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Ramansu Goswami[ae], Suprabhat Mukherjee[b], Vipin Singh Rana[c], Dhira Rani Saha[d], Rajagopal Raman[c], Pratap Kumar Padhy[e] & Shibnath Mazumder[a]

[a] Immunobiology Laboratory, Department of Zoology, University of Delhi, Delhi, India

[b] Parasitology Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan, India

[c] Gut Biology Laboratory, Department of Zoology, University of Delhi, Delhi, India

[d] Microscopy Laboratory, National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata, India

[e] Department of Environmental Studies, Visva-Bharati University, Santiniketan, West Bengal, India
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Isolation and Characterization of Arsenic-Resistant Bacteria from Contaminated Water-Bodies in West Bengal, India

RAMANSU GOSWAMI[1,5], SUPRABHAT MUKHERJEE[2], VIPIN SINGH RANA[3], DHIRA RANI SAHA[4], RAJAGOPAL RAMAN[3], PRATAP KUMAR PADHY[5], and SHIBNATH MAZUMDER[1]*

[1]*Immunobiology Laboratory, Department of Zoology, University of Delhi, Delhi, India*
[2]*Parasitology Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan, India*
[3]*Gut Biology Laboratory, Department of Zoology, University of Delhi, Delhi, India*
[4]*Microscopy Laboratory, National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata, India*
[5]*Department of Environmental Studies, Visva-Bharati University, Santiniketan, West Bengal, India*

Bengal Basin is known for severe arsenic contamination. In the present study, we have isolated six bacteria from the arsenic contaminated surface water of Bengal Basin. 16S rDNA sequence analysis identified them as *Microbacterium oleivorans*, *Acinetobacter soli*, *Acinetobacter venetianus*, *Acinetobacter junii*, *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*. All the isolates possess arsenic accumulation potential and high molecular weight plasmid (>10 kb). PCR amplification indicated the presence of arsenic-resistance genes (*arsB* and *aoxB*) either in the genome or plasmid or in both in the isolated bacteria (except in *Acinetobacter venetianus*). Exposure to arsenic affected bacterial growth and induced alteration in cytoplasmic membrane integrity.

Keywords: 16S rDNA, *arsB*, *aoxB*, arsenic, bacteria, surface water

Introduction

Arsenic is a toxic metalloid that is found in soil, air and water. Environmental arsenic exists in both organic and inorganic states. In the inorganic state, arsenic is present either as trivalent arsenite (As^{+3}) or pentavalent arsenate (As^{+5}). Both arsenite and arsenate are soluble over a wide range of pH and routinely observed, though arsenite is reported to be more toxic (Cervantes et al. 1994).

Microorganisms play important roles in the biochemical cycle and arsenic-speciation (Liu et al. 2012). Bacteria-induced arsenic transformation involves oxidation, reduction or methylation to overcome the toxic effects and survive in arsenic-rich environment. Earlier work suggested the involvement of *arr* genes, *ars* operon and *aox* genes in arsenic resistance and metabolizing systems in bacteria (Liu et al. 2012). In *Escherichia coli*, an ArsA-ArsB complex functions as a primary arsenite pump (Cervantes et al. 1994). In *Staphylococcus aureus*, ArsB alone is sufficient to act as a chemiosmotic secondary transport system for arsenite resistance without the presence of an ArsA-ATPase (Cervantes et al. 1994). An additional gene, *arsC*, has been shown to encode for an arsenate reductase that mediates reduction of arsenate prior to arsenite efflux (Liu et al. 2012).

Periplasmic respiratory arsenite oxidase (Cai et al. 2009) and respiratory arsenate reductase (Liu et al. 2012), have also been isolated and their genetic basis studied in bacteria. The arsenite oxidase (*aoxBA*) system contains two subunits encoded by the genes *aoxA* and *aoxB*, respectively, and converts highly toxic arsenite to less toxic arsenate (Cai et al. 2009), while respiratory arsenate reductase (*arrAB*) functions as a terminal electron acceptor, allowing heterotrophic anaerobic growth in the absence of oxygen (Liu et al. 2012). The arsenic-resistance genes in bacteria can be either genomic or plasmid encoded (Cervantes et al. 1994).

Severe arsenic contamination has been reported in Indo-Bangladesh Gangetic Basin (Bengal Basin) and the immediate source of arsenic being the land sediments (Islam et al. 2005). However, the mechanism of arsenic release from these contaminated sediments is not very clear (Islam et al. 2005). Several arsenic-resistant bacteria from aquifer-sediments of Bengal Basin (Chowdhury et al. 2009; Gault et al. 2005; Islam et al. 2005; Rowland et al. 2009; Salam et al. 2009) have been documented, but reports of such bacteria from the surface waters of this region are lacking. With the increased detection of arsenic from different parts of the globe, the local microbiota is expected to adapt to high concentrations and perhaps even gain the capability of maintaining the arsenic cycle also. In reference to this context, the present study has been aimed to i) isolate and characterize the arsenic-resistant bacteria from surface waters of contaminated aquatic bodies, and ii) study the effects of the toxicant on bacterial physiology and growth.

*Address correspondence to Shibnath Mazumder, Immunobiology Laboratory, Department of Zoology, University of Delhi, Delhi 110 007, India; Email: shibnath1@yahoo.co.in

Materials and Methods

Sampling

The Chakdah block (23° 3′ N 88° 35′ E) in West Bengal, India, is an arsenic-rich zone (http://www.soesju.org/arsenic/wb.htm) and was selected for sampling. Surface water samples in 1 mL aliquots were collected from a depth of 15 cm (approx.) from ponds of six different villages (Dakshin Panch Pota, Rasullapur, Deuli, Kundulia, Narayanpur and Chaugachha) and analyzed for arsenic content by hydride generation technique using atomic absorption spectrophotometer (AAS, Perkin Elmer). Citrus leaf (SRM 1577) was used as standard reference material to access the analytical efficacy of arsenic detection and the mean recovery and detection limit of arsenic was 89.2% and 0.75 ng mL^{-1}, respectively.

Isolation of Arsenic-Resistant Bacteria

Arsenite stock solutions (100 mM) were prepared by dissolving arsenic trioxide (As_2O_3) (Sigma, USA) as described earlier (Goswami et al. 2011). In a similar way, 1000 mM arsenate solution was prepared by dissolving sodium arsenate (Na_2HAsO_4, 7 H_2O) (Sigma, USA) in water and pH adjusted at 7.0. To obtain arsenic-resistant bacteria, aliquots of water samples were added to nutrient agar (NA) plates supplemented with 10 mg L^{-1} of arsenite and incubated at 30°C for 48 h. The isolated colonies were re-streaked and the process repeated to obtain pure cultures. The pure cultures were stored in 20% glycerol at − 80°C for future characterization.

16S rDNA Analysis

For isolation of genomic DNA, 100 μL of freshly prepared bacterial culture was removed and re-suspended in lysis buffer comprising 400 μL sodium-EDTA with 5 μL of 50 mg mL^{-1} lysozyme, 5 μL of 20 mg mL^{-1} proteinase K and 10 μL of 20% SDS. After 30 min of incubation, genomic DNA was extracted step wise with the addition of phenol, twice with phenol/chloroform (v/v), and once with chloroform. Finally the DNA was precipitated in 3 M sodium acetate (pH 5.2) and ice-cold ethanol, pelleted by centrifugation, ethanol washed and resuspended in 50 μL TE buffer (Sambrook et al. 1989). The plasmid DNA was isolated using Quiagen plasmid isolation kit (Cat. # 12125). The DNA was analyzed on 1% agarose gel in the presence of molecular weight marker (Fermentas).

The bacterial 16S rDNA gene was amplified with universal 16S rDNA primers (forward 5′-ccgaattcgtcgacaacA-GAGTTTGATCCTGGCTCAG-3′ and reverse 5′-cccgggatc-caagcttACGGCTACCTTGTTACGACTT-3′) and the cycling conditions consisted of 25 to 35 cycles at 95°C (2 min), 42°C (30 s) and 72°C (4 min), plus one additional cycle with a final 20 min chain elongation (Weisburg et al. 1991). The PCR products were purified by using the QIA quick PCR purification kit (Qiagen). The 16S rDNA were sequenced commercially (BioServe-India) by Sanger's method using a Genetic analysis system, Model CEQ-800 (Beckman Coulter, Inc.).

The BLASTn program (www.ncbi.nlm.nih.gov) was used for 16S rDNA based identification of the isolates and sequences submitted to GenBank. The 16S rDNA sequences were subjected to pairwise and multiple sequence alignment using ClustalW program (Thompson et al. 1994). An un-rooted phylogenetic tree was constructed using maximum parcimony method employing Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar 2000) using MEGA 5.1 (Tamura et al. 2011). Moreover, reliability of the phylogenetic tree was tested by the bootstrap method (500 replicates), using software MEGA 5.1.

PCR Amplification of Arsenic-Resistance Genes

The amplification of different arsenic-resistance genes in genomic and plasmid DNA were done using specific primers keeping the PCR conditions unaltered (Table 1).

Table 1. Primers used during amplification of arsenic-resistance genes

Primer	5′-3′	Gene name	Reference
darsB 1F	GGTGTGGAACATCGTCTGGAAYGCNAC		
darsB 1R	CAGGCCGTACACCACCAGRTACATNCC	*arsB* gene specific	Cai et al. 2009
dacr 1F	GCCATCGGCCTGATCGTNATGATGTAYCC		
dacr 1R	CGGCGATGGCCAGCTCYAAYTTYTT	*ACR3(1)* gene specific	Cai et al. 2009
dacr 5F	TGATCTGGGTCATGATCTTCCCVATGMTGVT		
dacr 4R	CGGCCACGGCCAGYTCRAARAARTT	*ACR3(2)* gene specific	Cai et al. 2009
aoxBM1-2F	CCACTTCTGCATCGTGGGNTGYGGNTA		
aoxBM2-1R	GGAGTTGTAGGCGGGCCKRTTRTGDAT	*aoxB* gene specific	Quemeneur et al. 2008; 2010
amlt-42-F	TCGCGTAATACGCTGGAGAT		
amlt-376-R	ACTTTCTCGCCGTCTTCCTT	*arsC* gene specific	Sun et al. 2004
arrA F	GTAGAAGGGACCTTATT		
arrA R	CTTAAGGGGCGAATTTCT	*arrA* gene specific	Freikowski et al. 2010

(M = A or C; N = A, C, G or T; R = A or G; V = A, C or G; Y = C or T); F, forward primer; R, reverse primer. PCR conditions as mentioned in each reference.

In silico Phylogenetic Analysis of ArsB

To study the sequence variability of *arsB* gene product, the amino acid sequences of ArsB protein of bacteria of interest along with other closest neighbors (www.ncbi.nlm.nih.gov) were subjected to both pairwise and multiple sequence alignment by ClustalW program provided by the software package MEGA 5.1 (Tamura et al. 2011) and an un-rooted phylogenetic tree was constructed using the SPR algorithm provided by the same software.

Arsenic Accumulation by Bacteria

First, 1-mL aliquots of actively growing bacterial cultures were either centrifuged and dried in speed-vac centrifuge to calculate the dry weight of bacterial pellet or re-suspended separately in sterile polypropylene tube containing 0.1 mg L^{-1} and 1 mg L^{-1} arsenite dissolved in water. The concentrations of arsenic selected have been reported from the Indo-Bangladesh region (http://www.soesju.org/arsenic/wb.htm). The samples were incubated at 30°C in a roller mixer for 1 h and 4 h, respectively, harvested and the amount of arsenic accumulated by bacteria determined by AAS using the method of standard addition (Surowitz et al. 1984).

Transmission Electron Microscopy

Bacteria grown in presence of 0.1 mg L^{-1} and 1 mg L^{-1} arsenic were prepared for TEM analysis as described previously (Goswami et al. 2011). Ultra-thin sections (60 nm) were cut, stained and examined under a Tecnai 12 Bio-Twin transmission electron microscope (FEI, The Netherlands) operating at 80 kV.

Determination of Minimum Inhibitory Concentration (MIC) and Effect of Arsenic on Bacterial Growth

The MIC of the isolates for arsenite and arsenate was determined by the agar dilution technique using an inocula of 1×10^5 cells ml^{-1} on Mueller–Hinton agar plates amended with variable concentrations of arsenite (0–1×10^3 mg L^{-1}) and arsenate (0–30×10^3 mg L^{-1}) respectively (Escalante et al. 2009). The diluted bacterial cultures (100 μL) were plated and incubated for 48 h at 30°C and MIC defined as the lowest concentration of arsenite or arsenate that causes no visible growth. An agar plate with bacteria and without the metalloid was used as a control.

To determine the effect of arsenic on bacterial growth, 10-μL cell suspensions from exponential phase cultures were added in BHI or minimal media containing < MIC concentrations of arsenite (1.0×10^2 mg L^{-1} for Ransu-1 and 6.0×10^2 mg L^{-1} for *Acenetobacter* sp.) and bacterial growth was monitored at 600 nm at 30°C (Rosenberger and Elsden 1960).

Statistical Analysis

Results have been expressed as mean $\pm$ SE calculated for each parameter considered in the present study and pairwise comparison was performed between the unexposed and arsenic-exposed groups. The value of $p < 0.05$ was considered statistically significant.

Results

Detection of Arsenic in Surface Water

AAS revealed the surface water concentration to be 0.150 ± 0.0208 mg L^{-1} for Dakshin Panch Pota, 0.144 ± 0.0197 mg L^{-1} for Rasullapur, 0.091 ± 0.0225 mg L^{-1} for Deuli, 0.081 ± 0.010 mg L^{-1} for Kundulia, 0.063 ± 0.013 mg L^{-1} for Narayanpur, and 0.058 ± 0.013 mg L^{-1} for Chaugachha village, respectively.

Phylogenetic Analysis of Arsenic-Resistant Bacteria

All six isolates grew on 10 mg L^{-1} arsenite containing media and possessed > 10 kb plasmid (Supplementary data: Figure S1). The 16S rDNA-based identification revealed six distinct bacteria- *Microbacterium oleivorans* (Ransu-1, GenBank accession # KC900892), *Acinetobacter soli* (IBL-1; GenBank accession # KC900893), *Acinetobacter venetianus* (IBL-2; GenBank accession # KC900894), *Acinetobacter junii* (IBL-3; GenBank accession # KC900895), *Acinetobacter baumannii* (IBL-4; GenBank accession # KC900896) and *Acinetobacter calcoaceticus* (IBL-5; GenBank accession # KC900897). Figure 1a describes the phylogenetic relationship between two groups of arsenic-resistant bacteria, i.e., *Acinetobacter* sp. and *M. oleivorans* with their neighbors. Among the five isolates belonging to genus *Acinetobacter*, closest species pairs were *A. baumannii- A. venetianus* and *A. soli- A. calcoaceticus* respectively.

These bacteria being members of the microbial consortium present at the sites of isolation, the phylogenetic relation with the other arsenic-resistant bacteria were studied. Furthermore, the bootstrap test also supported the result of maximum parsimony analysis. According to Hillis and Bull (1993), a bootstrap value (BV) greater than 70% indicated well-established monophyletic group. In this study a high BV (83%) for the pair *A. baumannii- A. venetianus* indicated a well-supported monophyletic group (Figure 1a). In contrast, some less-supported species pairs were also evident in the tree with BV less than 70% such as pairs constituting *A. calcoaceticus- A.baylyi*.

PCR Detection of Arsenic-Resistance Genes

The arsenic-resistance genes *arsB* and *aoxB* were detected by PCR within genomic and plasmid DNA by using gene specific primers. An amplicon of 750 bp corresponding to *arsB* gene was detected in both genomic and plasmid DNA of Ransu-1, plasmid DNA of IBL-1 and genomic DNA of IBL-3 and IBL-4 strains, respectively (Figure 2, Lane 2, 3, 6, 7
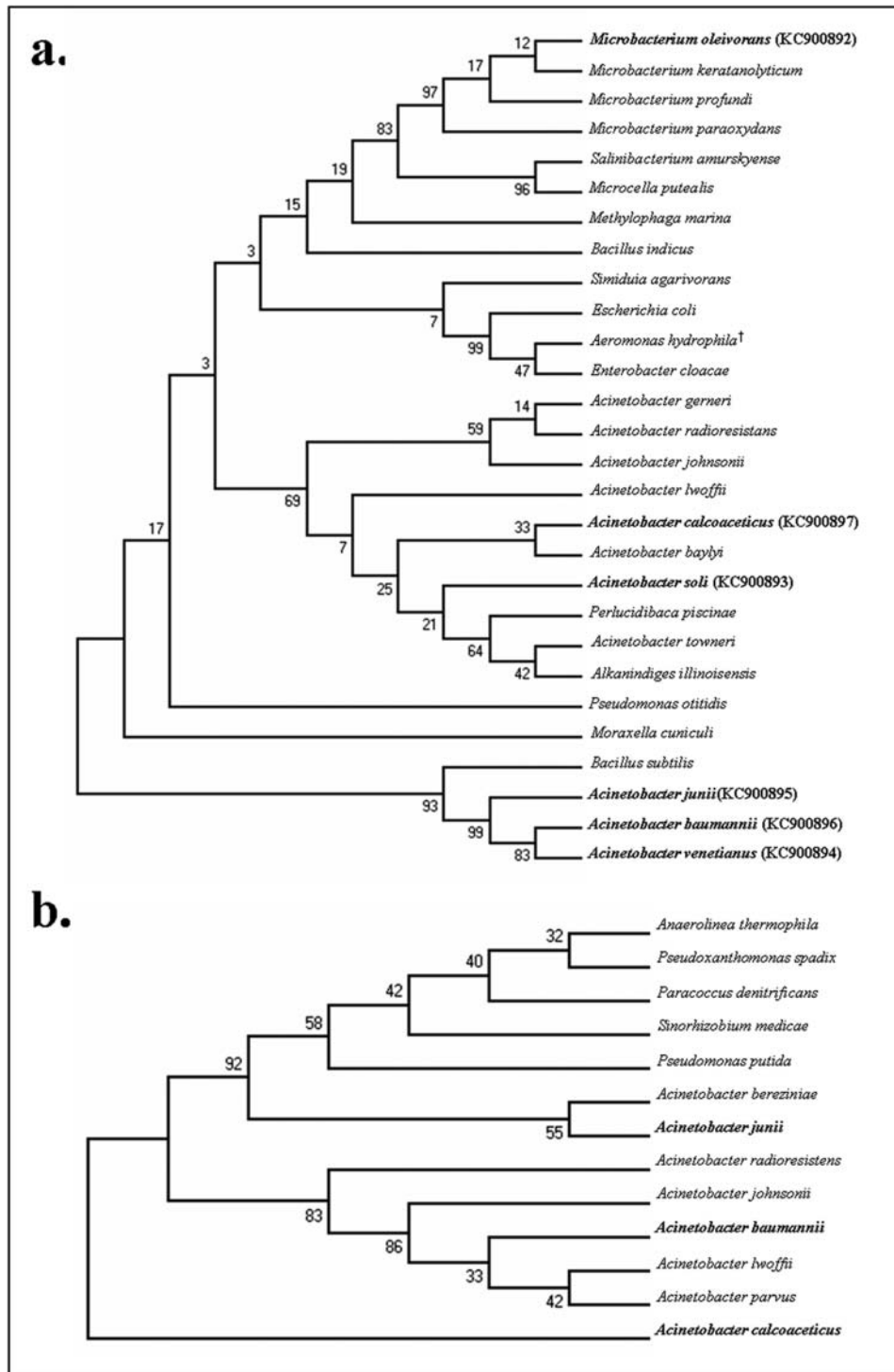
Fig. 1. Phylogenetic analysis of arsenic-resistant bacteria based on 16S rDNA and ArsB protein. a) Evolutionary relationship among arsenic resistant isolates. The most parsimonious tree with length = 230 is shown. The consistency index is 0.730435 (0.711628), the retention index is 0.840617 (0.840617), and the composite index is 0.614016 (0.598206) for all sites and parsimony-informative sites (in parentheses). The analysis involved 28 nucleotide sequences. There were a total of 114 positions in the final dataset. Values indicated in the tree represent% bootstrap values (BV). % BVs less than 10 were not shown. †indicates the bacteria previously isolated and reported for arsenic resistance (Goswami et al., 2011). b) Evolutionary relationship of ArsB in arsenic-resistant bacteria. Amino acid sequences were retrieved from Genbank (www.ncbi.nlm.nih.gov) and the evolutionary relationship among the sequences was inferred using the maximum parcimony method. The most parsimonious tree with length = 711 is shown. The consistency index is 0.856540 (0.783898), the retention index is 0.665574 (0.665574), and the composite index is 0.570091 (0.521742) for all sites and parsimony-informative sites (in parentheses). There were a total of 326 positions in the final dataset. Bacteria isolated in this study are shown in bold face. Values indicated in the tree represent% bootstrap values (BV). % BVs less than 10 were not shown.
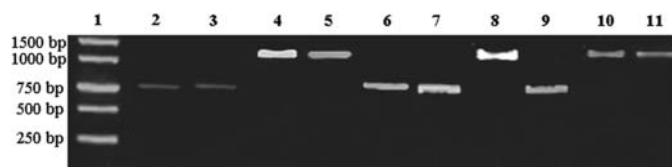
Fig. 2. PCR products from genomic and plasmid DNA. Lane 1, Molecular weight marker; Lane 2, amplified product of *arsB* gene specific primer in genomic DNA of strain Ransu-1; Lane 3, amplified product of *arsB* gene specific primer in the plasmid DNA of strain Ransu-1; Lane 4, amplified product of *aoxB* gene specific primer in the genomic DNA of IBL-1; Lane 5, amplified product of *aoxB* gene specific primer in the plasmid DNA of IBL-1; Lane 6, amplified product of *arsB* gene specific primer in the plasmid DNA of IBL-1; Lane 7, amplified product of *arsB* gene specific primer in the genomic DNA of IBL-3; Lane 8, amplified product of *aoxB* gene specific primer in the genomic DNA of IBL-3; Lane 9, amplified product of *ars B* gene specific primer in the genomic DNA of IBL-4; Lane 10, amplified product of *aoxB* gene specific primer in the genomic DNA of IBL-4; Lane 11, amplified product of *aoxB* gene specific primer in the plasmid DNA of IBL-5.

and 9). The distinct band with amplicon size of 1100 bp suggested the presence of *aoxB* gene in the genomic and plasmid DNA of IBL-1, genomic DNA of IBL-3 and IBL-4 and plasmid DNA of IBL-5 respectively (Figure 2, Lane 4, 5, 8, 10, and 11). No arsenic-resistance genes could be detected using any of the primers in IBL-2.

Accumulation of Arsenic

AAS revealed arsenite accumulation in all the bacterial isolates was time dependent with more arsenic recovered following 4 h of incubation ($p < 0.05$). However, concentration dependency in arsenite accumulation was also observed in

the isolates, as arsenic accumulation was significantly increased ($p < 0.05$) with the gradual enhancement of arsenic (from 0.1 mg L^{-1} to 1 mg L^{-1}) (Table 2).

Effect of Arsenic on Bacterial Growth

The MIC for Ransu-1 was 1.2×10^2 mg L^{-1} for arsenite and 25×10^3 mg L^{-1} for arsenate, respectively. For all members of *Acinetobacter* family the MIC for arsenite and arsenate were 6.25×10^2 mg L^{-1} and 25×10^3 mg L^{-1}, respectively.

To determine the effect of arsenite on growth, inoculates from actively growing bacterial cultures were incubated separately in BHI broth containing indicated concentrations of arsenite and monitored at regular intervals (Figure 3). The concentration of arsenite selected (1.0×10^2 mg L^{-1} for Ransu-1 and 6.0×10^2 mg L^{-1} for *Acinetobacter* isolates) was lower than the MIC values calculated for each isolate. The presence of arsenite-influenced bacterial growth (Figure 3) and among the different isolates studied, growth of Ransu-1 appeared to be most significantly affected ($p < 0.05$) as arsenite induced a shift in initiation of log phase, mid and late log phase from 12 h, 24–48 h and 72–96 h to 216 h, 240 h and 264 h, respectively, in exposed bacteria (Table 3). When the growth curves of different *Acinetobacter* isolates (IBL-1- IBL-5) were compared, IBL-2 and IBL-3 appeared to be more prone to arsenite (Table 3) (Figure 3).

In a parallel study, freshly prepared bacterial cultures were grown in minimal media containing arsenite and growth monitored. It was observed that all six isolates were able to grow in minimal media. However, the growth rates were slower in minimal media compared to BHI (data not shown).

Arsenic-Induced Ultra-Structural Changes

TEM studies revealed that arsenite affected the integrity of the plasma-membrane with maximum damage recorded with

Table 2. Arsenic accumulation potential of the isolates

		Arsenite (μg) absorbed mg^{-1} bacteria[a]	
		Hours of exposure to arsenite	
Bacterial isolate	Arsenite amendment (mg L^{-1})	1	4
Ransu-1	0.10	0.23 ± 0.005	0.80 ± 0.057*
	1.00	2.90 ± 0.057[†]	4.12 ± 0.075*[†]
IBL-1	0.10	1.90 ± 0.057	2.52 ± 0.069*
	1.00	2.52 ± 0.069[†]	3.79 ± 0.04*[†]
IBL-2	0.10	0.70 ± 0.065	1.40 ± 0.072*
	1.00	2.80 ± 0.041[†]	5.61 ± 0.051*[†]
IBL-3	0.10	0.91 ± 0.061	1.45 ± 0.068*
	1.00	1.81 ± 0.041[†]	4.40 ± 0.055*[†]
IBL-4	0.10	0.175 ± 0.003	0.70 ± 0.006*
	1.00	2.24 ± 0.07[†]	3.16 ± 0.022*[†]
IBL-5	0.10	0.176 ± 0.006	1.76 ± 0.04*
	1.00	1.41 ± 0.04[†]	3.20 ± 0.015*[†]

[a] Mean $\pm$ SE. Bacteria exposed to 0.1 mg L^{-1} and 1 mg L^{-1} arsenite for 1 and 4 h were harvested and bacterial arsenic accumulation was measured by AAS. Arsenite accumulation in the bacterial isolates showed time (*$p < 0.05$) and concentration dependency ([†]$p < 0.05$). The results represent mean $\pm$ SE of three independent observations.
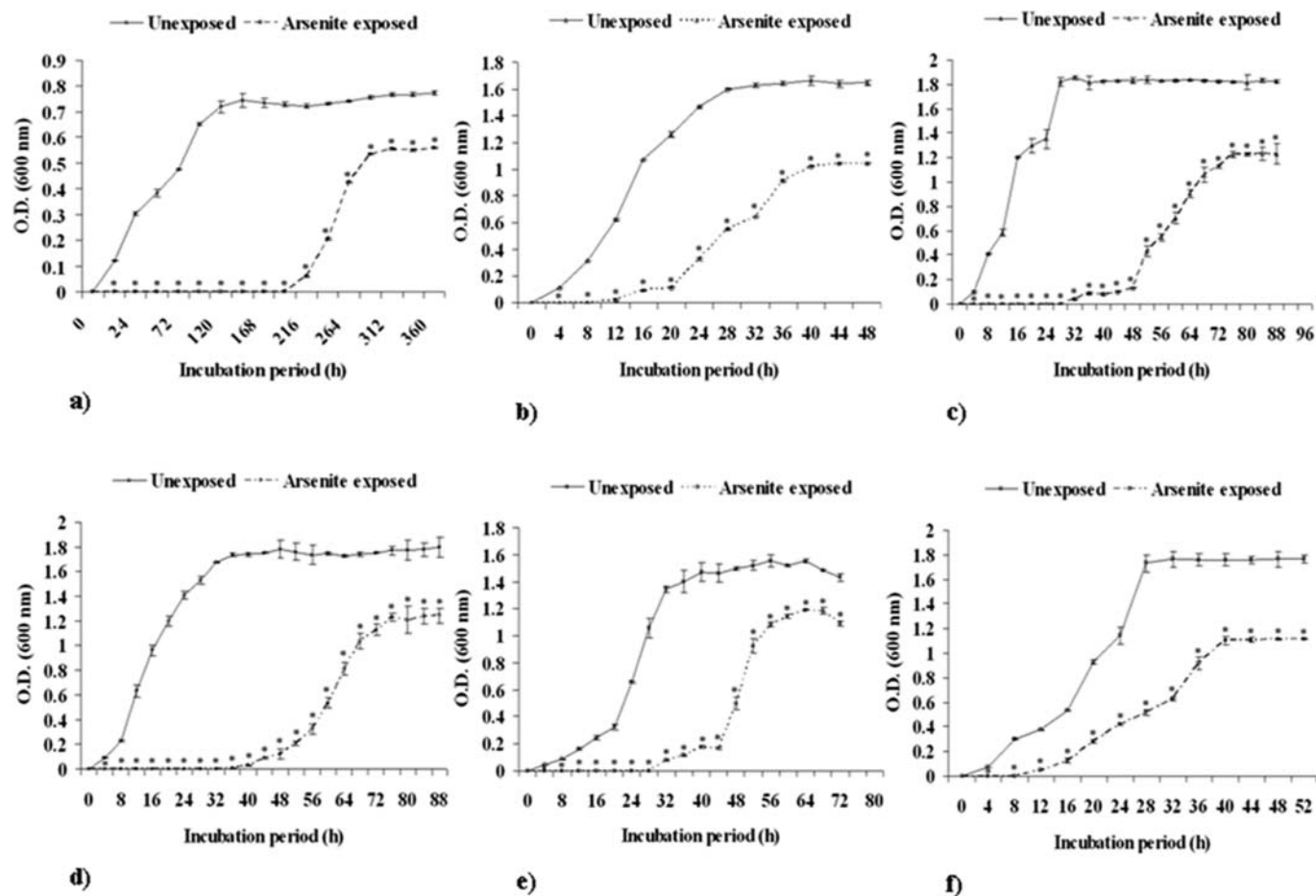
Fig. 3. Effect of arsenic on bacterial growth. Bacteria were grown in presence (arsenite exposed) or absence (unexposed) of arsenite and changes in O.D. monitored at regular intervals. Growth curve of a) Ransu-1 exposed (100 mg L^{-1}) or unexposed to arsenite, b-d) IBL-1, IBL-2, IBL-3, IBL-4 and IBL-5 exposed (600 mg L^{-1}) or unexposed to arsenite. The results represent mean $\pm$ SE of three independent observations. *p < 0.05.

Table 3. Time periods of different growth phases of unexposed and arsenic exposed bacterial isolates

Bacterial isolate	As^{+3} exposure	I.L. (h)	M.L. (h)	L.L. (h)	Stn. (h)
Ransu-1	Unexposed	12	24–48	72–96	288
	100 mg L^{-1}	216	240	264	312
IBL-1	Unexposed	4	8–12	20–24	36
	600 mg L^{-1}	12–16	24–28	32–36	40
IBL-2	Unexposed	4	12–16	24	44
	600 mg L^{-1}	32	56–60	64	76
IBL-3	Unexposed	4	12–16	24	44
	600 mg L^{-1}	40	60	68–72	80
IBL-4	Unexposed	4	24	32	56
	600 mg L^{-1}	32	48	52	64
IBL-5	Unexposed	4	16–20	24–28	32
	600 mg L^{-1}	12	28	32–36	40

I.L., initiation of log phase; M.L., mid log; L.L., late log; Stn., stationary phase of growth; Unexposed, bacteria grown in absence of arsenic; 100 and 600 mg L^{-1}, concentration of arsenite (As^{+3}) where bacteria were exposed. The results represent the average three independent observations.

1 mg L^{-1} arsenite in Ransu-1 and IBL-4. Isolates IBL-1, IBL-2, IBL-3, and IBL-5 exhibited distinct structural alterations characterized by loss of membrane integrity and cytoplasmic condensation which appeared to be more pronounced on exposure to 1 mg L^{-1} of arsenite (Figure 4).

Discussion

The objective of this study was to isolate arsenic-resistant bacteria from surface water of Bengal Basin and for that six villages in West Bengal, India, were selected. In the process we observed surface water arsenic concentrations in these villages to be significantly higher than the WHO permissible limit (10 μg L^{-1}). Such high concentration of arsenic from surface water further compounds the problem of arsenic toxicity in these areas. Several hypotheses have been proposed to explain the presence of arsenic in the Indo-Bangladesh region including anthropogenic activities and geogenic processes (Islam et al. 2005). Besides, the role of microbes on influencing the arsenic concentration in the Bengal basin water cannot be excluded. Hydrogeochemical analyses of water from
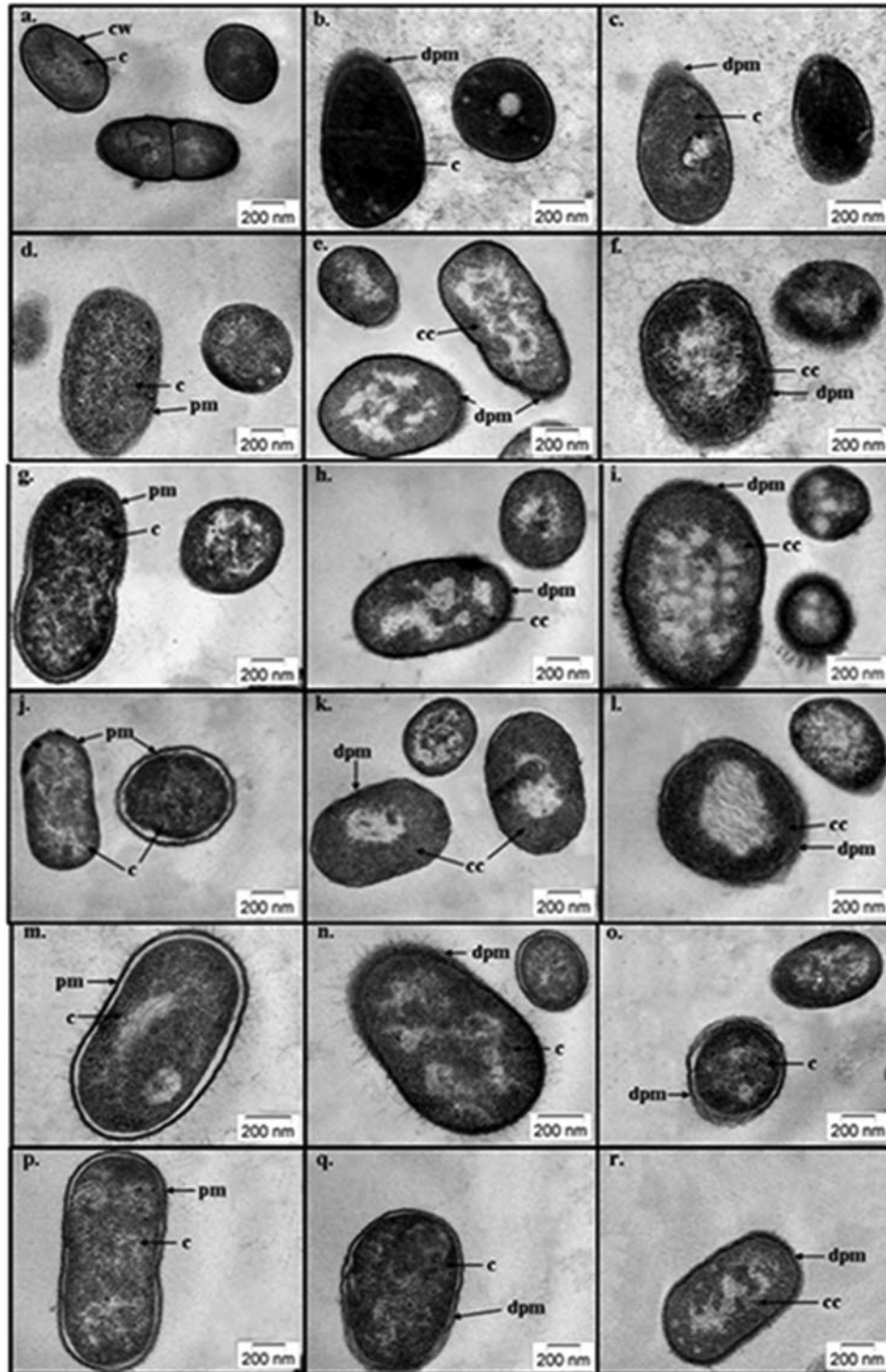
Fig. 4. Arsenic-induced ultra-structural changes. Bacteria were exposed to different concentrations of arsenite (0.1 and 1 mg L^{-1}) and structural alterations studied. a) unexposed Ransu-1; b) 0.1 mg L^{-1} exposed Ransu-1; c) 1 mg L^{-1} exposed Ransu-1; d) unexposed IBL-1; e) 0.1 mg L^{-1} exposed IBL-1; f) 1 mg L^{-1} exposed IBL-1; g) unexposed IBL-2; h) 0.1 mg L^{-1} exposed IBL-2; i) 1 mg L^{-1} exposed IBL-2. j) unexposed IBL-3; k) 0.1 mg L^{-1} exposed IBL-3; l) 1 mg L^{-1} exposed IBL-3; m) unexposed IBL-4; n) 0.1 mg L^{-1} exposed IBL-4; o) 1 mg L^{-1} exposed IBL-4; p) unexposed IBL-5; q) 0.1 mg L^{-1} exposed IBL-5; r) 1 mg L^{-1} exposed IBL-5. cw, cell-wall; c, cytoplasm; cc, condensed cytoplasm; pm, plasma-membrane, dpm, damaged plasma-membrane.

this area suggested the absence of dissolved oxygen, nitrates and enrichment of reduced solutes, implicating microbe-mediated, thermodynamically favored redox processes dominated by Fe/Mn-reduction to be an important reason for arsenic toxicity in the water from this region (Mukherjee et al. 2008). However, the absence of large-scale industrialization and practicing conventional agricultural methods suggest intensified arsenic in this region to be primarily geogenic.

Several arsenic-resistant bacteria have been isolated from ground water or arsenic-rich sediments in West Bengal and Bangladesh. In this study we report for the first time the presence of arsenic-resistant bacteria from the surface water of these areas, which includes *M. oleivorans, A. soli, A. venetianus, A. junii, A. baumannii,* and *A. calcoaceticus.* Although, arsenic-resistant strains of *Microbacterium* and *Acinetobacter* have been reported (Abou-Shanab et al. 2007; Achour et al. 2010; Sultana et al. 2011) this is the first report of *Microbacterium oleivorans* and *Acinetobacter soli* resistant to the metalloid. The six isolates were screened on the basis of ability to grow in presence of high levels of arsenite, as it is more toxic. Metal-stress affects bacterial growth (Bagde et al. 2008; Patel et al. 2006) and we observed that all the isolates grew in regular as well as minimal media supplemented with arsenite, albeit at slower rate. This suggested that the rich medium did not buffer the toxic effects of arsenic; rather, the presence of an innate system of arsenic-resistance in these bacteria helped in their growth.

The ability of the isolates to grow in minimal medium containing arsenic made us interested in studying the underlying mechanism. Earlier studies suggested that arsenic selected variants possess genetic resistance determinants which help in overcoming arsenic-toxicity (Cervantes et al. 1994; Goswami et al. 2011). Although, arsenic-resistant bacteria have been reported from the Bengal Basin, a complete picture of the genes responsible for this trait and their mechanism of action are incomplete. A PCR-based approach using specific primers was used to study the occurrence and diversity of arsenic-resistance genes in these bacteria.

When plasmid and genomic DNA from *M. oleivorans* were amplified, the *arsB* gene was detected from both the genomic and plasmid DNA, suggesting an arsenic-resistant trait in the isolate at plasmid and genomic level. The *arsB* gene or its product has not been reported in *M. oleivorans.* Hence, to the best of our knowledge, is the first report of *arsB* gene in this bacterium. However, as the isolate could withstand arsenate-stress, we could not detect arsenate-resistant genes in this isolate with the primers. Thus, identifying these genes would help understanding how *M. oleivorans* withstands arsenate stress.

When the plasmid and genomic DNA of the different *Acinetobacter* isolates were amplified we obtained a varied picture. In *A. soli, aoxB* was present on plasmid and genomic DNA whereas *arsB* was present on plasmid suggesting complementation of the arsenic-resistant trait in the isolate at plasmid and genomic level. In *A. junii* and *A. baumannii, arsB* and *aoxB* were located only in the genomic DNA. This suggested the survival mechanism of *A. soli, A. junii* and *A. baumannii* in both the arsenite and arsenate-contaminated environments. *A. calcoaceticus* only had plasmid-borne

aoxB, suggesting the arsenate-resistance trait to be plasmid-encoded for the isolate. In the absence of arsenite-resistant traits, it is intriguing how this isolate withstands arsenite-stress.

Interestingly, we failed to identify the presence of any arsenic-resistance gene either in the plasmid or genomic DNA of *A. venetianus,* suggesting the arsenic-resistance trait to be mediated by hitherto unknown mechanisms. It is important to note that often a single primer is unable to amplify a particular gene from every environmental isolate due to subtle variations in the conserved regions (Sun et al. 2004). Thus the failure of the primers to amplify *arsB, ACR3(1), ACR3 (2), arsC* and *arrA* genes in isolates could be due to sequence diversity compounded with the fact that the primers were designed from phylogenetically different arsenic-resistant bacteria. Although it is not possible to conclude from our study whether these bacteria are autochthonous or allochthonous, our observations suggest that the diversity of arsenic resistance genes is probably much greater and more complex than is apparent from studies on known arsenic-resistant isolates (Goswami et al. 2011).

The presence of *arsB* in four of the six isolates prompted us to study *in silico,* the phylogenetic relationship among the isolates. Although, *M. oleivorans* and *A. soli* possessed *arsB*, due to the unavailability of their protein sequence, were excluded from the analysis. We observed (Figure 1b) that *A. johnsonii* and *A. junii* as well as *A. baumannii* and *A. junii,* were most distantly related species in their *arsB* sequence, whereas significant similarities between *A. johnsonii- A. lwoffii* and *Pseudoxanthomonas spadix- Paracoccus denitrificans* were noted. The high degree of similarity among the *arsB* sequences suggests the gene was acquired through horizontal gene transfer. As the six sampling sites were closely located, we expect the mechanism of arsenic resistance to be similar. This probably accounts for the high degree of similarity among the *arsB* gene product. Although the amino acid sequence of ArsB is available in the NCBI database, we failed to identify the presence of *arsB* gene in *A. calcoaceticus* using the primer enlisted in Table 1. This may also be due to the sequence variability as mentioned earlier.

Despite being resistant to arsenic, this metalloid induced ultra-structural changes to varying extent in all the isolates. There could be several mechanisms responsible for inducing such changes in the bacteria. Metal ions form unspecific complexes when present at higher concentrations, which accumulate or bind onto bacterial membranes, altering their structure (Patel et al. 2006). It has been suggested that following the withdrawal of metal stress, these revert to normalcy (Surve and Bagde 2010; Tornabe and Edwards 1972). It is important to mention that the arsenic-resistant isolates were maintained and stored in arsenic-free media in the laboratory.

Thus, consequent to arsenic exposure, these changes are induced on the bacterial isolates. Moreover, metal-microbe interactions under natural conditions are a matter of speculation. There could be methods employed by these bacteria to overcome metal-stress that cannot be replicated under laboratory conditions. Hence exposure to arsenic under lab conditions led to acute stress, prompting alterations in bacterial

structures. We had measured the total arsenic content from the surface waters, not the relative concentrations of arsenite and arsenate. However to study the arsenic-induced structural changes we exposed the bacteria to elevated concentrations of arsenite. It is possible that the dose of arsenite selected is not what they are exposed to in nature, thus resulting in these structural changes. However, it is important to note that the ultra-structural changes observed in the present study are akin to lead-induced changes reported in *A. hydrophila* (Silverberg et al. 1976), suggesting these as a general response subsequent to metal-stress in bacteria.

Bio-remediation is an effective tool to reduce contaminant toxicity to levels that are innocuous to human health and ecosystem (Mukherjee et al. 2013). Bacteria have their own mechanisms for metal accumulation and helps in bio-remediation (Shuttleworth and Unz 1993). The advantages of using microbes for bio-remediation include natural occurrence, cheap production and easy availability to treat large volumes of waste water. We observed time and concentration dependent accumulation of arsenic in the isolates suggesting the usage of the isolates for bio-remediation in arsenic-prone areas.

Thus the present study provides valuable information of microbial species in surface waters of arsenic-affected areas, which could probably be responsible for natural arsenic speciation. It adds basic inputs regarding the possible mechanisms of arsenic tolerance in these bacteria and provides a platform for future study of arsenic-remediation through microbial route in endemic areas.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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