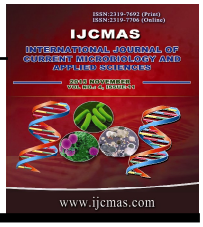
Int.J.Curr.Microbiol.App.Sci (2015) 4(11): 457-469

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 11 (2015) pp. 457-469**
http://www.ijcmas.com



Original Research Article

Mechanism of Arsenic Tolerance in *Klebsiella pneumoniae* (HQ857583)

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Keywords

Arsenic, *K. pneumoniae*, Resistance, SEM, AAS, Arsenate reductase

A B S T R A C T

The pollution of the environment with toxic arsenic is spreading throughout the world along with industrial progress. The ubiquity of arsenic in the environment has led to the evolution of the arsenic defense mechanism in certain microbes. The present study deals with characterization of arsenic tolerant bacteria *K. pneumoniae*. The arsenate reductase (*arsC*) gene present on both chromosome as well as on plasmid in *K. pneumoniae*, capable of reducing arsenate As(V) to arsenite As(III). The maximum arsenic accumulation was found during mid-log phase of *K. pneumoniae* under arsenic stress [1.92 ±0.04 mg As g^{-1} of As(V) treated dry bacterial pellet and 0.77± 0.03 mg As $g^{-1}$of As(III) treated dry bacterial pellet].

Introduction

Arsenic is a well-known toxic chemical and listed as a known carcinogen by the Environmental Protection Agency (EPA) and the World Health Organization (WHO, 2001). It is the 20th abundant element in the earth's crust but 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 ones, such as pesticides (e.g. calcium arsenate dimethylarsonate, disodium methylarsenate), production of paints and dyes, application of dusts from burning of fossil fuels, semiconductor industry, processing of pressure-treated wood [e.g. chromated copper arsenate (CCA)], coal combustion, high-temperature combustion and smelting of metals (Cullen and Reimer, 1989). In nature, the most common oxidation states for soluble arsenic exist as pentavalent arsenate [As(V)] and trivalent arsenite [As(III)] (Bhattacharjee and Rosen, 2007).

The ubiquity of arsenic in the environment has forced the evolution of arsenic defense mechanism in certain microbes to metabolize arsenic in an environment (Dhuldhaj *et al.*, 2013; Halter *et al.*, 2011; Poirel *et al.*, 2013). Arsenic metabolism genes mainly consists of *aio* genes (arsenite oxidase), *arr* genes (arsenate respiration) and *ars* genes (arsenate reduction and arsenate methylation) (Cai *et al.*, 2013). The *ars* operon, either chromosomally or plasmid encoded, is the most widespread

resistance mechanisms found in both Gram negative and Gram positive bacteria. The two most common types of these operons contain either five (*arsRDABC*) or three (*arsRBC*) genes (Silver and Phung 1996). This operon codes for (i) a regulatory protein (arsR), (ii) an arsenate permease (arsB), and (iii) a gene coding for an arsenate reductase (arsC) that converts arsenate to arsenite (Rosen, 2002). Arsenate (As V) is transported into the cell by the phosphate transporter (Pit system) and is subsequently reduced to arsenite (As III) by a cytoplasmic arsenate reductase enzyme encoded by the *arsC* gene. This (As III) is then effluxed from cells through the chemiosmotic gradient by the As (III)-specific transmembrane protein arsB encoded by the *arsB* gene.

Resistance against arsenic has been shown in various species of bacteria, including *K. pneumoniae*. *K. pneumoniae* is a group of gram negative, non- motile, rod shaped bacteria which have ability to oxidize As(III) to As(V) (Butt *et al.,* 2011; Abbas *et al.,* 2014) as well it is reports that *ars* operon is present in the *K. pneumoniae* which have ability to reduce As(V) to As(III). In view of above, the present study aims to describe a mechanism of arsenic resistance prevalent in *K. pneumoniae* which has been shown to have a high tolerance to many heavy metal ions like nickel, copper, cobalt, mercury, cadmium, lead, arsenate, arsenite etc (Daware *et al.,* 2015; Bar *et al.,* 2007; Essa *et al.,* 2002).

The ability of *K. pneumoniae* to oxidize or reduced arsenic was studied by transformation assay to verify the possible detoxification mechanism. The *arsC* gene was amplified from both plasmid and genomic DNA and sequenced. Further, to validate the mechanism, atomic absorption spectroscopy and scanning electron microscopy analysis was done.

Materials and Methods

Bacterial strain, media and growth conditions

The bacterial strain used in the present study is *K. pneumoniae* strain (HQ857583) (Daware *et al.,* 2012). It was maintained on Luria-Bertani (LB) medium. In the experiments 16 h old inoculums grown in LB broth were used. The cultures were maintained at 28°C for all experimental procedures (Daware *et al.,* 2015).

Growth curve of *K. pneumoniae* under different conditions

To study the effect of heavy metal concentration on growth of bacteria, 1.0% (v/v) inoculum was added in LB broth with varying concentration of arsenic [As(V) (100mM, 200mM, 250mM, 300mM) and As(III) (1mM and 2.5mM)] and without arsenic (control) and incubated at 28°C in shaker incubator (180 rpm). Bacterial samples were withdrawn at fixed intervals of time and growth of bacterium was monitored in terms of optical density (OD) at 600nm until the growth reached stationary phase (*i.e.* up to constant O.D. reading at 600nm). The growth curve was plotted and the generation time was determined.

Effect of arsenic on cellular morphology

To study the effect of arsenic on cellular morphology of *K. pneumoniae*, 1% inoculum was added into LB broth containing LD_{50} concentration of arsenic [either 200mM As(V) or 2.5mM As(III)] and without arsenic (control) and incubated at 28°C in shaker incubator (180 rpm). Samples were withdrawn at varying time periods (i.e. lag, log, late log), such as 30 min, 7h, 10h for control; 8h, 15h, 22h for As(III) and 7h, 16h, 26h for As(V).

Samples were prepared for SEM and EDS according to De *et al.* (2008). Following incubation, both control and arsenic stressed bacterial cells were harvested by centrifugation at 6,000g for 10 min, 4°C. The pelleted cells were washed twice with 0.1M potassium phosphate buffer solution (PBS; pH 7.2) and fixed overnight in 2 % glutaraldehyde in 0.1M PBS at 8°C. The cells were washed again with PBS and then distilled water before further processing. The cells were dehydrated through a series of different concentrations (10% to absolute) of ethanol by holding them in each concentration for half an hour. Finally, 2 µl of each sample was placed on 1mm clean and grease free glass slides. The glass slides were coated with platinum and examined under SEM with the acceleration voltage of 20 kV. Energy dispersive x-ray spectroscopy (EDS) was performed to detect the presence of arsenic over cell surface or in exopolymeric substance (EPS) produced by the cell.

Accumulation of arsenic by the *K. pneumoniae*

The glassware was washed with 30% nitric acid (HNO_3) and rinsed with distilled water. Three independent experiments were done. An aliquot of (1%) inoculum was added in each flask containing 250 ml LB broth with LD_{50} concentration of arsenic [either 200mM As(V) or 2.5mM As(III)] and incubated at 28°C in shaker incubator (180 rpm). Samples were withdrawn at varying time periods (i.e. lag, log, late log, stationary), such as 8h, 15h, 22h, 30h for As(III) and 7h, 16h, 26h, 40h for As(V) and cells were harvested by centrifugation at 10,000g for 10 min. The pellet was dried in oven at 60°C till constant weight and further processed for determination of total arsenic in the cell mass. For determination of total arsenic in the cells mass, dried cell pellets were digested with concentrated HNO_3 and 30% H_2O_2 (3:1) in microwave digester at 200°C for 15 min. The digested samples were brought to a constant volume by dissolving in distilled water before analysis and analyzed on ICP-AES. All the experiments were performed in triplicate and the average values were determined.

Accumulation capacity (mg/g) calculated by

$$Q = \frac{\text{arsenic reading of cells x volume of the reaction mixture (L)}}{\text{Dry weight of the cells (g)}}$$

Biotransformation of arsenic by the *K. pneumoniae*

To test the ability of the isolates to reduce As(V) or oxidize As(III), *K. pneumoniae* was grown in Tris Mineral Medium (TMM) with low phosphate content (Mergeay *et al.*, 1985). An aliquot (1 %) (v/v) of inoculum was added into two 250 ml flasks, each containing 100 ml of TMM with either 1 mM As(V) or 1 mM As(III) and incubated at 28°C in shaker incubator (180 rpm). One ml samples were withdrawn at various time intervals to assess the cell growth as well as to determine arsenic concentration spectrometrically (Bachate *et al.*, 2008). Flask containing 1 mM As(V) or 1 mM As(III) without cells were treated as control. The As(V) concentration was determined by acidifying 100 µl samples with 100 µl of HCl (24 mM). A 100 µl of the acidified sample was then added to 900 µl of the reaction mixture containing the following: ammonium molybdate (6 g l^{-1}), ascorbic acid (10.8 g l^{-1}), potassium antimonyl tartahrate (0.136 g l^{-1}) and concentrated H_2SO_4 (67.3 ml l^{-1}). Each component was stored as a separate solution. Samples were placed in a hot water bath at 78°C for 10

min subsequently placed on ice for 5 min. The absorbance at 865 nm was compared to acidified As(V) standards. The As(III) concentration was determined by oxidizing a second sample in 100 µl of KIO_3 (5 mM) and HCl (48 mM) for 10 min and then OD measured at 865 nm. Blanks of milli-Q water were used to calibrate the spectrophotometer. New plasticware was used each time to avoid contamination due to excess phosphate from detergents. Standard curves were prepared for concentrations of 0–100 µmol l^{-1} for both As(V) and As(III). The difference between oxidized and unoxidized samples represented the concentration of As(III).

Arsenic resistant gene in the *K. pneumoniae*

A polymerase chain reaction (PCR) based approach was performed to identify arsenate resistance gene. Chromosomal DNA was extracted by using Bacterial Genomic DNA isolation kit (Sigma Aldrich, USA) and plasmid of *K. pneumoniae* was extracted according to Mansi El-Mansi *et al.* (2000). The integrity of DNA was checked on gel electrophoresis and samples were stored at -20°C. The extracted samples were used to identify the presence of arsenate reductase (arsC) in *K. pneumoniae.* The oligo nucleotide primers used in this study were designed by comparing the known reported sequences of *arsC* (arsenate reductase gene) from *K. pneumoniae*. The consensus sequence conserved in an arsenic-resistant *K. pneumoniae* found in the GenBank database (retrieved from NCBI) were used for designing specific primers for the genes. Four primer sets were designed for *ars*C genes are

F 5'GAGCATCACCATTTACCATAA 3', arsC R 5' TATTTCACACGCTGCCCTGC 3';

F 5' GAGCAACATCACTCATTTATCAC AA 3', arsC R 5' ACTTTRTCYGTC TTC CTT 3; F 5' GAGCAACATTACCATTT ATCACAA 3', arsC R 5' TCTCACCGTC CTCTTTCGT 3; F 5' GAGCAACATM ACYATYTATCACAA 3', arsC R 5' TTTT CGCCATCTTCCTTR 3. These primer sets were used to amplify *arsC* gene. PCR reactions for *arsC* gene fragments selected in this work was performed in a final volume of 50 µl containing: 10 ng DNA, 0.2 mmol l^{-1} of dNTPs, 1.75 mmol l^{-1} $MgCl_2$, 0.4 µmol l^{-1} of each primer, 2 U of Taq polymerase and 1X PCR buffer. DNA amplification conditions for *arsC* were: initial denaturation at 95°C for 1.5 min, 40 cycles of 94°C for 45s, 55°C for 45s, 72°C for 45s and then a final extension step at 72°C for 10 min and then PCR products were checked on 1.8% agarose gel and visualized by ethidium bromide dye and documented by using the Gel Doc image analyzer system (Alpha Innotech) and then sequenced.

Statistical analysis

All experimental values represent the mean of at least three independent determinations.

Results and Discussion

Growth curve in presence of arsenic stress

Growth profile of *K. pneumoniae* in LB with different concentrations of As(V) (100mM, 200mM, 250mM, 300mM) and different concentration of As(III) (1mM and 2.5mM) were studied. The Growth profile in presence of above concentration of As(V) and As(III) showed significant difference than control (*K. pneumoniae* in LB without arsenic). The Log phase of *K. pneumoniae* in presence of As(V) and As(III) was observed after a prolong lag phase of 6h to

13h in different concentration of As(V), 6h to 9h in different concentration of As(III) compared to 1 hour in control without arsenic. The degree of growth in response to metal ions varied with the form of arsenic [either As(V) or As(III)] and the concentration of the metal ion supplemented in the medium (Fig. 1 and 2). The generation time of *K. pneumoniae* in control and experimental condition given in table 1.

Changes in cell morphology in presence of arsenic stress

The scanning electron micrographs of *K. pneumoniae* cells without arsenic (control) and with exposure to arsenic [As(III) and As(V)] were studied in different phases of growth cycle including lag, log and late log (Fig. 3). Cellular morphology of *K. pneumoniae* in the presence of As(V) and As(III) was unchanged in log and late log phases (Fig. 3D to I) where as in lag phase, cells became elongated (Fig. 3B and C). The energy dispersive X-ray spectroscopy (EDS) analysis showed a distinct EDS signal corresponding to arsenic peak was observed in presence of As(III) and As(V) loaded cells (Fig. 3K and L) however, no such peak was observed in control (Fig. 3J).

Accumulation of arsenic by *K. pneumoniae*

Since the growth phase is a biotic variable that can affect metal uptake by a bacterial population, the arsenic uptake [i.e. As(V) and As(III)] was determined during the growth cycle of *K. pneumoniae.* The maximum arsenic accumulation was found during mid-log phase of *K. pneumoniae* in arsenic stress [1.92 ±0.04 mg As g^{-1} of As(V) treated dry weight of bacterial pellet and 0.77± 0.03 mg As $g^{-1}$of As(III) treated dry weight of bacterial pellet] after this the reduction in intracellular arsenic concentration was observed in both which means the organism started to produce an efflux of the intracellular arsenic into the extracellular environment (Fig. 4 and 5).

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. The growth profile of *K. pneumoniae* in presence of 1mM As(V) and 1 mM As(III) supplemented in TMM medium is shown in figure 6.

Arsenate reductase gene (arsC) in *K. pneumoniae*

To know the presence of arsenic resistant gene, arsenate reductase (arsC) in *K. pneumoniae* and check whether this genes present on chromosome or plasmid; we designed different sets of primers that allow for the specific amplification of the arsC gene in *K. pneumoniae*. However, there was positive PCR amplification for arsC gene (nearly 400bp expected) in *K. pneumoniae* (Fig. 7).

>*arsC* sequence in plasmid

GAGCATCACCATTTACCATAACCCTG
AATGCGGCACCTCGCGTAATACCCTG
GCGCTGATCCGCAACAGCGGCGCTGA
GCCCACCATTATCTATTATCTGGAGA
CGCCGCCGTCGCGCGATGAGCTGCGT
CAGCTGATCGCCGCGATGGCGATCCC
GGTGCGGGCGCTGCTGCGGCAAAAC
GTCGAACCCTACGATGCGCTGGGCCT
GGCGGAAGACCGGTTTACAGATGACC
AGTTAATCGACTTTATGCTCCAGCAT

CCGATCCTGATTAACCGCCCGATCGT
GGTGACGCCGCGGGGCACCCGGCTCT
GCCGCCCGTCAGAGGTGGTGCTGGAG
ATCCTGCCGGCGCCGCAAAAAGGCG
CCTTCGTGAAGGAAGACGGTGAGCG
GGTCATTGATCGGGCAGGGCAGCGTG
T

>2308 *arsC* sequence in chromosome
TGGAGCATCACCATTTACCATAACCC
TGAATGCGGCACCTCGCGTAATACCC
TGGCGCTGATCCGCAACAGCGGCGCT
GAGCCCACCATTATCTATTATCTGGA
GACGCCGCCGTCGCGCGATGAGCTGC
GTCAGCTGATCGCCGCGATGGCGATC
CCGGTGCGGGCGCTGCTGCGGCAAA
ACGTCGAACCCTACGATGCGCTGGGC
CTGGCGGAAGACCGGTTTACAGATGA
CCAGTTAATCGACTTTATGCTCCAGC
ATCCGATCCTGATTAACCGCCCGATC
GTGGTGACGCCGCGGGGCACCCGGCT
CTGCCGCCCGTCAGAGGTGGTGCTGG
AGATCCTGCCGGCGCCGCAAAAAAG
GCGCCCTTTTTCGTGGT

This amplified product shows 99.9% similarity with *arsC* gene of *K. pneumoniae* 342 and *K. pneumoniae* KCTC 2242. The *arsC* gene present on chromosome as well as on plasmid and there is 95% sequence similarity.

Microbes encounter metals and metalloids in the environment and it is therefore expected them to interact with metals (Ehrlich, 1997). The species *K. pneumoniae* is gram-negative, non-motile, rod-shaped bacterium and has been known for its 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), mercury, silver, cadmium (Zeroual *et al.,* 2001). However, little is known about tolerance and adaptation of *K. pneumoniae* to arsenic.

The growth curve of *K. pneumoniae* showed a prolonged lag phase at different concentrations of arsenic [As(V) and As(III)]. It was observed that there was reduced growth with increase in the As(V) concentration in the medium due to the toxicity of arsenic at higher concentration. Also, there was depression in the log phase and delay in attainment of stationary phase in the presence of arsenic. The prolonged lag phase in presence of metal can be due to its affect on membrane permeability to transport the nutrient for normal growth of the organism. Guo *et al.* (2010) and Srinath *et al.* (2002) reported that the lag phase prepares the cellular metabolism against toxic elements of the environment such as metals.

Morphological changes of *K. pneumoniae* under arsenic were studied by scanning electron microscopy. In the presence of arsenic, in the late loge phase there is no change in cell morphology in the presence of As(V) and As(III) whereas cells become elongated in lag phase indicating possible interference of these metals with regulation of bacterial cell wall biosynthesis (Vaituzis *et al.,* 1975). Cell elongation could result from incomplete reproduction process because of the starvation related lack of structural molecules (Fulladosa *et al.,* 2006).

It was also supported by growth curve data that prolong lag phase of *K. pneumoniae* under arsenic stress as compared with control. From the EDS signal it was evident that the arsenic was mostly entrapped in the EPS of *K. pneumoniae*. This could be due to efflux commonly seen in gram-negative bacteria as a detoxification measure as reported by Nies (1999). Extracellular matrices with negative charges form metal complexes so as to confer resistance to the cell.

Table.1 Generation time of *K. pneumoniae* at different concentration of arsenic

Arsenic (mM)	Generation time (min)
Control (Only LB)	46
100 As(V)	180
200 As(V)	235
250 As(V)	266
300 As(V)	290
1 As(III)	128
2.5 As(III)	268

Fig.1 Growth profile of *K. pneumoniae* in LB with different concentrations of arsenate (100mM, 200mM, 250mM, 300mM). The Growth profile in presence of arsenate shows significant difference than control (only LB)

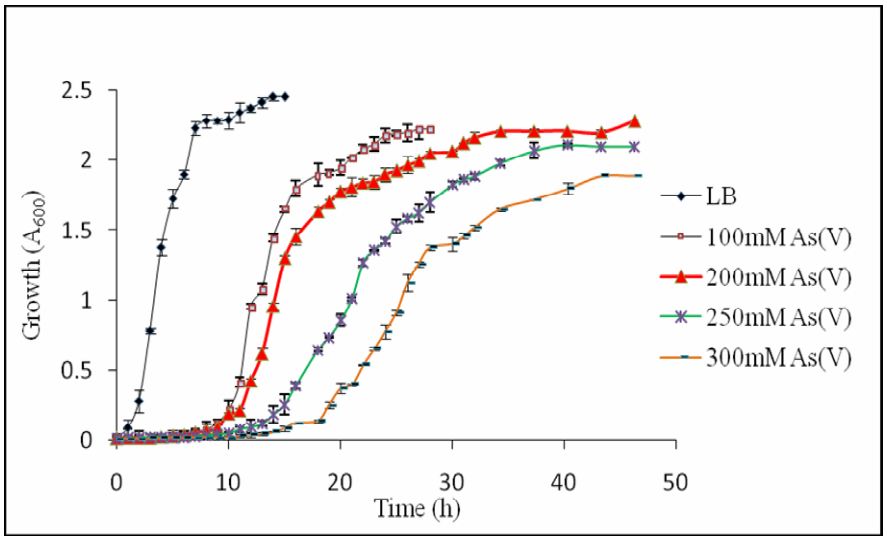


Fig.2 Growth profile of *K. pneumoniae* in LB with different concentrations of arsenite (1mM, 2.5mM). The Growth profile in presence of arsenite shows significant difference than control (only LB)

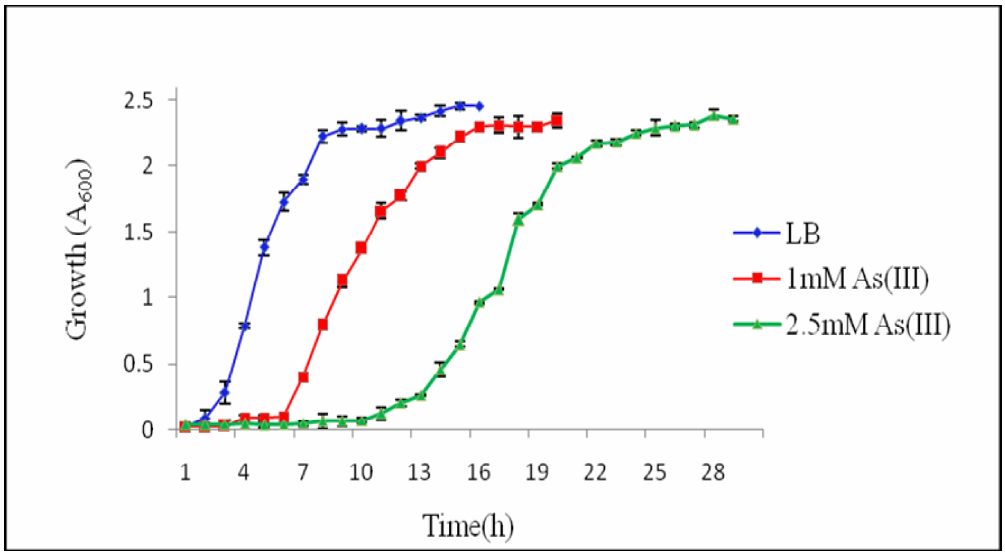
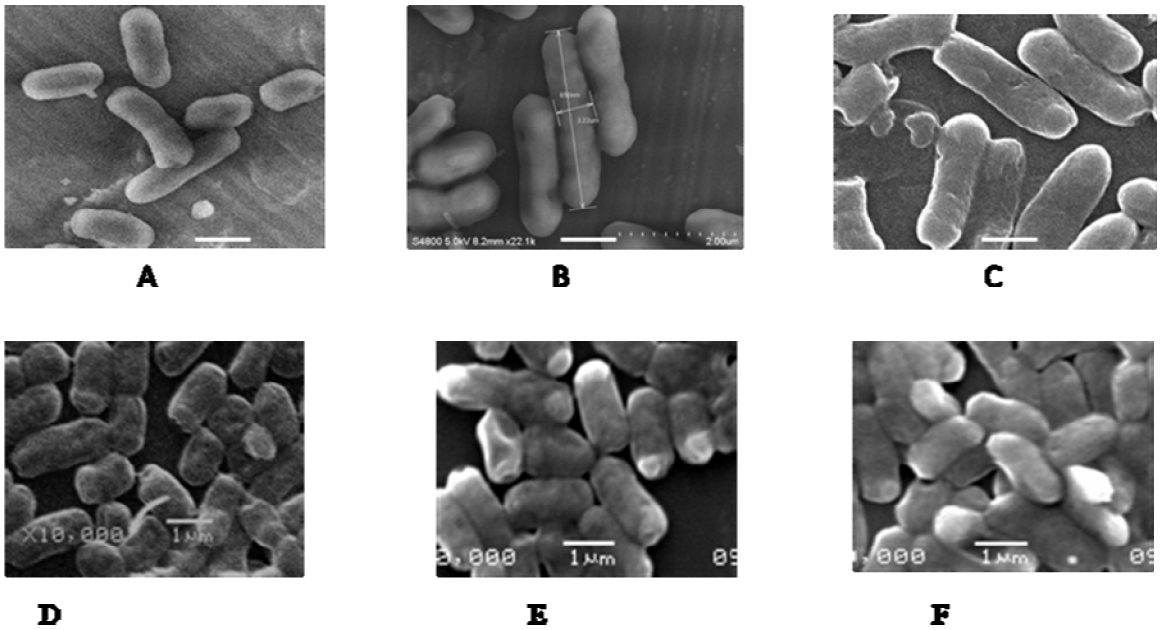


Fig.3 Scanning electron micrographs of *K. pneumoniae* in control [A (lag phase), D (log phase) and G (late log phase)]; in the presence of 2.5mM As(III) [B (lag phase), E (log phase) and H (late log phase)]; in the presence of 200mM As(V) [C (lag phase), F (log phase) and I (late log phase)] (magnification 10,000 X; bar at the base centre represents 1µm), representative energy dispersive X- ray spectrum of late log phase SEM image of samples shown in G, H and I in the absence of arsenic (J), in the presence of 2.5mM As(III) (K) and in the presence of 200mM As(V) (L)



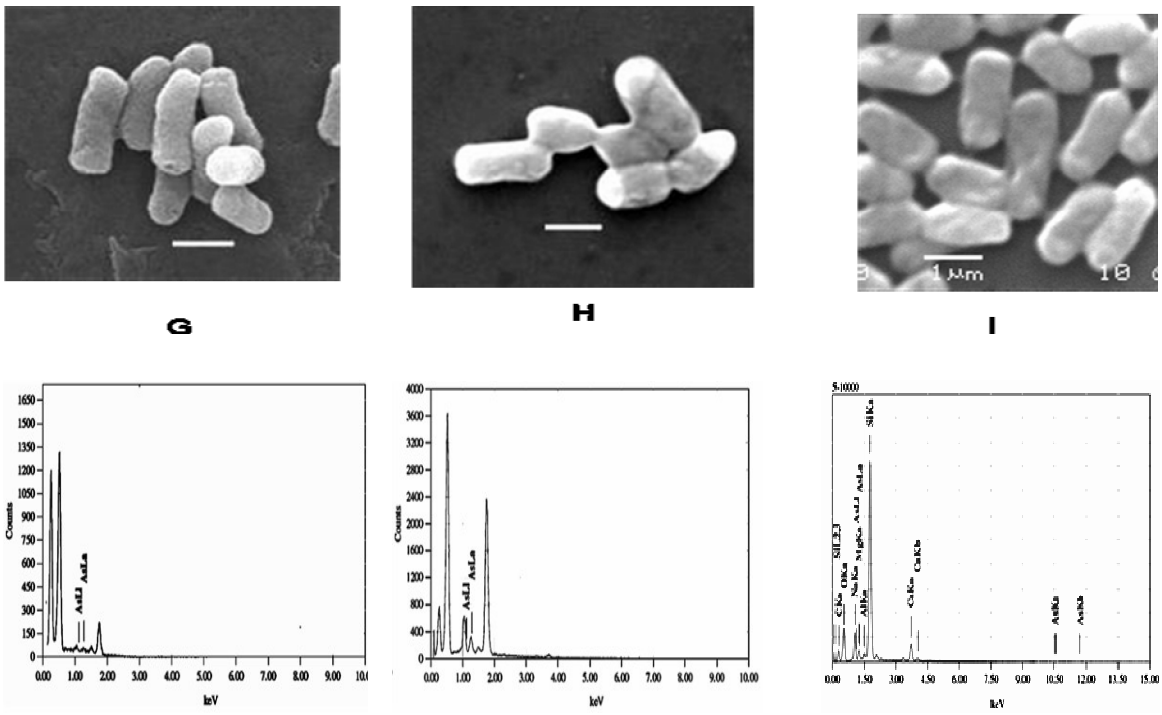


Fig.4 Arsenic accumulation in *K. pneumoniae* in different growth phases in presence of 200mM As(V). (7h- lag phase; 16h- mid log phase; 26h- late log phase; 40h- stationary phase)

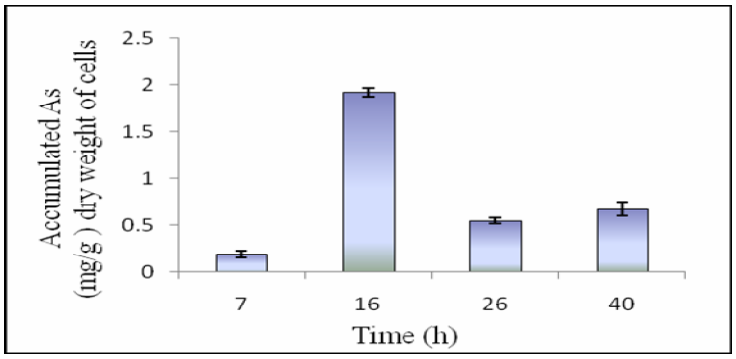


Fig.5 Arsenic accumulation in *K. pneumoniae* in different growth phases in presence of 2.5mM As(III). (8h- lag phase; 15h- mid log phase; 22h- late log phase; 30h- stationary phase)

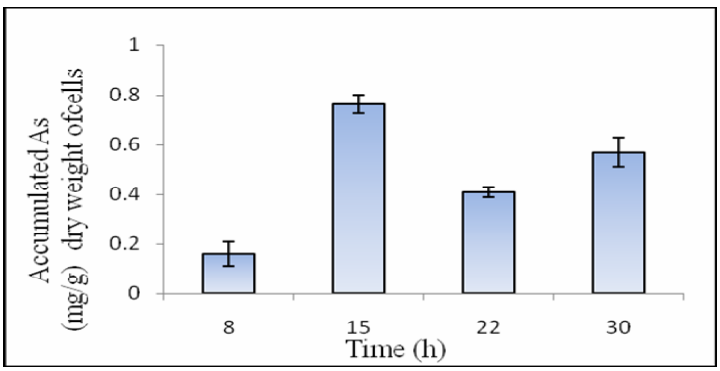


Fig.6 Biotransformation of arsenic by *K. pneumoniae* (A) Biotransformation of 1mM As(V) in TMM medium (B) Biotransformation of 1mM As(III) in TMM medium. (: represents growth; (: represents biotransformation

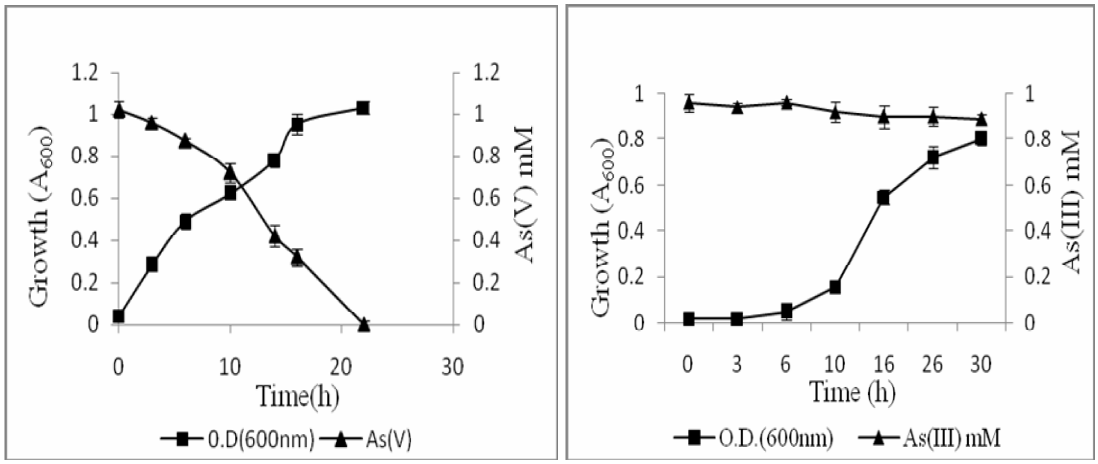
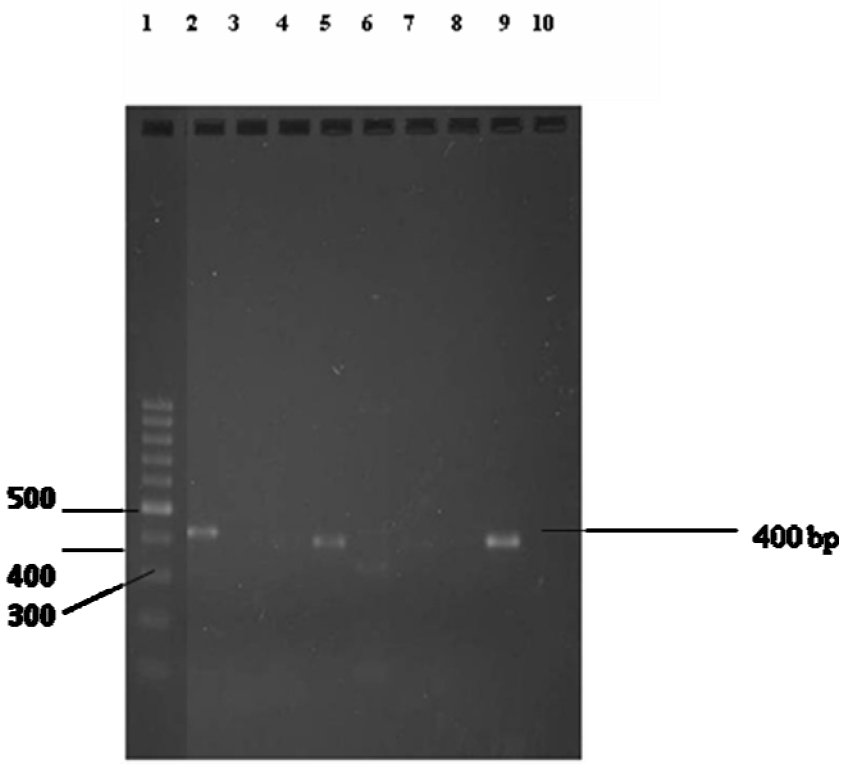


Fig.7 PCR amplification of arsC gene fragments, lane 1: DNA ladders (100-1000bp), lane 2, and 5: PCR amplification of arsC gene fragment from genomic DNA using 1st and 4th primer sets respectively, lane 9: PCR amplification of arsC gene fragment from plasmid using 4th primer sets, lane 10: Negative control.



Due to the abundance of arsenic in the environment, bacteria have evolved a variety of mechanisms for coping with arsenic toxicity and few of them can tolerate higher

concentration of arsenic (Baker-Austin *et al.,* 2007; Anderson and Cook, 2004). As a detoxification mechanism, As(V) is converted to 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) extrusion pump is sufficient for As(III) resistance while ArsC is required for resistance to As(V) (Slyemi and Bonnefoy, 2011). Also, some microbes have arsenite oxidase which converts As(III) into As(V). This enzyme was present in various bacterial strains including *H. arsenicoxydans* (Weeger *et al.,* 1999), *K. pneumoniae* (Butt *et al.,* 2011). Some microbes also sustain the arsenic inside the cell via accumulation in the cytoplasm (Joshi *et al.,* 2008). Arsenic accumulation in some bacteria such as *Pseudomonas* (Cai *et al.,* 1998) and *M. communis* (Takeuchi *et al.,* 2007) was reported which lack *ars* operon.

In this study, to understand arsenic tolerance in *K. pneumoniae*, PCR for arsenate reductase (*arsC*) gene, biotransformation assay and bioaccumulation were done. PCR results for *arsC* gene suggest that, the *arsC* gene is present on chromosome and plasmid in *K. pneumoniae* and spectrophotometric biotransformation assay data confirms that *ArsC* converts As(V) into As(III). In bioaccumulation study, it was found that under arsenic stress, maximum arsenic accumulates in mid-log phase of *K. pneumoniae* and after mid-log phase, there was decrease in the cellular arsenic content indicating that the organism efflux the intracellular arsenic into the extracellular environment. Thus from the results of these studies it is concluded that, in *K. pneumoniae* under As(V) stress, As(V) enter into the cell and accumulate till mid-log phase, and the *ArsC* converts As(V) into As(III) and pumped out of the cell by an As(III) efflux pump. Under As(III) stress, As(III) also enter into the cell and accumulate till mid-log phase further pumped out of the cell by an As(III) efflux pump. Concluding, isolated *K. pneumoniae* is tolerant to high concentration of As(V) due to presence of classical *ars* operon system.

Acknowledgments

V.D. is thankful to UGC, New Delhi, India for providing research fellowship in science for meritorious students (RFSMS).

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