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Effects of arsenite stress on growth and proteome of *Klebsiella pneumoniae*

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**ABSTRACT**

In the present study an arsenite, As(III), tolerating bacterium, MR4, was isolated from Mulla River Pune, India, capable of reducing arsenate to arsenite and identified as *Klebsiella pneumoniae* (HQ857583). Comparative proteomic analysis using two-dimensional gel electrophoresis (2-DGE) and matrix assisted laser desorption ionization-time of flight-time of flight (MALDI-TOF/TOF) was used to monitor the proteins undergoing changes in expression levels under 2.5 mM As(III) stress. The 2-DGE protein map has shown that 60 proteins were differentially expressed under As(III) stress, of which 39 proteins were successfully identified with a Mascot score greater than 70 (p < 0.05). Among the identified proteins, membrane transport/binding proteins, porins, and amino acid metabolism enzymes were down-regulated while stress responsive proteins and antioxidant enzymes were up-regulated. Proteins involved in carbohydrate metabolism, particularly those in pentose phosphate pathway were also up-regulated. These findings provide new insights into the probable mechanisms by which *K. pneumoniae* (HQ857583) could be adapting to As(III) stress.

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1. Introduction

Arsenic is widely distributed in the environment being released from both natural sources, e.g. by volcanic action, during weathering of rocks and mining as well as from anthropogenic sources, such as pesticides, high-temperature combustion and smelting of metals. In the environment, arsenic exists in several oxidation states viz., arsenite [As(III)], arsenate [As(V)], arsenite [As(III)], and arsenate [As(V)], with the most common oxidation states being the pentavalent arsenate, As(V) and the trivalent arsenite, As(III) (Cullen and Kenneth, 1989). Of these two, As(III) is 100 times more toxic than As(V) to living organisms (Neef, 1997) and has the ability to bind sulfhydryl groups of amino acids thereby inactivating a wide range of enzymes. In bacteria, no As(V) extrusion pump has been identified to date. As a detoxification mechanism, the less toxic As(V) is generally converted to the more toxic As(III) by the cytoplasmic arsenate reductase (ArsC), which is then pumped out of the cell by As(III) efflux pumps. This As(III) extrusion pump is sufficient for As(III) resistance while ArsC is required for resistance to As(V) (Slyemi and Bonnefoy, 2011).

Proteomics has widely been used in a variety of basic and applied research and its analysis provides valuable information about changes in the synthesis, degradation, post translation modifications, and interactions among proteins. An important aspect of proteomics is to study the expression levels and characterization of proteins in cells when exposed to different environmental conditions. There are very few reports of proteomic approaches being undertaken to study arsenite stress on microorganisms. Weiss et al. (2009) and Parvatiyar et al. (2005) have used this approach to examine arsenic stress in *Herminiimonas arsenicoxidans* and *Pseudomonas aeruginosa*, respectively and have reported the role of antioxidant enzymes, superoxide dismutase (SOD) and glutathione reductase, in the mechanism of As(III) resistance. Baker-Austin et al. (2007) have shown the importance of chaperone proteins, DnaK and HSP60, in the mechanism of As(III) resistance in *Ferroplasma acidarmanus*.

The genus *Klebsiella pneumoniae* is a group of gram-negative, non-motile, rod-shaped bacteria and have been known for their resistance and survival in the presence of several toxic compounds such as cyanide (Tang et al., 2009), tetracyanonickelate (Chen et al., 2010) and heavy metals like cobalt and lead (Bar et al., 2007). However, little is known about tolerance, adaptation
and the protein expression changes arising in *K. pneumoniae* in response to As(III) stress. In view of this, we have used a proteomic approach to analyze the differences in protein expression patterns arising in *K. pneumoniae* due to As(III) stress. This knowledge is important because it enables us to understand the physiological responses elicited by microbes during adaptation to arsenite stress. In this study, we have identified 39 differentially expressed proteins belonging to various functional classes and discuss their probable role in As(III) resistance.

2. Materials and methods

2.1. Isolation of arsenite tolerant bacteria

The arsenite tolerant bacteria were isolated from river Mullu, Pune, Maharashtra, India as described previously (Bar et al., 2007). The minimum inhibitory concentration (MIC) of arsenite metal was established with macrodilution method by using sodium salt of arsenite in Luria-Bertani (LB) broth (Hassen et al., 1998). Of 12 isolates screened, MR4 showing the highest MIC was identified using 16S ribotyping and was thus selected for further study.

2.2. Growth curve of *K. pneumoniae* (MR4)

To study the effect of metal concentration on growth of bacteria, *K. pneumoniae* (1.0%, v/v) was inoculated in LB broth with and without 2.5 mM As(III), and incubated for varying time periods at 28 °C under shaking conditions at ISOrpm. Growth was monitored spectrophotometrically at 600 nm until constant readings were observed in control and test samples.

2.3. Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM/EDS)

*K. pneumoniae* were grown in LB media for control (without As III) and test (with 2.5 mM As(III)) samples for 7 and 22 h, respectively, and harvested by centrifugation at 10,000 x g at 8 °C for 10 min. Samples were prepared for SEM and EDS analysis according to De et al. (2008).

2.4. Biotransformation of arsenite and arsinite

To test the ability of the isolates to reduce As(V) or oxidize As(III), *K. pneumoniae* was grown to mid-exponential phase in Tris Mineral Medium (TMM) with low phosphate content (Mergeay et al., 1985), and 1% was then inoculated into two 250 ml flask, each containing 100 ml of TMM with either 1 mM As(V) or As(III) to obtain an OD 600 nm of 0.05. Flask containing 1 mM As(V) or As(III) without cells were treated as control. Biotransformation of As(V) and As(III) was then determined according to the method described by Batche et al. (2009).

2.5. Sample preparation for 2-DGE

Cells were grown under control (7 h) and experimental conditions [22 h; 2.5 mM As(III)] in 250 ml of LB medium and harvested by centrifuging at 10,000 x g at 4 °C for 10 min. The pellets were washed twice with distilled water and resuspended in lysis buffer (3 ml/g wet cells) containing 2 M thiourea, 8 M urea, 4% CHAPS, 50 mM DTT, 150 μl of 1% (v/v) protease inhibitor cocktail and 1 M PMSF. Cells were then sonicated at 20 kHz for 8 min. in cycles of 2 s on and 5 s off. Cell debris was removed by centrifugation for 30 min at 10,000 x g and 4 °C. Proteins were precipitated by TCA (Nandakumar et al., 2003) and the protein content was determined by Bradford assay using bovine serum albumin as standard (Bradford, 1976).

2.6. Two-dimensional gel electrophoresis (2-DGE) and image analysis

Isoelectric focusing (IEF) was carried out using immobilized pH-gradient (IPG) strips (11 cm, linear gradient pH 4-7, ReadyStrip, Bio-Rad). Briefly, 450 μg of total protein was diluted with a rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 1 μl amphotolys) to 200 μl. The samples were loaded on the IPG strips in the IEF tray and were passively rehydrated for 16 h and focused (250V, 30 min; linear increase to 8000 V, 150 min; final focusing at 8000 V for a total of 23,000 Vh) at 20 °C in a PROTEINIEF CELL (Bio-Rad). For the second dimension electrophoresis, IPG strips were equilibrated for 30 min in buffer (6 M urea, 0.375 M Tris–HCL, pH8.8, 20% glycerol, 2% SDS, and 2% DTT) and equilibrated again for another 30 min in the same buffer, except that DTT was replaced by 2.5% (w/v) iodoacetamide. The proteins were subsequently separated on 12% SDS-PAGE in a Bio-Rad Protein cell.

For protein detection, gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R250 overnight. The destained wet gels were scanned with a densitometer (GS-800, Bio-Rad) and analysis of the gel images was performed using the PDQuest software (8.0.1; Bio-Rad). Spots were automatically detected on the basis of the spot parameters such as the faintest, the smallest and the largest spot on the gel scan. After the initial automatic spot detection, each gel scan was controlled visually in order to eliminate artifacts from the spot list and to mark undetected spots. After the initial spot detection the final recorded changes in protein levels were based upon densitometric analysis of three different sets of control and As(III) treated samples and only those spots that were detectable on all gels of a sample set were considered for evaluation. Fold change above 1.5 or below 0.75 with a p-value less than or equal to 0.05 were considered significant and calculated according to Jin et al. (2008).

2.7. Proteins identification by MALDI-TOF/TOF mass spectrometry

Protein spots of interest were excised from the gels and subjected to *in situ* digestion with trypsin as described previously (Weiss et al., 2009). The resulting 1 μl supernatant was mixed with 1 μl MALDI matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) saturated solution with 50% acetonitrile in 0.1% TFA). The total 0.8 μl solution was spotted on MALDI target disk and allowed to air-dry. The tryptic peptides were analyzed by MALDI-TOF/TOF with a 4800 Proteomics Analyzer (Applied Biosystems MDS SCX) which was fully automated with predefined laser pattern, probe motion pattern and peak intensity threshold for switching from the MS survey scan mode to MS/MS, and from one precursor mass to another. The mass spectra have been processed for noise removal, baseline correction, deisotoping, and then the Mascot (http://www.matrixscience.com) search engine was used for obtaining protein identities and peptide sequences using the entire protein database of NCBI and MSDB.

2.8. Cell extract preparation, non-denaturing gel electrophoresis, and superoxide dismutase (SOD) enzyme assays

Cells were grown under control (7 h) and experimental conditions [22 h; 2.5 mM As(III)] in 250 ml of LB medium and harvested by centrifuging at 10,000 x g at 4 °C for 10 min. The pellets were washed twice and resuspended in 100 mM phosphate buffer (pH 7.8). Cells were then sonicated and cell debris was removed by centrifugation for 30 min at 10,000 x g and 4 °C. Supernatant was used as source of crude enzyme for determination of SOD activity.
SOD activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm and by activity staining (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT. Native gel-electrophoresis was performed to determine SOD isozymes which were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide (KCN) and 5 mM hydrogen peroxide (H$_2$O$_2$) (Fridovich, 1986). Where applicable, statistics were performed using Student’s t-test, with all experiments being performed in triplicates.

3. Results and discussion

3.1. Identification of isolate MR4

The As(III) tolerating bacteria were isolated from the Mulla river in Pune, Maharashtra, India where industrial effluents are discharged, using the spread plate method. A total of 12 As(III) tolerant bacteria capable of growing in media containing 1 mM As(III) were isolated and it was noted that isolate MR4 exhibited maximal tolerance to As(III), as seen by monitoring growth at 600 nm and was thus chosen for further study. Using 16S rRNA sequencing, the As(III)-resistant bacterium, MR4, was identified as K. pneumoniae (Gene Bank database accession number HQ857583).

3.2. Effect of arsenite on growth of K. pneumoniae

The minimal inhibitory concentration (MIC) of sodium arsenite was determined to be 5 mM in LB broth for K. pneumoniae, where 99% inhibition of cell growth as seen by colony forming units (CFU) was noted (Fig. 1). Thus, the LD$_{50}$, 2.5 mM As(III), was selected for further studies. The growth curve of isolate in the presence and absence of As(III) is shown in Fig. 2. In the presence of As(III), K. pneumoniae showed a prolonged lag phase of 13 h as compared to 1 h for control without As(III). However, comparable levels of cell growth were observed during the exponential phase which was attained in 7 h for control and 22 h in As(III) stress. Cells grown on LB media have a doubling time (t$d$) of 1 h and 15 min whereas cells grown on 2.5 mM As(III) containing LB broth were found to have a t$d$ of 4 h and 28 min. Thus, it is likely that this differential growth rate would be reflected in the protein expression patterns of K. pneumoniae under As(III) stress.

3.3. Changes in cell morphology

The scanning electron micrographs of K. pneumoniae cells without (control) and with exposure to As(III) are shown in Fig. 3A and B, respectively. No change in the morphology of cells was observed in the presence or absence of As(III). The energy dispersive X-ray spectroscopy (EDS) analysis showed a distinct EDS signal corresponding to arsenic peak was observed in presence of As(III) loaded cells (Fig. 3D), however, no such peak was observed in control (Fig. 3C).

3.4. Arsenic transformation by K. pneumoniae

The ability of K. pneumoniae to oxidize or reduce arsenic was studied in order to verify the possible detoxification mechanism. It was found that K. pneumoniae was capable of reducing 1 mM As(V) into As(III) but could not oxidize As(III) to As(V) under aerobic conditions. However, As(V) reduction did not occur in controls (without cells) indicating that As(V) reduction was a microbial process. Thus the data suggests that K. pneumoniae has arsenate reductase which converts As(V) into As(III). It is likely, that the As(III) thus produced could be pumped out of the cell by an As(III) efflux pump as suggested by Slyemi and Bonnefoy (2011).

3.5. Proteomic analysis and functional classification of identified proteins

Any change in the environmental conditions would affect the physiological response given by the cell. This would in turn be reflected in the protein expression levels of the cell. Therefore, it was relevant to ascertain the expression levels of proteins which could be responsible for As(III) tolerance in K. pneumoniae. A proteomic analysis was carried out to investigate the stress response of K. pneumoniae to As(III). For each spot, the pixel volume was calculated based on spot intensity and area followed by normalization with the total pixel volume of all the spots in the gel image. Using the spot pixel as the basis for comparison we could calculate the intensity and compare the protein expression changes in control and As(III) grown cells.

Approximately 350 protein spots in the pl range from pH 4 to pH 7 and molecular weight from 6.5 to 116.25 kDa were separated on 2D gels in the absence (Fig. 4A) and presence of 2.5 mM As(III) (Fig. 4B). Of these, the 60 most intense protein spots present on all triplicate gels were excised from both control and test gels and 39 differentially expressed protein spots were successfully identified by MALDI-TOF-TOF with a significant mascot score >70 (p-value <0.05). The remaining 20 identified proteins had mascot scores <70 with p-value >0.1 and were considered as not statistically significant. These statistically significant spots were labeled on the 2D gel as shown in Fig. 4A and B. Each protein’s spot number, protein name, experimental and theoretical pl and molecular.
weight, mascot score, percentage of sequence coverage, accession number and the fold change in expression are given in Table 1. As shown in Fig. 5, these identified proteins were classified into the following functional categories viz., (A) outer membrane proteins, membrane transport and binding proteins; (B) antioxidant proteins; (C) stress proteins; (D) translation factor; (E) carbohydrate metabolism; (F) amino acid metabolism.

3.5.1. Outer membrane proteins (OMP), membrane transport and binding proteins

The exposure of K. pneumoniae to 2.5 mM As(III) resulted in an altered expression of several outer membrane proteins, particularly, porins and membrane transporter-binding proteins. Among the outer membrane proteins, three porins viz., OmpR, OmpK36 precursor, and OmpA precursor were down-regulated. The OmpR is a cytoplasmic transcription factor and have been shown in Escherichia coli to controls the expression of OmpF and OmpC which are homologous to OmpK35 and OmpK36, respectively, in K. pneumoniae (Martinez-Martinez et al., 1996). Hence, as OmpR is down-regulated, expression of OmpK36 is also concomitantly down-regulated. Antibiotic resistant strains of K. pneumoniae are known to be associated with metal resistance (Seyfried et al., 1989) while a porin deficient mutant of K. pneumoniae has been shown to exhibit antibiotic resistance (Martinez-Martinez et al., 1996). In our studies, as K. pneumoniae has been found to be resistant to antibiotics such as vancomycin (30 μg), sulphatriod (200 μg) and ampicillin (10 μg), it is likely that this organism increases As(III) resistance by down-regulation of OmpK36 porin. Another porin,
12

Table 1
Differentially expressed proteins (i.e., those that show changes in spot intensity) identified in K. pneumoniae grown under arsenite As(III) stress conditions.

<table>
<thead>
<tr>
<th>Spot No</th>
<th>Gene name</th>
<th>Protein name</th>
<th>pl observed/calculated</th>
<th>Mw (kDa) observed/calculated</th>
<th>Accession number (NCBI)</th>
<th>Mascot score</th>
<th>% SC</th>
<th>Protein level ratio (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0003</td>
<td>Crr</td>
<td>Phosphotransferase system enzyme II, glucose-specific factor III</td>
<td>4.73/4.82</td>
<td>18.24/19.20</td>
<td>QWECF3</td>
<td>238</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>0301</td>
<td>ompK36</td>
<td>OmpK36 protein precursor</td>
<td>4.52/4.46</td>
<td>40.73/38.28</td>
<td>Q9X393</td>
<td>91</td>
<td>46</td>
<td>1</td>
</tr>
<tr>
<td>4206</td>
<td>ompA</td>
<td>Outer membrane protein A precursor</td>
<td>6.00/5.9</td>
<td>38.02/3.69</td>
<td>J5058</td>
<td>542</td>
<td>76</td>
<td>1</td>
</tr>
<tr>
<td>7704</td>
<td>oppA</td>
<td>Oligopeptide transport periplasmic binding protein</td>
<td>5.95/6.35</td>
<td>60.97/50.84</td>
<td>Q2VQ07</td>
<td>82</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>8102</td>
<td>ompR</td>
<td>Omsensor response regulator ompR</td>
<td>6.04/6.6</td>
<td>27.24/29.68</td>
<td>RCECOR</td>
<td>128</td>
<td>46</td>
<td>1</td>
</tr>
<tr>
<td>9186</td>
<td>hiV</td>
<td>Probable cystine-binding periplasmic protein hiV</td>
<td>7.77/7</td>
<td>29.10/27.57</td>
<td>AC0225</td>
<td>75</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>9601</td>
<td>dppA</td>
<td>ABC transporter, periplasmic dipeptide binding protein</td>
<td>5.75/6.5</td>
<td>60.27/55.60</td>
<td>Q6644C</td>
<td>355</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>9505</td>
<td>atpD</td>
<td>H+-transporting two-sector ATPase beta chain</td>
<td>4.90/5.0</td>
<td>50.29/47.36</td>
<td>A93742A</td>
<td>294</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>7801</td>
<td>atpA</td>
<td>H+-transporting two-sector ATPase alpha chain</td>
<td>5.86/6.2</td>
<td>55.19/52.06</td>
<td>C65716</td>
<td>151</td>
<td>41</td>
<td>1</td>
</tr>
</tbody>
</table>

**Antioxidant enzymes**

| 0007 | Tpx       | Thiol peroxidase | 5.10/4.94 | 17.58/20.08 | AGO285 | 128 | 11 | 1 | 2.04 |
| 6007 | sodB      | Superoxide dismutase [Fe] | 5.58/6.1 | 21.12/22.81 | PO4D5 | 254 | 24 | 1 | 0.37 |
| 7003 | dps       | Dps protein | 5.66/6.3 | 17.84/16.43 | Q4B70 | 760 | 64 | 1 | 0.09 |
| 9106 | sodA      | Superoxide dismutase [Mn] | 6.44/6.28 | 22.93/24.55 | P66828 | 394 | 46 | 1 | 2.78 |

**Stress proteins**

| 0607 | Tig       | Trigger factor | 4.81/5.01 | 48.16/53.24 | D64773 | 321 | 31 | 1 | 1.69 |
| 0707 | HSFP60    | Heat shock protein [HSFP60] | 4.84/5 | 57.00/60.90 | AAC8741 | 149 | 73 | 1 | 1.97 |
| 0803 | dnaK      | Chaperone protein DnaK | 4.80/4.97 | 68.98/66.20 | A0057 | 600 | 27 | 1 | 2.29 |
| 2902 | htpG      | Chaperone protein HtpG (Heat shock protein htpG) | 5.03/5.25 | 70.74/69.33 | P58482 | 81 | 16 | 1 | 3.79 |

**Translation**

| 4504 | rpsA      | Ribosomal protein S1 | 4.89/5 | 61.12/61.96 | F64830 | 611 | 43 | 1 | 1.71 |
| 3903 | fusA      | Elongation factor G | 5.24/4.44 | 77.37/78.72 | Q837C | 171 | 39 | 1 | 1.52 |

**Carbohydrate metabolism**

**Glycolysis**

| 3501 | Eno       | Phosphoenolpyruvate hydratase/Enolase | 5.32/5.49 | 45.63/45.00 | G6059 | 633 | 43 | 1 | 0.67 |
| 9301 | fabB      | Fructose-bisphosphate aldolase class I (FBP aldolase) | 6.79/6.8 | 37.92/39.30 | Q7N3D6 | 178 | 18 | 1 | 0.31 |

**Pyruvate metabolism**

| 4912 | aceE      | Pyruvate dehydrogenase | 5.47/5.7 | 99.55/97.40 | AE0415 | 86 | 10 | 1 | 0.25 |
| 7302 | adhE      | Alcohol dehydrogenase | 6.14/6.25 | 39.49/41.08 | Q32G20 | 271 | 31 | 1 | 0.41 |
| 7407 | ackA      | Acetate kinase | 5.85/6.27 | 43.24/44.44 | Q33G80 | 216 | 37 | 1 | 0.65 |

**Pentose phosphate pathway**

| 1202 | talB      | Transaldolase B | 5.10/5.16 | 35.07/34.92 | P0A871 | 416 | 31 | 1 | 1.59 |
| 1506 | EC5342    | Transketolase I isomerase | 5.48/5.88 | 72.11/69.33 | Q83CQ4 | 90 | 11 | 1 | 1.56 |

**TCA cycle**

| 2503 | acnB      | Acetil-CoA/CoA ligase | 5.28/5.35 | 93.43/94.37 | Q7UD9R | 100 | 18 | 1 | 2.17 |
| 3201 | Mdh       | Malate dehydrogenase (Fragment) | 5.81/5.6 | 28.46/31.00 | Q5X9X | 966 | 56 | 1 | 0.68 |

| 7612 | lpdA      | Dihydrolipoamide dehydrogenase | 5.79/6.4 | 50.66/53.62 | S45155 | 377 | 39 | 1 | 2.26 |
| 7812 | fndA      | Fumarate reductase | 6.18/6.27 | 66.10/66.20 | S05017 | 318 | 21 | 1 | 0.25 |

| 8204 | sucD      | Succinate-CoA ligase (ADP-forming) | 6.32/6.6 | 29.76/31.00 | A90499 | 150 | 54 | 1 | 1.83 |
| 9101 | EC0749    | Succinate dehydrogenase | 6.32/6.66 | 26.71/28.96 | E90722 | 127 | 41 | 1 | 0.65 |

**Amino acid metabolism**

| 2604 | aspA      | Aspartate ammonia-lyase | 5.19/5.35 | 52.32/49.71 | A01159S563 | 334 | 21 | 1 | 0.72 |
| 7004 | wrbA      | Trp repressor-binding protein | 5.59/6.3 | 20.83/23.08 | B64842 | 263 | 22 | 1 | 0.40 |
| 7609 | hpaB      | 4-Hydroxyphenylacetate 3-monooxygenase | 5.89/6.35 | 58.81/58.00 | Q31520 | 215 | 38 | 1 | 0.45 |

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C: sequence coverage.
L: control/grown in LB.

* Spot number as stated in Fig. 4A and B.
* Scores in MAQC/MS greater than 70 were considered significant (p < 0.05).
* Protein expression level in As(III) treated compared with control (without stress) (p < 0.05). Expression level in control was set at 1.

OmpA, K. pneumoniae, amino acids, down-regulation, arsenic, amino acid catabolism.
important mechanism of tolerance during environmental stress against toxic compounds such as phenol (Rodrigues et al., 2010). In this study, two ABC transporter proteins were down-regulated and identified as ABC periplasmic dipeptide binding protein precursor (DppA) and Oligopeptide transport periplasmic binding protein (OppA) which are involved in the transport of dipeptides and oligopeptides, respectively. DppA has previously been shown to be down-regulated, as a mechanism of zinc resistance in E. coli (Easton et al., 2006). Thus, it is likely that in our study, down regulation of OppA, DppA, and OmpA could be a mechanism of As(III) resistance.

As shown in Table 1, the H+ transporting two-sector alpha chain and beta chain ATPase (ATP synthase), which generates energy in the form of ATP was up-regulated. This enzyme has been shown to play an important role in Candidatus Pelagibacter ubique under nutrient stress (Sowell et al., 2008) as well as in Deinococcus geothermalis under radiation stress (Tian et al., 2010).

One membrane transport and binding protein namely a cystine-binding periplasmic protein (IIY), which catalyzes the transport of cystine into the cytoplasm, was found to be up-regulated. In a significant study, Iwao et al. (2010) showed that an inducible l-cysteine-l-cystine shuttle system plays an important role in the detoxification of hydrogen peroxide (H2O2) in E. coli. Since catalase does not occur in the periplasm, the H2O2 generated by the electron transport chain in the inner membrane of the E. coli is detoxified by the reducing equivalents provided by this shuttle system. Thus, it is likely that such a cystine binding and transport protein in K. pneumoniae plays a similar role in the detoxification of H2O2 generated during As(III) stress. This, in turn, is supported by the observation that superoxide dismutase was also found to be up-regulated during As(III) stress (Section 3.5.2).

A phosphotransferase system (PTS) enzyme, glucose-specific factor III PTS enzyme II (hexose permease) was down-regulated. Earlier studies suggest that As(III) enters into the cell via an aquaglyceroporin in E. coli (Meng et al., 2004) or by hexose permease as in Saccharomyces cerevisiae (Liu et al., 2004). Since, we were unable to identify the aquaglyceroporin homologous protein on the K. pneumoniae proteome map, it seems that K. pneumoniae responds to As(III) stress by reducing hexose permease through which As(III) may be entering into the cell.

3.5.2. Antioxidant enzymes

Four antioxidant proteins identified on the proteome map of K. pneumoniae, under As(III) stress were Dps protein, superoxide dismutase (Mn and Fe SOD), and thiol peroxidase. Collectively, they function to keep the concentrations of ROS generated during metal stress at a low level, thereby minimizing damage to the cell components.

The protective role of Dps is achieved via binding to DNA leading to its compaction. It reduces the production of oxidative radicals by sequestering metal ions such as iron that engage in fenton reaction. Dps is also known to neutralize toxic peroxides through its ferroxidase activity. Surprisingly, in our study, a significant decrease in the concentration was noted. Li et al. (2008) have also reported that Dps protein is down-regulated in streptomycin (SM) resistant E. coli and this negative regulation was functionally linked to its SM resistance. It is likely that a similar phenomenon could be occurring in K. pneumoniae under As(III) stress.

Among the antioxidant enzymes, Mn-SOD was up-regulated while Fe-SOD was down-regulated. However, an up-regulation of Fe-SOD has been reported in H. arsenicoxidans (Weiss et al., 2009) and P. aeruginosa (Parvatiyar et al., 2005) under As(III) stress. Earlier studies, however, have shown that in heavy metal resistant Streptomyces sp., Fe SOD was repressed (Schmidt et al., 2007) while in Enterobacter liquefaciens under Co(II) stress Mn-SOD was
up-regulated (Marrero et al., 2004), similar to the results observed in this study. Up-regulation of Mn-SOD is in concurrence with up-regulation of htpG protein mentioned earlier in Section 3.5.1. Both are required for detoxification of ROS. Thioredoxin, a coenzyme, was up-regulated which play a role in the detoxification of exogenous peroxides produced via the electron transport chain during As(III) stress. These observations suggest that the above antioxidant enzymes may be required to protect the K. pneumoniae from oxidative damage resulting from exposure to As(III).

3.5.3. Stress proteins

In this study, five stress proteins were up-regulated under As(III) stress and identified as chaperon protein htpG, DnaK type molecular chaperones, trigger factor, Hsp60 and a protein similar to GroES protein. The heat shock protein htpG, which is a homolog of Hsp90, has been shown to play a role in the acclimation to oxidative stress in C. freundii and Synechococcus sp. PCC 7942 (Hossain and Nakamoto, 2003). Molecular chaperone DnaK, a key factor for correct protein folding, renaturation or degradation and protein–protein interactions was also found to be up-regulated under As(V) and As(III) stress in F. acidarmanus (Parvatiyar et al., 2005). In K. oxytoca, also an up-regulation of DnaK under tetracyanonickelate (II) (TCN) stress has been reported (Chen et al., 2010). The trigger factor, another highly conserved molecular chaperone is a ribosome-associated peptidyl-prolyl cis-trans isomerase, central to protein biogenesis and bacterial survival under environmental stress, operates by binding to nascent polypeptides (Deuerling et al., 1999). Thus, in K. pneumoniae As(III) resistance may be enhanced by the up-regulation of these stress proteins which are known to protect proteins and cells during oxidative and heavy metal stress.

3.5.4. Protein involved in translation

Two proteins involved in translation were up-regulated under As(III) stress and identified as 30S ribosomal protein S1 and elongation factor EF-G (Table 1), thereby reflecting a need for synthesis of proteins during As(III) stress.

3.5.5. Carbohydrate metabolism

Proteins involved in carbohydrate metabolism [glycolysis, pyruvate metabolism, TCA cycle, and pentose phosphate pathway (PPP)] showed altered responses under As(III) stress. Among the glycolytic enzymes, fructose bisphosphate aldolase class I and enolase were down-regulated. Glyceraldehyde-3-phosphate dehydrogenase (GPDH) was up-regulated. CPDH, a central glycolytic enzyme catalyzes the interconversion of glyceraldehyde-3-phosphate and glyceraldehyde 1, 3 bisphosphate and NADH. This enzyme was also found to be up-regulated under osmotic stress in Lactobacillus rhamnosus, resulting in the formation of glycerol-modified sugars (Prasad et al., 2003). Thus, the increased expression of GPDH and reduced expression of enolase implies that during glycolysis a probable accumulation of reducing equivalents (NADH) and glyceraldehyde-3-phosphate, a precursor for glycerol and amino acid biosynthesis, occur.

Pyruvate metabolism is the link between glycolysis and TCA cycle and determines the routes for carbon flux. In K. pneumoniae, four proteins involved in pyruvate metabolism and six proteins involved in the TCA cycle were differentially expressed under As(III) stress (Table 1). Among the pentose phosphate pathway (PPP), three proteins were up-regulated in As(III) stress and identified as transaldolase B, transketolase 1 isozyme, and phosphoglucomutase. These enzymes are involved in the conversion of carbohydrates to 5-C sugar phosphates and the 3-C glyceraldehyde-phosphate, which in turn are precursors for biosynthesis of amino acids, purine and pyrimidine nucleotides. Similarly, Henne et al. (2009) have also reported that transaldolase and transketolase were up-regulated in Arthrobacter sp. under chromate stress and provide a link between the glycolytic and PPP.

3.5.6. Amino acid metabolism

Under arsenite stress, three proteins involved in amino acid metabolism were down-regulated in K. pneumoniae, and were identified as Trp repressor-binding protein (wrbA), 4-hydroxyphenylacetate 3-hydroxylase (hpaA) and aspartate ammonia-lyase (aspA). Trp repressor-binding protein is a transcription factor involved in controlling amino acid metabolism (Zhang et al., 1987). The aspA cleaves carbon–nitrogen bonds and participates in alanine, aspartate, and nitrogen metabolism while hpaA is involved in tyrosine catabolism. K. pneumoniae has been shown to metabolize 4-hydroxyphenylacetate (4-HPA) through a meta-cleavage pathway with succinate and pyruvate as the final products (Martin et al., 1991). As outer membrane porins involved in transport of amino acids and peptides were down-regulated, it is possible that the cellular levels of amino acids such as tryptophan, tyrosine, and aspartate need to be maintained by repression of their catabolic enzymes.

3.6. Spectrophotometric assay and activity staining of SOD

Superoxide dismutase (SOD) constitutes one of the first line of defense against ROS, which is crucial for the removal of O$_2^-$ in the compartments where O$_2^-$ radicals are formed. Cell extracts were examined for total SOD activity as well as isozyme profiles. The total SOD specific activity detected in control is 7.85 U/mg protein and 9.49 U/mg protein in As(III) treated cells. Fig. 6A). Activity staining revealed the expression of two SOD isozymes in K. pneumoniae under As(III) stress and control condition (Fig. 6B). The gel data suggests that Fe-SOD was down-regulated (Fig. 6C) while Mn-SOD was
Fig. 7. Probable mechanisms of arsenic tolerance in Klebsiella pneumoniae. (1) Up-regulation and (2) down-regulation.

up-regulated (Fig. 6C and D) under As(III) stress. This data supports the proteomic data (Section 3.5.2), wherein up-regulation of Mn-SOD and down-regulation of Fe-SOD has been seen. The present study suggests that SOD may play a protective role on exposure of K. pneumoniae cells to As(III) stress.

Thus, the results obtained in this study suggest that multiple factors, which include down-regulation of outer membrane proteins, and up-regulation of stress and antioxidant proteins, play an important protective role during arsenite stress. It is also noted that up-regulation of GDPH and PPP would direct the carbon flux between glycolysis, central metabolism and anaerobiosis (Fig. 7).

4. Conclusions

We report here for the first time a profile of the K. pneumoniae (HQ857583) proteome under 2.5 mM As(III) stress. Identification of proteins showing differential expression in response to As(III) revealed that the As(III) stress response of K. pneumoniae is a complex process.

The expression of some proteins appears to be induced while others are repressed. Several key changes were observed in cellular physiology, including alterations in porins, membrane transport and solute binding proteins, antioxidant proteins, stress proteins and the enzymes of the PPP. Further experiments to confirm the findings are currently under way.

The present work provides early insight into the probable mechanisms by which K. pneumoniae may be adapting to As(III) stress and may facilitate further investigations into the physiological aspects of this response.

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References


