Isolation and Identification of Arsenic Resistant *Providencia rettgeri* (KDM3) from Industrial Effluent Contaminated Soil and Studies on its Arsenic Resistance Mechanisma

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Abstract

A novel strain of *Providencia rettgeri* was isolated from metal contaminated industrial soil in Mumbai and was identified and characterized. This isolate could survive in a medium containing up to 10,000 $\mu\text{g mL}^{-1}$ of arsenate (133.3 mM sodium arsenate). Growth parameter studies revealed that only at 5000 and 10000 $\mu\text{g mL}^{-1}$ arsenic growth was retarded as compared to control cells. TEM studies indicated no major morphological changes or damages when cells were grown in presence of 10,000 $\mu\text{g mL}^{-1}$ of arsenate. Arsenic treated cells as compared to untreated cells showed over expression of a protein, arsenical pump driving ATPase identified by MALDI TOF-Mass Spectrometry. Arsenate, once taken inside the cells, was presumably reduced to arsenite and further extruded out of the cells. The arsenic extrusion from the cells resulted in reduced accumulation of arsenic inside the cells as confirmed by TEM-EDX and XPS analysis. The high resistance to arsenic by *Providencia rettgeri* was found to be plasmid mediated. Plasmid cured cells were sensitive to arsenic stress and could not grow in arsenic containing medium. Reduction of arsenate (V) to arsenite (III) by the present bacterial strain can contribute to mobility and bioavailability of arsenic in the soils. This is the first report of such high arsenic tolerance in *Providencia sp.* and it may have a very high potential for incorporating in an arsenic bioremediation strategy.

Keywords: *Providencia rettgeri*; Arsenic resistance; Bioremediation; Protein identification

Highlights

1. Arsenic (V) resistance till 10,000 $\mu\text{g mL}^{-1}$ in *Providencia rettgeri* is reported for the first time.
2. *Providencia rettgeri* can reduce As (V) to As (III) and can efflux it out of the cell.
3. Resistance to high concentrations of arsenic in *Providencia rettgeri* is plasmid mediated and by ATP dependent metal extrusion phenomenon.
4. Reduction of arsenate (V) to arsenite (III) can contribute to mobility and bioavailability of arsenic in the soils, which can be useful in phytoremediation of arsenic contaminated soils.

Introduction

Arsenic, a class one carcinogen, is distributed ubiquitously in the environment. Contamination of water and soil by arsenic poses a major threat to the public health and the environment worldwide. Local concentrations of arsenic may vary depending on its use and geological history of the region. Himalayan river sediments are the main sources of ground water arsenic contamination in large areas of

South and Southeast Asia [1]. World health organization has set the acceptable limit in drinking water as 10 $\mu\text{g L}^{-1}$. At present, arsenic is estimated to affect more than 150 million people worldwide with its increasingly elevated concentrations in drinking water. In some areas of Bangladesh and West Bengal in India, groundwater arsenic concentration has exceeded 2000 $\mu\text{g L}^{-1}$ [2]. Besides the natural processes, human interventions have been an important contributor to the mobilization of arsenic through anthropogenic factors such as mining operations, disposal of industrial wastes, combustion of fossil fuels, use of arsenical pesticides, herbicides and poultry feed additives

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[3,4]. Arsenic occurs in nature in four oxidation states (+5, +3, 0 and -3), with pentavalent arsenate (+5, AsV) and trivalent arsenite (+3, AsIII) predominating in aquatic and soil environments [5]. Arsenate (V) is present as negatively charged oxyanions ($\text{H}_2\text{AsO}_4^- / \text{HASO}_4^{2-}$) at moderate pH levels and these oxyanions are strongly adsorbed to the surface of common soil minerals such as Fe & Al (hydroxides). However As (III) primarily exists as uncharged H_3AsO_3 with pKa of 9.2 and is therefore less adsorptive and more mobile than As (V) in most environments [6]. In aerobic environments, As (V) is found to be the predominant species and is immobilized in solid phase. In contrast, As (III) is more prevalent in anoxic environments, and that leads to mobilization into aqueous phase [7]. As a result of irrigation with As-contaminated water, arsenic can find its way into human food chain through different food crops including rice, wheat, vegetables, fruits etc. Among the food crops, rice is the major crop in areas where arsenic contamination occurs. Accumulation in rice to the extent of 2 mg As/kg of grain and 92 mg As/kg of straw has been reported [8]. The rice straw is the most common feed for cattle in West Bengal and Bangladesh. That leads to arsenic contamination of meat and milk. Therefore the diet of many people in West Bengal and Bangladesh is under severe threat of arsenic contamination [9].

Microorganisms are involved in metal biogeochemistry with a variety of processes influencing the mobility and bioavailability of many metals [10]. In nature, microbes respond to arsenic in a variety of different ways. Depending on the species of different microorganisms, the responses could be chelation, compartmentalization, exclusion, and immobilization [11]. Understanding the arsenic metabolism at molecular and biochemical level will be important for developing efficient and selective arsenic bioremediation approaches, which have so far been considered as a cost-effective and environments friendly way for heavy-metal removal [12]. Microbe mediated reductive dissolution of arsenic in biomining has been reported [13]. Rhizoremediation, involving both plants and rhizospheric microbes, is an efficient bioremediation process for arsenic contaminated soil [14,15]. The present study reports isolation of an arsenic resistant bacteria from an effluent contaminated soil and its subsequent characterization. In the present isolate, the arsenic resistance is a plasmid mediated phenomenon, and the bacterium has the ability to reduce arsenate to arsenite, and that leads to arsenite exclusion from the cell. Reduction of arsenate (V) to arsenite (III) by this bacterial isolate can contribute to higher mobility and bioavailability of arsenic to plants, and therefore can be used as a supplementary strategy for phytoextraction, for its remediation.

Materials and Methods

Field sampling, enrichment and isolation of arsenic resistant bacteria

Samples were collected from metal contaminated soils from industrial area in Mulund, Mumbai where paint industries are located. The Arsenic concentration in the metal contaminated soil was 2 µg/gm of soil. Soil samples (0-15 cm depth) were collected in sterile container and preserved at 4°C till further processing. One gram soil sample was suspended in minimal media spiked with 100, 200 and 300 µg gm⁻¹ of arsenate (V) as sodium arsenate salt ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$) and was incubated for 48 hrs at room temperature. Arsenic tolerant cultures were isolated using serial dilution technique. Cultures which could grow at high concentrations of arsenic (300 µg gm⁻¹) were maintained by repeated sub culturing and pure cultures were used for further studies.

Identification of the isolated bacterial culture

The pure culture of arsenic – resistant isolate was initially characterized by colony morphology (color, shape, size) and by microscopic observations (Gram stain, spore stain, motility). Various biochemical tests like oxidase, catalase, citrate, indole, starch hydrolysis, nitrate reduction, MR-VP test, Glucose - Lactose utilization and high salt tolerance test were performed as per standard protocol [16]. Antibiotic sensitivity tests with various antibiotics were performed using ready to use combi octodiscs (make-HiMedia, India) of antibiotics. Further, for 16S rRNA gene sequencing, genomic DNA was isolated from the culture using Genei Ultrapure™. Bacterial Genomic DNA Purification kit KT162. Using forward (FP 5' AGA GTT TGA TCM TGG CTC AG 3') and reverse (RP 5' TAC GGY TAC CTT GTT ACG ACT T 3') primers the 1.5kb 16 S rDNA fragment was amplified by *Taq* DNA polymerase. The PCR product was sequenced and the sequence data was aligned and analyzed for finding the nearest taxonomic neighbour.

Confirmation of arsenic tolerance by agar well diffusion method

To confirm the arsenic tolerance by the bacteria, Luria Bertani (LB) agar plates were spread with *P. rettgeri* and 10mm diameter wells were formed using sterile metal borer. The wells were filled with sodium arsenate solution of concentration 100, 200, 500, 750, 1000, 2000, 5000, 7000 and 10,000 µg mL⁻¹ of arsenic. *E-coli* (BW25113) strain was used for comparison as arsenic sensitive culture. The plates were kept for incubation at 30°C for 24 hrs and observed for zone of inhibition due to arsenic toxicity.

Growth kinetics

To study the effect of arsenic on growth, inoculum from an exponentially growing culture was added to 50 mL LB broth to a resultant OD₅₃₀ of 0.05. This culture was grown in different concentrations of arsenic as sodium arsenate viz. 0, 50, 100, 200, 500, 1000, 5000 and 10,000 µg mL⁻¹ of arsenic as arsenate at 30°C at 140 rpm. The growth of the bacteria was measured after every 1 h till it reached stationary phase by measuring the absorbance at 530 nm using JASCO-V-530 spectrophotometer. Bacterial growth was also monitored at higher concentrations of arsenic as 500 and 1000 µg mL⁻¹ at different pH viz. 5, 6 and 7.

Transmission electron microscopy (TEM), TEM-EDX and X-ray photoelectron spectroscopy (XPS)

Cells grown in media supplemented with 1000 µg mL⁻¹ arsenate and without arsenate (control) were harvested by centrifugation at 12000 g. Cells were washed twice with 50 mM cacodylate buffer (pH 7.4) and fixed in a solution (2.5% glutaraldehyde + 0.5% para-formaldehyde) overnight at 4°C. Following three washes with cacodylate buffer, cells were embedded in 2% noble agar and dehydrated in a graded series of ethanol (30%, 60%, 75%, 90%, and 100%). After removal of ethanol by treatment with propylene oxide, blocks were subsequently infiltrated with spurr reagent on incubation with 1:3, 3:1 and 1:1 (v/v) propylene oxide: spurr reagent for 2 h each. The samples were finally infiltrated with spurr resin for 16 h and embedded in it by incubation at 60°C for 72 h. Thin sections of samples were prepared with a microtome (Leica, Germany), placed on 200 mesh formvar-coated copper grids and viewed with the Libra 120 plus Transmission Electron Microscope (Carl Zeiss). TEM-EDX and XPS (VG Scienta made spectrometer provided with single channeltron detector) analysis were performed

on cells grown in medium containing 10,000 $\mu\text{g mL}^{-1}$ of arsenic as per the standard protocol to study the bioaccumulation of arsenic by *Providencia rettgeri* [17].

Protein extraction and identification

Total cellular protein extraction and SDS-PAGE analysis: Overnight grown culture was used to inoculate 100 mL fresh LB broth with and without 100 $\mu\text{g mL}^{-1}$ arsenic ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$) for 24 h at 30°C. The cells (from 10 mL culture) were washed twice with sterile milli-Q water after harvesting. The pellet was resuspended in 1 mL lysis buffer (100mM NaCl, 25mM Tris HCl: pH8.0, 200mM PMSF, 0.2 mg mL^{-1} lysozyme, 0.2% tritonX-100 and 10% glycerol) followed by sonication on ice for three cycles of 10 sec each. Sonicated samples were centrifuged at 15000 g for 20 min. at 4°C. The supernatant containing all the soluble proteins were collected and further stored at -20°C for proteomics analysis. Protein concentration was determined by BCA (Bicinchoninic acid) method using Bovin Serum Albumin standard (GeNei™). For separating proteins, 12% Tris glycine linear pre cast gel of Invitrogen was used. Approximately 100 μg of total protein of both control and arsenic treated samples were loaded on to the gel and proteins were separated at 25mA constant current for 180 min. The protein bands were visualized by staining with Coomassie Brilliant Blue dye (R250).

Tryptic In gel digestion: After separation of the protein bands by SDS-PAGE, the gel was washed with sterile double distilled water (two changes, 10 min. each). The protein bands were carefully excised from the gel and further cut into 1 mm pieces in eppendorf tube and processed by standard protocol for mass spectrometry (Bruker Daltonik GmbH). Gel particles were washed with 1:1 (V/V) 50mM $(\text{NH}_4)_2\text{HCO}_3$: acetonitrile for 15 min for destaining. The tubes were incubated for 30 min. at room temperature. This step was repeated several times till the gel pieces became completely colourless. For reduction and alkalization, 10mM dithiotreitol/50 mM $(\text{NH}_4)_2\text{HCO}_3$ (freshly prepared) was added and incubated for 45 min at 56°C. Tubes were cooled down on ice and excess liquid was removed and replaced quickly by 55 mM iodoacetamide. In-gel digestion was done by freshly prepared 5 ng/ μL Trypsin solution in 25 mM $(\text{NH}_4)_2\text{HCO}_3$. The gel pieces in that trypsin solution were kept on ice for 30 min. and were further incubated at 37°C overnight. Peptides were extracted from gel sequentially by 0.1%TFA, followed by 0.1% TFA in 50% Acetonitrile and finally Acetonitrile. Supernatant was collected after vortexing for 30 min. in each solvent followed by centrifugation at 15000 g. All the collected supernatant extracts were pooled and dried in speed vacuum and stored at -20°C till further mass spectrometry analysis.

Protein identification by MALDI-TOF-MS/MS: The extracted peptide samples were reconstituted in 5 μL of 0.1% TFA in 50% acetonitrile. 2 μL digested peptides were mixed with 2 μL matrix (4-hydroxy- α -cyanocinnamic acid) in 1:1 ratio and loaded on MALDI plate and dried for 2 h. The analysis was done by Bruker Ultra Flex II mass spectrometer by calibrating BSA protein standard. Mass spectra were obtained using nitrogen laser shots for 10 sec. positively charged ions were analyzed in the reflector mode, using delayed extraction. The mass spectrometer was operated under 19 kv accelerating voltage in the reflection mode and an m/z range 600-4000. Typically 100 shots were accumulated per spectrum in MS mode and 400 shots in MS/MS mode. The spectra were processed using the Flex analysis 2.2 and BioTools 2.2 software tools. Based on the mass signals, protein identification was performed using the Mascot server (<http://www.matrixscience.com>). The mass signals were taken for searching the NCBI nr protein database within Eubacteria taxonomy group.

Arsenate reduction assay by differential pulse cathodic stripping voltammetry

The Reduction of As(V) to As(III) by *Providencia rettgeri* was studied by measuring concentration of arsenite in the growth medium by voltammetric measurements using differential pulse cathodic stripping voltammetry (Potentiostat/Galvanostat Autolab model PG-Stat20; Eco-chemie, interface with multimode electrode stand; 663 VA stand, Metrohm) composed of HMDE/ Ag-AgCl/ Pt-rod electrode system. As (V) is electrochemically inactive. Starting with an O.D.₅₃₀ of 0.05 the cells were grown in Erlenmeyer flask at different concentrations of As (V) viz. 0, 100, 200, 400, 500, 750 and 1000 $\mu\text{g mL}^{-1}$ at 140 rpm and 30°C. After 18-20 h of growth the culture was centrifuged at 8000 g for 15 min. Supernatant was passed through Whatman filter paper (no.44), and analyzed for presence of As (III). Sample solution was made 0.1M with respect to HCl to facilitate reduction of As(III) to As(0) and 5 $\mu\text{g mL}^{-1}$ of Cu(II) was added for easy preconcentration of As(0) onto the electrode surface. The solution was scanned in cathodic direction from -0.45V to -0.9V after deposition for 30 sec. at -0.45V. Reduction peak for As (III) appears at -0.75V. Quantification was carried out by standard addition method. Kinetic study of the reduction assay was carried out by growing the culture at 1000 $\mu\text{g mL}^{-1}$ As (V), and by measuring the arsenite As (III) concentration every 2 h as stated above.

Plasmid isolation, curing and transformation

Plasmid curing of the cells was performed by repeated sub-culturing at 42°C. Plasmid was isolated from wild type and transformed culture using Roche-High pure plasmid isolation kit. Transformation of cured culture by the wild type plasmid was done using standard protocol [18]. Transformed cells were selected on arsenic supplemented (1000 $\mu\text{g mL}^{-1}$) media. Restriction digestion using HIND III was performed as per the standard protocol from the manufacturer (Bangalore Genie).

Results and Discussion

Identification of the isolated bacterial strain

Among the various arsenic tolerant isolates obtained from the enrichment experiment which could grow in presence of 300 $\mu\text{g mL}^{-1}$, one pure isolate was selected and studied for its taxonomic identification and detailed arsenic tolerance mechanisms. The selected isolate studied in detail here is a Gram negative non sporulating short rod. The biochemical results (Table 1) indicate that it belong to Enterobacteriaceae family and *Providencia* genus. The partial 16S rRNA gene sequence of this isolate was submitted in genbank (Accession number KC247668.1 GI: 432140652). BLAST search using this partial16S rRNA gene sequence as query, generated a large number of hits with very high sequence identity. On the basis of multiple sequence alignment followed by phylogenetic analysis (Figure 1) we identified the present isolate as *Providencia rettgeri*. The morphological features of the organism, the results of biochemical tests and the 16SrRNA sequence are in agreement with this taxonomic assignment. This isolate was designated as *Providencia rettgeri* KDM3.

Effect of arsenic on growth kinetics of *P. rettgeri*

Growth studies indicated that 100 $\mu\text{g mL}^{-1}$ of As concentration in medium did not show any inhibitory effect of arsenic on the growth of this organism (Fig. 2). We noticed a marginal growth enhancement at 20 $\mu\text{g mL}^{-1}$ of arsenic compared to that of control culture. Mild stress induced growth enhancement as a result of stress adaptive response has been reported earlier [19]. At 5000 and 10,000 $\mu\text{g mL}^{-1}$ arsenic concentration, growth was significantly decreased as compared to

that of control. Growth beyond 10,000 µg mL⁻¹ was severely affected. So we did not increase conc. more than 10,000 µg mL⁻¹. Arsenic contaminated soils are primarily acidic in nature and therefore growth of this organism at acidic condition was tested. Both in presence and absence of arsenic, the growth at pH 5 was comparable to that observed at pH 7 (Figures 2a,2b,2c). While there is no effect on doubling time during the log phase of growth till 500 µg mL⁻¹ of As (V) concentration, the doubling time increased by two fold at 1000 µg mL⁻¹. Although an isolate of *Pseudomonas* (17) could tolerate As (V) up to 3800 µg mL⁻¹ and an isolate of *Micrococcus roseus* could tolerate As (V) up to 3746 µg mL⁻¹ [20], the present isolate is the highest As (V) tolerant *Providencia sp.* reported. Agar well diffusion studies confirmed that there was no growth inhibition at 10,000 µg mL⁻¹ arsenic concentration (Figure 3). The experiment was performed with *E-coli* (BW25113) as a control (sensitive) culture for comparison, when a clear zone of inhibition was observed at 1000 µg mL⁻¹ of arsenic (Figure 3) and with subsequent increase in arsenic, increase in radii of zones of inhibition also was observed.

TEM, TEM-EDX and XPS analysis

Cells growing in presence of different concentrations of arsenic

Morphological Test		Antibiotics sensitivity test	
Shape	Circular	Penicillin - G	+
Colour	creamy	Clindamycin	+
Margin	Even	Gentamycin	+
Opacity	Opaque	Fusidic acid	+
Elevation	Elevated	Erythromycin	+
Consistency	Sticky	Trimethoprim	+
Gram Staining	Gram negative Short Rods	Sulphamethoxazole	+
Motility	Actively motile	Tetracycline	+
Spore Staining	Non sporulating	Ciprofloxacin	-
Size	4 mm in diameter	Ofloxacin	-
Biochemical test		Sparfloxacin	-
D-Glucose		Aztreonam	+
Acid production	+	Azithromycin	+
Gas production	-	Vancomycin	+
Lactose		Doxycycline Hydrochloride	+
Acid production	+	Cephalothin	+
Gas production	-	Clindamycin	+
High Salt (6.5 % NaCl)	+	CO-Trimoxazole	+
Indole Test	+	Kanamycin	+
MR Test	+	Amphicillin	+
VP Test	-	Chloramphenicol	+
Nitrate Test	+	Gatifloxacin	-
Catalase Test	+	Negative -	Positive +
Oxidase Test	+		
Citrate Test	+		
Starch Test	+		

Table 1: Morphological and Biochemical characteristics of isolated bacterial strain KDM3.

Figure 1: Phylogenetic Tree made using neighbor joining method.

and in absence of arsenic were observed using transmission electron microscopy to study the intracellular changes induced by arsenic. Our isolate *Providencia rettgeri* could tolerate arsenate up to 10,000 µg mL⁻¹ with no major cellular damage or morphological changes (Figure 4a). TEM-EDX and XPS analyses indicated no major accumulation of arsenate in the cells even when they are growing at 10,000 µg mL⁻¹ of As (Figure 4b, 4c). We conclude that the observed arsenic resistance of the present isolate is most probably due to arsenic efflux from the system. This is in contrast to observation that intracellular accumulation of As in *Pseudomonas* was responsible for resistance as reported by Joshi et al. [17].

Identification of an arsenic induced protein

Several proteins were differentially expressed at 100 µg mL⁻¹ arsenic treatment (Figure 5). A prominent band at approx 63 kDa, was identified by MALDI TOF MS/MS as arsenical pump driving ATPase among various differentially expressed proteins. Arsenical pump driving ATPase (ArsA protein), is a membrane-associated ATPase

attached to the ArsB inner-membrane protein and it energizes the arsenite efflux pump by ATP hydrolysis [21,22]. Ars C gene of the operon codes for a protein that belongs to thioredoxin super family and it can reduce arsenate (V) to arsenite (III) [24]. Arsenite can relatively effluxed from the cell easily. The function of most of the resistance systems is based on the energy-dependant efflux of toxic ions [23]. The present observation is in agreement with our earlier observation that no noticeable arsenic accumulation happens in the cell in spite of being grown in presence of high concentration of arsenic. Previously it was shown that prokaryotes have *ars* operon that codes for different proteins required for detoxification of arsenate, arsenite and antimonite [12]. Ars A and Ars B protein together forms the membrane embedded pump that enables arsenite efflux. Identification of Ars A as one of the over expressed proteins in response to arsenic treatment and absence of arsenic accumulation corroborates our hypothesis that this isolate of *Providencia rettgeri* also uses *ars* operon for detoxification of arsenic.

Figure 5: Peptide Mass Fingerprint (PMF) of over expressed 63 kDa Protein. The possible small peptides generated by trypsin digestion have been shown in red. Using black arrows, some of the peptides have been matched to individual mass peaks.

Figure 6a: Kinetics of arsenate reduction by *Providencia rettgeri* treated at 1000 µg mL⁻¹ arsenate.

Arsenate reduction assay by differential pulse cathodic stripping voltammetry

Pulse cathodic stripping voltammetry is a voltammetric method for quantitative determination of specific ionic species. The hypothesis

Figure 6b: Arsenate reduction assay at different arsenate concentrations (µg mL⁻¹).

that the present isolate is taking in arsenate, reducing it to arsenite and pumping it outside the cell was confirmed by studying the buildup of arsenite in the growth supernatant using this technique. It was observed that there is a steady increase in As(III) concentrations in the medium

when the cells were grown at 1000 $\mu\text{g mL}^{-1}$ As (V) over a period of 24 h (Figure 6a). To verify whether this As reduction is dependent on the initial As (V) concentration in the media the experiment was repeated taking different As (V) concentrations, starting with as low (100 $\mu\text{g mL}^{-1}$) concentration of As. It was found that As(V) to As(III) reduction is effective at low (100 $\mu\text{g mL}^{-1}$) as well as high (1000 $\mu\text{g mL}^{-1}$) concentration of As(V) as shown in Figure 6b. Arsenic (V) reduction as a strategy for As tolerance in bacteria has been reported earlier [23] and such As (V) reducers have been isolated from diverse sources including As hyperaccumulating Fern *Pteris vittata* [24].

Plasmid mediated arsenic resistance

In prokaryote many different survival mechanisms including antibiotic resistance [25] and metal tolerance [26] are plasmid encoded. To identify the possible existence of a plasmid involvement in As tolerance in the present isolate of *P. rettgeri*, presence of plasmid DNA was confirmed by agarose gel electrophoresis (Figure 7). After confirming the presence of plasmid, the culture was grown successively for five generations in LB broth at 42°C [27] for plasmid curing. This cured culture was found to be arsenic sensitive and could not grow beyond 500 $\mu\text{g mL}^{-1}$ of arsenate concentration. When this culture was transformed with wild type plasmid, the resultant culture could grow in medium having 1000 $\mu\text{g mL}^{-1}$. Transformed colonies were selected. Plasmids were isolated from the transformed colonies. Hind III digestion clearly showed that in wild type there are two plasmids while only one of them was required for arsenate tolerance in the transformed cells. HIND III digestion cuts the plasmid related to tolerance to 2 pieces of 1.5 kb and 3kb while the other wild type plasmid remains undigested.

The present study reports arsenic resistance upto 10,000 $\mu\text{g mL}^{-1}$ by a local isolate of *Providencia rettgeri*. It is established that many microorganisms survive in the presence of toxic metals or metalloids by inducing the expression of an array of proteins that are involved in resistance mechanism [28]. In case of arsenic resistance, role of *ars* operon has been indicated [29]. In the present study one of the up regulated proteins was identified to be Ars A, which is the ATPase component of the arsenite efflux pump. The TEM-EDX & XPS data clearly indicated that intracellular arsenic accumulation is not part

of the stratagem for this bacterium. Using differential pulse cathodic stripping voltammetry, it has been shown that with time, arsenite concentration in the media increases. Up regulation of *ars* operon component protein Ars A, absence of accumulated intracellular arsenic and concomitant extracellular arsenite build up led us to hypothesize that the present isolate of *Providencia rettgeri* takes up arsenic using one of the known transporters, possibly phosphate transporter [8] and that in turn induces *ars* operon. While Ars C reduces As (V) to As (III), the pump component of ArsA and Ars B protein throws out As (III) into the medium. Identification of the 63kDa up regulated protein as ArsA by MALDI-TOF strongly suggests this hypothesis.

To further investigate the possible role of a plasmid in arsenic tolerance in the present organism, plasmid was isolated from the organism and was reintroduced into the cured culture. Plasmid mediated arsenic resistance was studied in *Staphylococcus xylosus* (plasmid pSX267), *P. aeruginosa* (plasmid pUM310) and *Acidiphilium multivorium* (plasmid AIU301) [30]. In the present study it has been clearly shown that the isolated bacteria has two plasmid, one of which is cut into 2 pieces by Hind III digestion while the second plasmid remains unaffected. The plasmid bearing Hind III target site is the one that imparts arsenic resistance upon transformation of the cured culture. (Graphical Abstract)

Conclusion

A novel strain of *Providencia rettgeri* isolated from industrial effluent contaminated soil of Mumbai shows hyper resistance (up to 10,000 $\mu\text{g mL}^{-1}$) against arsenic. It shows the ability to reduce arsenate to arsenite and efflux the arsenite outside the cell. By curing and reintroduction of the wild type plasmid, it was shown that one or more arsenic resistant factors are plasmid encoded. Because of higher bioavailability of arsenite to plants, this bacterium can be a potential contributor in an arsenic phytoremediation strategy.

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