al. [16] also detected this protein only in chromate exposed cells, with this fact being validated by Ackerly et al. [29] and Hu et al. [25] who observed this in chromate challenged *E. coli* and *Caulobacter crescentus* respectively. Contrastingly, Brown et al. [10] did not observe this in chromate challenged *S. oneidensis* MR-1 cells. **UvrABC** (up-regulated in media with  $50$ ,  $100$  and  $150 \mu\text{g mL}^{-1}$  Cr), is part of a damage recognition complex composed of two UvrA and UvrB subunits that forms a repair system that scans the DNA for abnormalities, processing the recognized DNA lesions. Since it is up-regulated in almost all conditions, it is an important protein involved in superoxide

removal. Additionally, thioredoxin reductase and alkyl hydroperoxide reductase were also up-regulated, their role in reduction systems as a response to oxidative stress corroborated by Carmel-Harel and Storz [19]. Similarly, **AHPC** and **CLPC** were up-regulated in media with 100 and 150  $\mu\text{g mL}^{-1}$  Cr stating their importance in oxidative stress relief as a whole. Since this was a discovery experiment, experimental validation was not performed and additionally, biological replicates were not included as the data was generated by high-throughput techniques.

*S. xylosus* expressed and/or up-regulated numerous DNA repair related and peptide transport related proteins when grown in medium with 100 and 150  $\mu\text{g mL}^{-1}$  Cr. An increase in Cr (VI) concentration leads to an increase in Cr induced oxidative stress, indirectly correlating to increased DNA damage. As a result, *S. xylosus* responds with an increase in number of up-regulated repair associated proteins in comparison to the response generated at 50  $\mu\text{g mL}^{-1}$  Cr. Similarly, the increase in number of peptide transport related proteins suggest that these proteins are in high demand to cope with the larger number of mis-folded proteins generated as a direct result of oxidatively modified proteins not seen in *S. xylosus* grown in medium with 50  $\mu\text{g mL}^{-1}$  Cr (described by Gregerson and Bross, [30]). Since chaperone protein DnaK and 60 kDa chaperonin were up-regulated in all media containing Cr above 50  $\mu\text{g mL}^{-1}$  Cr (in comparison to the control), it is safe to say they are important and play a role in Cr tolerance, albeit indirectly. Fold changes show that chaperone protein DnaK is most active in medium with 100  $\mu\text{g mL}^{-1}$  Cr with a gradual decrease in its importance as Cr concentration rises. The lower fold changes for both at 200  $\mu\text{g mL}^{-1}$  Cr could be due to either overall suppression of *S. xylosus*'s proteomic output.

Exogenous chromate ( $\text{CrO}_4^{2-}$ ) shares a structural similarity to sulfate ( $\text{SO}_4^{2-}$ ) competing with it for transport across bacterial cell membranes [17, 31 and 32], and has been shown to competitively inhibit sulfate transport [33 and 34]. This is supported by the observation of up-regulated genes/proteins with annotated functions in sulfur transport and metabolism as observed by Thompson et al. [16]. In this study, we observed many proteins with annotated functions in amino-acid metabolism, but only one protein, up-regulated at 100  $\mu\text{g mL}^{-1}$  with a sulfate transport related annotated function. From this it is evident that Cr tolerance of *S. xylosus* does not rely on competitive inhibition of sulfur transport and metabolism, although we cannot be certain owing to non-detection (either due to extraction constraints or due to detection limits of the instrument).

Iron related proteins were up-regulated in medium with 100, 150 and even 200  $\mu\text{g mL}^{-1}$  Cr as was reported by Thompson et al. [16], who observed the up-regulation of genes/proteins with annotated functions in iron acquisition and homeostasis. These were TonB-dependant receptors for high-affinity iron chelators in response to chromate challenge. Reports [35, 36 and 37] revealed a distinct link between cell sensitivity to chromate and iron availability; with Brown et al. [10] describing the up-regulation of genes

involved in iron storage and transport. Henne et al. [38] postulated that the iron sequestration properties of certain iron transport proteins protect cellular macromolecules against oxidative damage resulting from this type of redox. Here, up-regulation of two siderophores, siderophore staphylobactin biosynthesis protein SbnC (SBNC) and putative siderophore biosynthesis protein (PSBP) (both having annotated functions in the siderophore biosynthetic process) in concentrations above  $50 \mu\text{g mL}^{-1}$  Cr were observed. This was similar to reports of Cortese [39] and Visca [40] who observed siderophores produced by certain *Pseudomonas* species that were able to bind exogenous transition metals other than Fe (III) with appreciable affinity producing conditions of low internal iron. Similarly, Zawadzka [40 and 42] showed siderophore pyridine-2,6-bis (thiocarboxylic acid, ptdc), from *Pseudomonas stutzeri* KC to detoxify extracellular chromium(VI), selenium, and tellurium oxyanions. This suggests that it not only functions in iron acquisition but also as an initial line of defense against metal toxicity. **SBNC** was up-regulated in both media with 100 and  $200 \mu\text{g mL}^{-1}$  Cr. The up-regulation of iron related proteins in media with Cr higher than  $50 \mu\text{g mL}^{-1}$  Cr show that these proteins may have a minor role in aiding *S. xylosus* tolerate higher concentrations of Cr.

We observed the up-regulation of alkaline shock protein 23 in media with 150 and  $200 \mu\text{g mL}^{-1}$  Cr. Kuroda et al. [43] suggested the role of alkaline shock protein 23 in alkaline tolerance mechanism since it was up-regulated on alkaline pH shock. This suggests that this protein is up-regulated when *S. xylosus* may be experiencing pH shock possible at higher Cr concentrations ( $150\text{-}200 \mu\text{g mL}^{-1}$  Cr) and may confer some sort of protection.

Extracellular and intracellular sequestration of metal, reduced permeability, active efflux, and enzymatic alteration to a less toxic form are strategies bacteria use to deal with metal [44]. Identifying proteins that play a part in any of these cell functions, will allow for enhanced toxic metal tolerance. Although proteins involved in response to oxidative stress are part of the cell's natural defense system, and are more viable to study, proteins such as Sulfite reductase [NADPH] hemoprotein beta-component (SRHB), **SBNC** and **PSBP** are part of more specific but poorly understood processes (like cysteine biosynthesis and iron transport). These need to be targeted by 'omics' approach.

36 proteins were not expressed in media containing Cr with general strategies of cellular resource reduction/suppression induced by heavy metals as the underlying cause, proposed by Yung et al. [13]. Importantly many proteins with general functions were expressed displaying *S. xylosus*'s ability to function and grow under Cr stress.

A total of 613 proteins observed in the entire data set, corresponds to 25% of the predicted 2,499 protein-coding ORF's within *S. xylosus* proteome (Proteome ID: UP000027230) with over 48% of the 613 uncharacterized. This highlights that there are still many functionally unknown proteins that may play

important roles in response to metal stress. Characterizing such unknown proteins and their functions by either improving on MS/MS search or by directly accessing the transcriptome may hold the key to relating stress response and Cr tolerance

In our study, responses to Cr metal ion stress can be grouped as oxidative stress responses (with superoxide removal, enhancement of free-radical detoxifying activities and DNA repair) and activation of amino acid synthesis. Up-regulation of proteins involved in peptide transport (and folding) as well as iron-related proteins suggests that different systems contribute simultaneously. The next step would be to deduce the overall mechanisms involved and to identify which of these proteins are primary sensors of Cr damage. This can be achieved using computational and/or other microanalytical methods.

## **CONCLUSION**

The protein profiles generated using **LC-MS-QToF** analysis reveal several insights into differential expression of proteins under Cr stress. The proteins common to all conditions either up or down-regulated suggest that, although some functions are affected such as those of enzymatic nature, the overall translation (62), transcription (13) and replication (15) state seem to be unaffected. This might imply that the isolate is capable of growing under considerable chromium stress. In this regard, the response of salt tolerant *S. xylosus* to Cr-stress will help better understanding of possible tolerance strategies in marine bacterial communities, and highlight their potential in heavy metal detoxification.

## **LIST OF ABBREVIATIONS**

**ACNA:** aconitate hydratase A

**ADDA:** ATP-dependent helicase/nuclease subunit A

**ADDB:** ATP-dependent helicase/deoxyribonuclease subunit **AHPC:** alkyl hydroperoxide reductase subunit C

**Cd (II):** Cadmium (II)

**CLPC:** ATP-dependent Clp protease ATP-binding subunit ClpC

**COG:** Clusters of Orthologous groups

**COME A:** Competence protein

**Cr (VI):** Chromate/hexavalent chromium

**DNA:** Deoxyribonucleic acid

**FE:** Iron

**IMP B:** ImpB/MucB/SamB family protein

**KDA:** kilodalton

**KEGG:** Kyoto Encyclopedia of Genes and Genomes

**LC-MS-QToF:** Liquid chromatography mass spectrometry quadrupole

**MPP:** Mass Profiler Professional

**NB:** Nutrient broth

**OD:** Optical density

**OPPD:** oligopeptide transport ATP-binding protein

**OPPB:** oligopeptide transport system permease protein

B

**ORF:** open reading frame

**OSMC:** osmotically inducible protein C

**PBS:** Phosphate buffered saline

**PSBP:** putative siderophore biosynthesis protein

**ROS:** Reactive Oxygen Species

**RPM:** revolutions per minute

**SBNC:** siderophore staphylobactin biosynthesis protein

**SOD:** superoxide dismutase

**SPI:** spectral peak intensity

**SRHB:** Sulfite reductase [NADPH] hemoprotein beta-component

**SWNA:** Sea water nutrient agar

**TE:** Tris-EDTA

**TUF:** Translation elongation factor

**UvrABC:** Subunit composition of UvrABC Nucleotide Excision Repair Complex = [UvrB][UvrC][UvrA]

## **CONFLICT OF INTEREST**

The authors declare that they have no competing interests. Elroy Joe Pereira was provided research fellowship by the University Grant Commission (UGC) (Sanction letter No: 18-12/2011(ii) EU-V); and

this study is funded by project No: PSCO0206. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## ACKNOWLEDGEMENTS

EJP designed this study, carried out experiments, analyzed data and wrote and revised this manuscript. SD and NR supervised experimental design and helped revise this manuscript. BF helped in carrying out proteomic experiments and analysis of data. All authors read and approved the final manuscript. We thank the Director, NIO for the facilities and encouragement. Elroy Joe Pereira was provided research fellowship by the University Grant Commission (UGC) (Sanction letter No: 18-12/2011(ii) EU-V); and this study is funded by project No: PSC0206 (MARBIOTECH).

## SUPPLEMENTARY MATERIAL

Supplemental File 1 contains the complete protein/ peptide summary of all entities. Supplemental File 2 contains all the files pertaining to the auto validation of all MS searches.

## REFERENCES

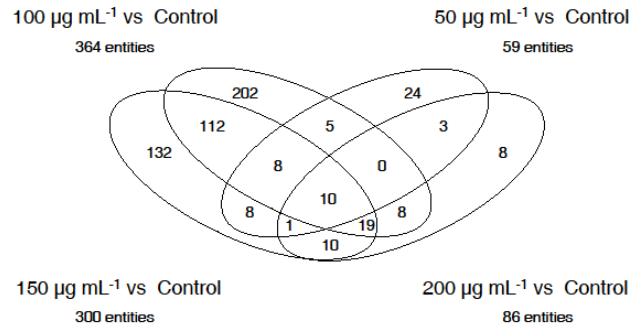
- [1] Hill, J.W.; Petrucci, R.H. *General chemistry: An integrated approach*, 2nd ed.; Upper Sadle River: Prentice Hall, **1999**.
- [2] Cotton, F.A.; Wilkinson, G.; Murillo, C.A.; Bochmann, M. *Advanced Inorganic Chemistry*, 6th ed.; Wiley & sons: New York, **1999**.
- [3] Macaskie, L.E.; Dean, A.C. Microbial metabolism, desolubilization, and deposition of heavy metals: metal uptake by immobilized cells and application to the detoxification of liquid wastes. *Adv. Biotechnol. Processes*, **1989**, *12*, 159–201. doi: PMID 2647096.
- [4] Yassi, A.; Nieboer, E. In: *Chromium in Natural and Human Environments*; Niragu, J.O.; Nieboer, E., Ed. Wiley & sons: New York, **1988**, pp. 443–495.
- [5] Crossland, C.J.; Kremer, H.H.; Lindeboom, H.; Marshall Crossland, J.I.; Le Tissier, M.D.A., Ed., *Coastal fluxes in the Anthropocene*; Springer: Berlin, **2005**.
- [6] Silver, S. Genes for all metals - a bacterial view of the periodic table. The 1996 Thom Award Lecture. *J. Ind. Microbiol. Biotechnol.*, **1998**, *20*(1), 1-12. doi: PMID 9523453.
- [7] Margesin, R.; Schinner, F. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Appl. Microbiol. Biotechnol.*, **2001**, *56*, 650–63. doi: 10.1007/s002530100701.
- [8] De, J.; Ramaiah, N.; Mesquita, A.; Verlekar, X.N. Tolerance to various toxicants by marine bacteria highly resistant to mercury. *Mar. Biotechnol.*, **2003**, *5*, 185-93. doi: PMID 12876655.
- [9] Dash, H.R.; Mangwani, N.; Chakraborty, J.; Kumari, S.; Das, S. Marine bacteria: potential candidates for enhanced bioremediation. *Appl. Microbiol. Biotechnol.*, **2013**, *97*(2), 561-71. doi: PMID: 23212672.
- [10] Brown, S.D.; Thompson, M.R.; Verberkmoes, N.C.; Chourey, K.; Shah, M.; Zhou, J.; Hettich, R.L.; Thompson, D.K. Molecular dynamics of the *Shewanella oneidensis* response to chromate stress. *Mol. Cell Proteomics*, **2006**, *5*(6), 1054-1071. doi: PMID 16524964.
- [11] Chourey, K.; Thompson, M.R.; Morrell-Falvey, J.; Verberkmoes, N.C.; Brown, S.D.; Shah, M.; Zhou, J.; Doktycz, M.; Hettich, R.L.; Thompson, D.K. Global molecular and morphological

- effects of 24-hour chromium(VI) exposure on *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.*, **2006**, 72(9), 6331-6344. doi: PMID 16957260.
- [12] Thompson, M.R.; Thompson, D.K.; Hettich, R.L. Systematic assessment of the benefits and caveats in mining microbial post-translational modifications from shotgun proteomic data: the response of *Shewanella oneidensis* to chromate exposure. *J. Proteome Res.*, **2008**, 7(2), 648-658. doi: PMID 18171020.
- [13] Yung, M.C.; Ma, J.; Salemi, M.R.; Phinney, B.S.; Bowman, G.R.; Jiao, Y. Shotgun proteomic analysis unveils survival and detoxification strategies by *Caulobacter Crescentus* during exposure to uranium, chromium, and cadmium. *J. Proteome Res.*, **2014**, 13(4), 1833-1847. doi: 10.1021/pr400880s.
- [14] Magnuson, T.S.; Swenson, M.W.; Paszczynski, A.J.; Deobald, L.A.; Kerk, D.; Cummings, D.E. Proteogenomic and functional analysis of chromate reduction in *Acidiphilium cryptum* JF-5, an Fe(III)-respiring acidophile. *Biometals*, **2010**, 23(6), 1129-1138. doi: PMID 20593301.
- [15] Jilic, N.K.; Stensballe, A.; Otzen, D.E.; Dönmez, G. Proteomic changes in response to chromium(VI) toxicity in *Pseudomonas aeruginosa*. *Bioresour. Technol.*, **2010**, 101(7), 2134-2140. doi: PMID 19945860.
- [16] Thompson, D.K.; Chourey, K.; Wickham, G.S.; Thieman, S.B.; VerBerkmoes, N.C.; Zhang, B.; McCarthy, A.T.; Rudisill, M.A.; Shah, M.; Hettich, R.L. Proteomics reveals a core molecular response of *Pseudomonas putida* F1 to acute chromate challenge. *BMC Genomics*, **2010**, 11, 311-26. doi: 10.1186/1471-2164-11-311.
- [17] Cervantes, C.; Campos-García, J.; Devars, S.; Gutiérrez-Corona, F.; Loza-Tavera, H.; Torres-Guzmán, J.C.; Moreno-Sánchez, R. Interactions of chromium with microorganisms and plants. *FEMS Microbiol. Rev.*, **2001**, 25(3), 335-347. doi: 10.1016/S0168-6445(01)00057-2.
- [18] Singh, J.; Carlisle, D.L.; Pritchard, D.E.; Patierno, S.R. Chromium induced genotoxicity and apoptosis: relationship to chromium carcinogenesis (review). *Oncol. Rep.*, **1998**, 5(6), 1307-1325. doi: PMID 9769362.
- [19] Carmel-Harel, O.; Storz, G. Roles of the glutathione-and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Ann. Rev. Microbiol.*, **2000**, 54, 439-461. doi: PMID 11018134.
- [20] Messer, J.; Reynolds, M.; Stoddard, L.; Zhitkovich, A. Causes of DNA single-strand breaks during reduction of chromate by glutathione in vitro and in cells. *Free Radic. Biol. Med.*, **2006**, 40(1), 1981-1992. doi: PMID 16716899.
- [21] Lloyd, J.R.; Lovley, D.R.; Microbial detoxification of metals and radionuclides. *Curr. Opin. Biotechnol.*, **2001**, 12(3), 248-253. doi: PMID 11404102.
- [22] Friedman, D.B. Quantitative proteomics for two-dimensional gels using difference gel electrophoresis. *Methods Mol. Biol.*, **2007**, 367, 219-239. doi: PMID 17185779.
- [23] Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **1951**, 193(1), 265-275. doi: PMID 14907713.
- [24] Kinter, M.; Sherman, N.E. *Protein sequencing and identification using tandem mass spectrometry*; Wiley & sons: New York, **2000**.
- [25] Hu, P.; Brodie, E.L.; Suzuki, Y.; McAdams, H.H.; Andersen, G.L. Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. *J. Bacteriol.*, **2005**, 187(24), 8437-8449. doi: PMID 16321948.
- [26] Joutey, N.T.; Sayel, H.; Bahafid, W.; El Ghachtouli, N. Mechanisms of hexavalent chromium resistance and removal by microorganisms. *Rev. Environ. Contam. Toxicol.*, **2015**, 233, 45-69. doi: PMID 25367133.
- [27] Bose, R.N.; Moghaddas, S.; Gelerinter, E. Long-lived chromium (IV) and chromium (V) metabolites in the chromium(VI) glutathione reaction – NMR, ESR, HPLC, and kinetic characterization. *Inorg. Chem.*, **1992**, 31(11), 1987-1994. doi: 10.1021/ic00037a004
- [28] Viti, C.; Marchi, E.; Decorosi, F.; Giovannetti, L. Molecular mechanisms of Cr(VI) resistance in bacteria and fungi. *FEMS Microbiol. Rev.*, **2014**, 38(4), 633-659. doi: PMID 24188101

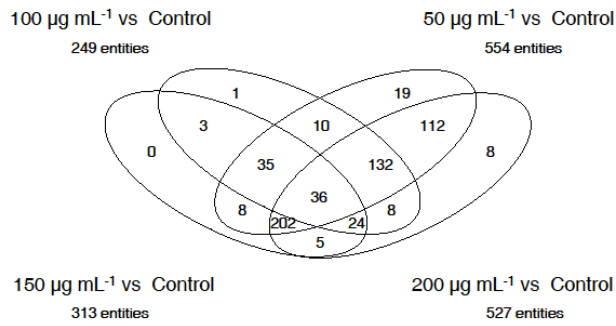
- [29] Ackerley, D.F.; Barak, Y.; Lynch, S.V.; Curtin, J.; Matin, A. Effect of chromate stress on *Escherichia coli* K-12. *J. Bacteriol.*, **2006**, *188*(9), 3371-3381. doi: PMID 16621832.
- [30] Walker, J.M. *Methods of molecular biology*, Vol. 648. Humana press: New York, **2010**.
- [31] Cervantes, C.; Ohtake, H.; Chu, L.; Misra, T.K.; Silver, S. Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. *J. Bacteriol.*, **1990**, *172*(1), 287-291. doi: PMID 16524964.
- [33] Nies, A.; Nies, D.H.; Silver, S. Cloning and expression of plasmid genes encoding resistance to chromate and cobalt in *Alcaligenes eutrophus*. *J. Bacteriol.* **1989**, *171*(9), 5065-5070. doi: PMID 2549011.
- [34] Cervantes, C.; Silver, S. Plasmid chromate resistance and chromate reduction. *Plasmid*, **1992**, *27*(1), 65-71. doi: PMID 1741461.
- [31] Ohtake, H.; Cervantes, C.; Silver, S. Decreased chromate uptake in *Pseudomonas fluorescens* carrying a chromate resistance plasmid. *J. Bacteriol.*, **1987**, *169*(8), 3853-3856. doi: PMID 3112130.
- [35] Guterman, S.K. Colicin B: mode of action and inhibition by enterochelin. *J. Bacteriol.*, **1973**, *114*(3), 1217-1224. doi: PMID 4576402.
- [36] Pugsley, A.P.; Reeves, P. Iron uptake in colicin B-resistant mutants of *Escherichia coli* K-12. *J. Bacteriol.*, **1976**, *126*(3), 1052-1062. doi: <http://j.b.asm.org/content/126/3/1052.short>.
- [37] Wang, C.C.; Newton, A. Iron transport in *Escherichia coli*: relationship between chromium sensitivity and high iron requirement of *Escherichia coli*. *J. Bacteriol.*, **1976**, *98*(3), 1135-1141. doi: PMC315307.
- [38] Henne, K.L.; Turse, J.E.; Nicora, C.D.; Lipton, M.S.; Tollaksen, S.L.; Lindberg, C.; Babnigg, G.; Giometti, C.S.; Nakatsu, C.H.; Thompson, D.K.; Konopka, A.E. Global proteomic analysis of the chromate response in *Arthrobacter* sp. strain FB24. *J. Proteome Res.* **2009**, *8*(4), 1704-1716. doi: PMID 19231868.
- [39] Cortese, M.S.; Paszczyński, A.; Lewis, T.A.; Sebat, J.L.; Borek, V.; Crawford, R.L. Metal chelating properties of pyridine-2,6-bis(thiocarboxylic acid) produced by *Pseudomonas* spp. and the biological activities of the formed complexes. *Biometals*, **2002**, *15*(2), 103-120. doi: 10.1023/A:1015241925322.
- [40] Visca, P.; Colotti, G.; Serino, L.; Verzili, D.; Orsi, N.; Chiancone, E. Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes. *Appl. Environ. Microbiol.*, **1992**, *58*(9), 2886-2893. doi: PMID 1444402.
- [41] Zawadzka, A.M.; Crawford, R.L.; Paszczyński, A.J. Pyridine-2,6-bis(thiocarboxylic acid) produced by *Pseudomonas stutzeri* KC reduces and precipitates selenium and tellurium oxyanions. *Appl. Environ. Microbiol.*, **2006**, *72*(5), 3119-3129. doi: PMID 16672449.
- [42] Zawadzka, A.M.; Crawford, R.L.; Paszczyński, A.J. Pyridine-2,6-bis(thiocarboxylic acid) produced by *Pseudomonas stutzeri* KC reduces chromium(VI) and precipitates mercury, cadmium, lead and arsenic. *Biometals*, **2007**, *20*(2), 145-158. doi: PMID 16900399.
- [43] Kuroda, M.; Ohta, T.; Hayashi, H. Isolation and the gene cloning of an alkaline shock protein in methicillin resistant *Staphylococcus aureus*. *Biochem. Biophys. Res. Commun.*, **1995**, *207*(3), 978-984. doi: PMID 7864904.
- [44] Hobman, J.L.; Yamamoto, K.; Oshima, T. Transcriptomic responses of bacterial cells to sublethal metal ion stress In: *Molecular Microbiology of Heavy metals*; Nies, D.H.; Silver, S., Eds. Springer-Verlag Berlin. Heidelberg, 2007; pp. 74-107.
- [45] Pradel, P.; Hernández, C.; Tello, M.; Villarreal, M.P.; Corsini, G.; González, A. *Staphylococcus equorum* isolated from seabed as potential biotool to Cr(VI) remediation. *Adv. Mat. Res.*, **2013**, *825*, 524-527. doi: 10.4028/www.scientific.net/AMR.825.524.
- [46] Thompson, M.R.; VerBerkmoes, N.C.; Chourey, K.; Shah, M.; Thompson, D.K.; Hettich, R.L. Dosage-dependent proteome response of *Shewanella oneidensis* MR-1 to acute chromate challenge. *J. Proteome Res.*, **2007**, *6*(5), 1745-1757. doi: PMID 17385904.



- [47] Viamajala, S.; Peyton, B.M.; Sani, R.K.; Apel, W.A.; Petersen, J.N.; Toxic effects of chromium(VI) on anaerobic and aerobic growth of *Shewanella oneidensis* MR-1. *Biotechnol. Prog.*, **2004**, *20*(1), 87–95. doi: PMID 14763828.
- [48] Zhang, X.; Krumholz, L.R.; Yu, Z.; Chen, Y.; Liu, P.; Li, X. A novel subspecies of *Staphylococcus Aureus* from sediments of Lanzhou Reach of the Yellow river aerobically reduces hexavalent chromium. *J. Bioremed. Biodeg.*, **2013**, *4*, 1-10. doi: 10.4172/2155-6199.1000188.
- [49] Arias, Y.M.; Obratsova, A.; Tebo, B.M. Natural attenuation of Cr(VI) contamination in laboratory mesocosms. *Geomicrobiol. J.*, **2003**, *20*(4), 389-01. doi: 10.1080/01490450303902.
- [50] Cheung, K.H.; Gu, J.D. Reduction of chromate (CrO<sub>4</sub><sup>2-</sup>) by an enrichment consortium and an isolate of marine sulfate-reducing bacteria, *Chemosphere*, **2003**, *52*(9), 1523-1529. doi: PMID 12867184.
- [51] Cheung, K.H.; Lai, H.Y.; Gu, J.D. Membrane-associated hexavalent chromium reductase of *Bacillus megaterium* TKW3 with induced expression. *J. Microbiol. Biotechnol.*, **2006**, *16*(6), 855-862. doi: <http://hdl.handle.net/10722/73246>.
- [52] Henne, K.L.; Nakatsu, C.H.; Thompson, D.K.; Konopka, A.E. High-level chromate resistance in *Arthrobacter* sp. strain FB24 requires previously uncharacterized accessory genes. *BMC Microbiol.* **2009**, *16*(9), 199. doi: 10.1186/1471-2180-9-199.
- [53] Kloos, W.; Schleifer, K. Isolation and Characterization of Staphylococci from Human Skin, I. Amended Descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and Descriptions of Three New Species: *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, and *Staphylococcus xylosus*. *Int. J. Syst. Evol. Microbiol.* **1975**, *25*(1):50-61. doi:10.1099/00207713-25-1-50.
- [54] Soge, O.O.; Meschke, J.S.; No, D.B.; Roberts, M.C. Characterization of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococcus* spp. isolated from US West Coast public marine beaches. *J. Antimicrob. Chemother.* **2009**, *64* (6): 1148-1155. doi: PMID 19837712.
- [55] Gunn, B.; Colwell, R. Numerical Taxonomy of Staphylococci Isolated from the Marine Environment. *Int. J. Syst. Evol. Microbiol.* **1983**, *33*(4): 751-759. doi: 10.1099/00207713-33-4-751.



**A**



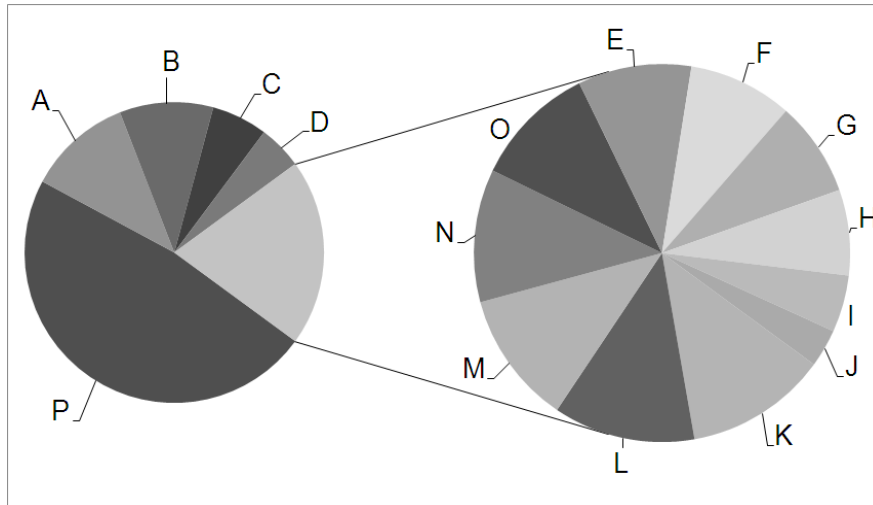
**B**

**Figure 1.**

Distribution of differentially expressed proteins in response to various Cr conditions.

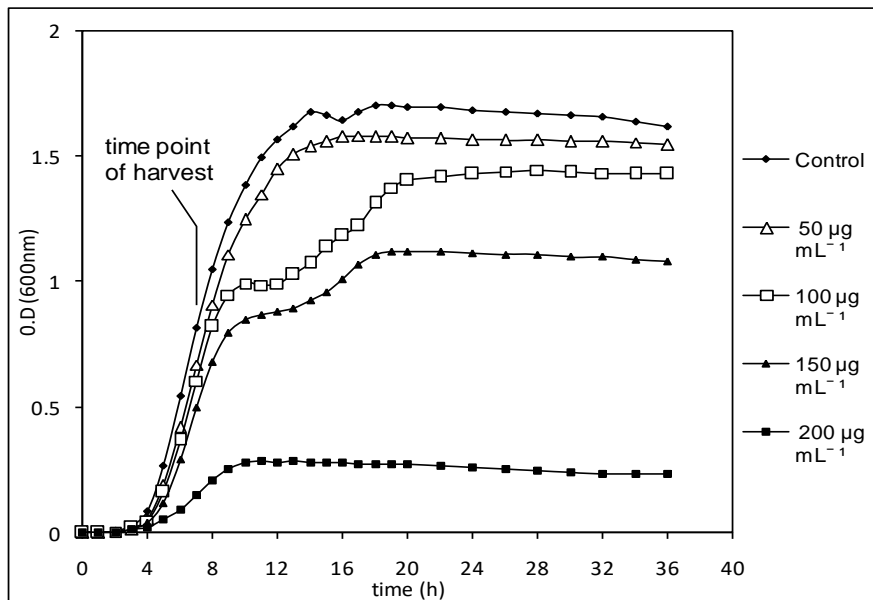
A. Venn diagram showing the number of up-regulated proteins in response to the different Cr conditions

B. Venn diagram showing the number down-regulated proteins in response to the different Cr conditions



**Figure 2.**

Differentially expressed proteins as a result of Cr treatment were categorized into *a* enzymes, *b* translation, *c* other metabolic processes, *d* membrane transport, *e* stress, *f* amino acid metabolism, *g* nucleotide metabolism, *h* miscellaneous, *i* protein folding, sorting and degradation, *j* cellular processes, *k* replication, *l* repair, *m* carbohydrate metabolism, *n* metal/antibiotic related, *o* transcription, *p* uncharacterized. The proteins corresponding functions were categorized according to COG functional categories.



**Figure 3.**

Optical densities of *Staphylococcus xylosus* cultures grown aerobically (110 rpm) in media with 0, 50, 100, 150 and 200  $\mu\text{g mL}^{-1}$  Cr. The arrow shows point of cell harvest for protein processing.

**Table 1. Up-regulated proteins common to all Cr stress conditions.**

Genes based on protein ID	Annotation	Known function	Fold change in 50 µg mL <sup>-1</sup> Cr*	Fold change in 100 µg mL <sup>-1</sup> Cr *	Fold change in 150 µg mL <sup>-1</sup> Cr *	Fold change in 200 µg mL <sup>-1</sup> Cr *
<i>tuf</i>	Translation elongation factor Tu	protein biosynthesis	1.138	6.853	5.394	2.057
SXYLSMQ121_1549	Translation elongation factor Ts	protein biosynthesis	1.113	7.085	4.771	1.767
HMPREF1208_00854	DNA-binding protein HU	DNA condensation	1.296	1.148	1.037	7.573
<i>rpsA</i>	30S ribosomal protein S1	structural constituent of ribosome	1.283	7.014	6.029	2.101
-	Small GTP-binding protein	GTP binding	3.413	14.275	12.971	7.391
C273_07362	Carboxyl-terminal protease	membrane component with peptidase activity	-	-	-	-
<i>groS</i>	10 kDa chaperonin	stress response and chaperone	-	-	-	-
ERYG_03274	YSIRK family Gram-positive signal peptide	signaling	-	-	-	-
<i>rplW</i>	50S ribosomal protein L23	structural constituent of ribosome	-	-	-	-
-	Uncharacterized protein	-	-	-	-	-

\*- in comparison to the fold change observed in control (system generated)

**Table 2. Proteins differentially expressed at 50 µg mL<sup>-1</sup> Cr stress.**

	<b>Genes based on protein ID</b>	<b>Annotation</b>	<b>Known function</b>	<b>Fold Change*</b>
Amino acid metabolism	SS7213T_09152	Aspartokinase	lysine and threonine biosynthesis	-
	<i>arcA</i>	Arginine deiminase	arginine catabolic process to ornithine	-
	<i>ilvB</i>	Acetolactate synthase	isoleucine and valine biosynthesis	-
	SXYLSMQ121_1052	D-alanine aminotransferase	D-amino acid metabolic process	-
Translation	<i>rpsQ</i>	30S ribosomal protein S17	structural constituent of ribosome	-
	<i>mmaA</i>	tRNA-specific 2-thiouridylase MmaA	tRNA modification	-
Stress	C273_01260	OsmC-like protein	response to oxidative stress	-
DNA repair	<i>uvrA</i>	UvrABC system protein A	SOS response and nucleotide-excision repair	-
Other metabolic process	SSP1416	Transketolase	transketolase activity	1.045
	<i>sucC</i>	Succinate--CoA ligase [ADP-forming] subunit beta	carbohydrate metabolism	1.287

\* - in comparison to the fold change observed in control (system generated)

**Table 3. Proteins differentially expressed at 100 µg mL<sup>-1</sup> Cr stress.**

	<b>Genes based on protein ID</b>	<b>Annotation</b>	<b>Known function</b>	<b>Fold change*</b>
Translation	<i>tuf</i>	Elongation factor Tu	elongation factor	4.27
	<i>fusA</i>	Elongation factor G	elongation factor	15.02
	<i>rpsE</i>	30S ribosomal protein S5	structural constituent of ribosome	20.72
	<i>rpsD</i>	30S ribosomal protein S4		9.73
	<i>rplE</i>	50S ribosomal protein L5		13.2
	<i>rpsG</i>	30S ribosomal protein S7		5.14
Stress	HMPREF0793_2441	Universal stress family protein	stress response	1.346
	SXYLSMQ121_1932	Thioredoxin reductase	removal of superoxide radicals	-
	SXYLSMQ121_0647	Oxidoreductase	reduction of molecular oxygen	-
	<i>ahpC</i>	Alkyl hydroperoxide reductase subunit C	peroxidase activity	-
	<i>clpC</i>	ATP-dependent Clp protease ATP-binding subunit ClpC	stress response and chaperone	-
	<i>sodA</i>	Superoxide dismutase	stress response and oxidoreductase activity	-
DNA Repair	SAGN_06134	DNA repair protein radA	DNA damage and repair	-
	HMPREF0798_00143	DNA-3-methyladenine glycosylase	base-excision repair	-
	<i>recN</i>	DNA repair protein RecN	DNA recombination and repair	-
	<i>uvrA</i>	UvrABC system protein A	SOS response and nucleotide-excision repair	-
	SEVCU012_1547	ImpB/MucB/SamB family protein	damgaged DNA binding and repair	-
Protein folding and transport	SCHR_04972	Bifunctional preprotein translocase subunit SecD/SecF	intracellular protein transport	-
	SPSINT_0670	Oligopeptide transport ATP-binding protein OppD	peptide transport	-
	<i>dnaK</i>	chaperone protein DnaK	stress response and chaperone	8.757
	<i>groL</i>	60 kDa chaperonin	chaperone	13.431
	HMPREF0793_1590	ABC transporter, ATP-binding protein	peptide transport	-
Metal or antibiotic response	ERS140167_00643	Arsenate reductase	Arsenic resistance	-
	SXYLSMQ121_1888	Arsenical-resistance protein ACR3	Arsenic resistance	-
	<i>czrC</i>	Cadmium and zinc resistance protein C	cation-transporting ATPase activity	-
	<i>tcaA</i>	Membrane-associated protein TcaA	Antibiotic resistance	-
	SAGN_02662	Antibiotic resistance-related transmembrane efflux protein	Antibiotic resistance	-
Iron-related	SEQMU2_08605	Iron-siderophore ABC transporter, substrate-binding protein	signal	-
	SOJ_03710	Siderophore staphylobactin biosynthesis protein SbnC	siderophore biosynthetic process	-
Metabolic process	<i>cysI</i>	Sulfite reductase [NADPH] hemoprotein beta-component	sulphur metabolism	-
	SSP1416	Transketolase	transketolase activity	16.28
	<i>sucC</i>	Succinate--CoA ligase [ADP-forming] subunit beta	carbohydrate metabolism	11.6

\* - in comparison to the fold change observed in control (system generated)

**Table 4. Proteins differentially expressed at 150 µg mL<sup>-1</sup> Cr stress.**

	<b>Genes based on protein ID</b>	<b>Annotation</b>	<b>Known function</b>	<b>Fold change*</b>
Translation	<i>tuf</i>	Elongation factor Tu	elongation factor	2.87
	<i>fusA</i>	Elongation factor G	elongation factor	4.79
	<i>rpsE</i>	30S ribosomal protein S5	structural constituent of ribosome	5.49
	<i>rpsD</i>	30S ribosomal protein S4		4.27
	<i>rplE</i>	50S ribosomal protein L5		6.78
	<i>rpsG</i>	30S ribosomal protein S7		1.88
Stress	<i>sodA</i>	Superoxide dismutase	stress response and oxidoreductase activity	-
	<i>clpC</i>	ATP-dependent Clp protease ATP-binding subunit ClpC	stress response and chaperone	-
	<i>ahpC</i>	Alkyl hydroperoxide reductase subunit C	peroxidase activity	-
	SARL_04221	Alkyl hydroperoxide reductase subunit F	alkyl hydroperoxide reductase activity	-
DNA Repair	SEVCU012_1547	ImpB/MucB/SamB family protein	damgaged DNA binding and repair	-
	<i>uvrA</i>	UvrABC system protein A	SOS response and nucleotide-excision repair	-
	<i>uvrB</i>	UvrABC system protein B	SOS response and nucleotide-excision repair	-
	C273_00955	DNA repair protein	DNA binding and repair	-
	<i>addA</i>	ATP-dependent helicase/nuclease subunit A	double-strand break repair via homologous recombination	-
	<i>addB</i>	ATP-dependent helicase/deoxyribonuclease subunit B	double-strand break repair via homologous recombination	-
Protein folding and Transport	SAGN_10870	Oligopeptide/dipeptide ABC transporter	peptide transport	-
	SS7213T_13162	Oligopeptide transport system permease protein oppB	Transport	-
	HMPREF0793_1590	ABC transporter, ATP-binding protein	peptide transport	-
	<i>dnaK</i>	chaperone protein DnaK	stress response and chaperone	7.944
	<i>groL</i>	60 kDa chaperonin	chaperone	32.532
Alkaline shock protein	SSIM_01695	Alkaline shock protein 23		-
Metal or antibiotic response	SXYLSMQ121_1888	Arsenical-resistance protein ACR3	Arsenic resistance	-
	<i>czrC</i>	Cadmium and zinc resistance protein C	cation-transporting ATPase activity	-
Iron-related	SARL_03166	Iron-regulated ABC transporter ATPase component	transport	-
	<i>iucC_2</i>	Putative siderophore biosynthesis protein	siderophore biosynthetic process	-
Metabolic process	SSP1416	Transketolase	transketolase activity	16.28
	<i>sucC</i>	Succinate--CoA ligase [ADP-forming] subunit beta	carbohydrate metabolism	7.835
	<i>yfkN_3</i>	5'-nucleotidase	nucleotide metabolism	2.39
	<i>pdhB</i>	Pyruvate dehydrogenase E1 component subunit beta	carbohydrate metabolism	5.48

\* - in comparison to the fold change observed in control (system generated)

**Table 5. Proteins differentially expressed at 200 µg mL<sup>-1</sup> Cr stress.**

	<b>Genes based on protein ID</b>	<b>Annotation</b>	<b>Known function</b>	<b>Fold change*</b>
DNA repair	<i>comEA</i>	Competence protein comEA	DNA repair	-
Protein folding and transport	<i>rimM</i>	Ribosome maturation factor RimM	chaperone	-
	<i>dnaK</i>	Chaperone protein DnaK	stress response and chaperone	1.152
	<i>groL</i>	60 kDa chaperonin	chaperone	5.967
Alkaline shock protein	SSIM_01695	Alkaline shock protein 23		-
Iron-related	SOJ_03710	Siderophore staphylobactin biosynthesis protein SbnC	siderophore biosynthetic process	-
Metabolic process	<i>yfkN_3</i>	5'-nucleotidase	nucleotide metabolism	1.71
	<i>pdhB</i>	Pyruvate dehydrogenase E1 component subunit beta	carbohydrate metabolism	1.72

\* - in comparison to the fold change observed in control (system generated)