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High arsenic tolerance in *Brevundimonas aurantiaca* PFAB1 from an arsenic-rich Indian hot spring

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ABSTRACT

Background: Arsenic contamination in the ground water of rural India is a recurrent problem and decontamination is mostly based on the chemical or physical treatments until now. Microbial bioremediation is eco-friendly, cheap, time-efficient and does not produce any toxic by-products.
Result: In the present study, a high arsenic tolerant bacteria *Brevundimonas aurantiaca* PFAB1 was isolated from Panifala hot spring located in West Bengal, India. Previously Panifala was also reported to be an arsenic-rich hot spring. *B. aurantiaca* PFAB1 exhibited both positive arsenic reductase and arsenite oxidase activity. It was tolerant to arsenite up to 90 mM and arsenate up to 310 mM. Electron microscopy has proved significant changes in cellular micromorphology and stalk appearance under the presence of arsenic in growth medium. Bioaccumulation of arsenic in As (III) treated cells were 0.01% of the total cell weight, while 0.43% in case of As (V) treatment.
Conclusions: All experimental lines of evidence prove the uptake/accumulation of arsenic within the bacterial cell. All these features will help in the exploitation of *B. aurantiaca* PFAB1 as a potent biological weapon to fight arsenic toxicity in the near future.

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1. Introduction

Arsenic (As) is one of the most abundant heavy metals easily encounterable in nature, which is also carcinogenic [1]. On the other side, it is a vital trace element to some animals, even in humans, but shows lethal effect above the threshold value (as low as 0.01 mg/day). There are several reports on chronic arsenic exposure-mediated elevation of blood pressure resulting into cardiovascular diseases, lung cancer, bladder cancer [1] pulmonary disorders [2] prostate cancer [3] hypertension, type 2 diabetes mellitus [4] acute coronary syndrome [5] or DNA damage in blood cells of children in arsenic mining area [6]. Even low arsenic exposure for a long term has deleterious effects on humans including skin lesions [7] hypothyroidism [8] risk of myocardial infarction and anaemia in pregnant women [9].

Arsenic may exist in the environment in four different oxidation states in between which, pentavalent arsenate (V) and trivalent arsenite (III) are most abundant in air as well as in surface and groundwater [10,11]. Arsenate is an inorganic form of arsenic in aerobic aqueous environments, which has high solubility, thus is difficult to separate from contaminated water. It is a phosphate structural analogue which enters a bacterial cell using phosphate transport system [12]. Few microbes also show enhanced phosphate uptake in addition to arsenic [12]. Toxicity of arsenate (V) is due to its interference with normal phosphorylation processes by replacing cellular phosphates [13]. It is also described that at neutral pH, arsenite (III) enters into the bacteria, yeasts or mammal cells via aqua-glyceroporins. By binding to the sulfhydryl groups of cysteine residues followed by inactivating them, arsenite exhibits its cytotoxicity. It is also considered to be on average 100 times more toxic than arsenate and can be oxidized to arsenate both chemically or microbiologically [13].

Conventional arsenic remediation techniques are expensive and chemicals dependent. Phytoremediation is reported as efficient but is a time dependent process [14] whereas microbial bioremediation is eco-friendly [15] cheap, time-efficient and does not produce toxic by-products [11]. Arsenate (V) reducing bacteria like *Desulfitobacterium* sp. DJ-3, *Exiguobacterium* sp. DJ-4 [16] and *Micrococcus*

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luteus strain AS2 [17] are predominant in arsenic contaminated water. Arsenates are actually salts consisting of AsO_4^{3-} ion that is required as a respiratory electron acceptor for oxidation of different organic substrates. Fortunately, microorganisms can induce the speciation and mobility to As; therefore, they can be used in detoxification of As and as environmental clean-up agents [18]. *Pseudomonas* group of rhizospheric bacteria can cause the conversion of arsenite (III) to arsenate (V) or vice versa [12]. There is information on arsenic biposorption by *Bacillus cereus*, where maximum biosorption capacity of live *B. cereus* cells for arsenic (III) has been 32.42 mg/g at pH 7.5 [19]. Arsenic resistance is described on different microorganisms that include *Desulfohalophilus alkaliarsenatis* [20] *Microbacterium* [21] *Klebsiellia*, *Acinetobacter*, *Enterobacter*, *Comamonas* [22] etc.

A recent pilot scale study in West Bengal, India assessed occupancy of toxic metals in groundwater. Prevalent presence of As has resulted into unintentional exposure of As to the mass [23]. So in the present scenario of India and also the usefulness of bacterial arsenic remediation as discussed earlier, it is fundamental to find a uniquely efficient arsenic-tolerant bacteria that may be used in future for possible bioremediation in order to make safe human habitation and uncontaminated water consumption. Interestingly, in our present study, extreme arsenic tolerance is reported from a novel thermotolerant bacteria *Brevundimonas aurantiaca* isolated from an arsenic-rich hot spring of India. The bacteria efficiently biosorbed arsenic inside the cell with some visible micromorphological changes.

2. Material and methods

2.1. Sampling, study site and water analysis

Water sample was collected from Panifala hot spring that is situated solitarily in a coalmine region of Asansol, West Bengal, India (23°45′33″N, 86°58′54″E) in sterilized polythene bottles according to the standard protocols to avoid exposure to the environment [24]. The maps of the study site have been prepared through supervised classification using the Maximum Likelihood method and the satellite images were imported in ArcGIS 10.1. Temperature, pH, electrical conductivity (EC), total dissolved solid (TDS), oxidation–reduction potential (ORP) and salinity of the immediately collected water sample were measured by a HORIBA multi-parameter meter, whereas other parameters viz. acidity, alkalinity, total hardness, calcium(Ca)-hardness, magnesium(Mg)-hardness, calcium and magnesium were measured following standards of APHA [25]. YSI multi-parameter meter was used for determining ammonia and nitrate concentration. Rest of the parameters, i.e., sulphate, phosphate, chloride and COD were measured according to turbidometric, stannous chloride, argentometric and open reflux methods, respectively.

2.2. Bacteriological analysis

Collected water sample was cultured in nutrient agar (NA) (Himedia®) media after serial dilution and was incubated in 45°C for 24 h. Fast growing colonies were isolated to test their arsenic resistance potential in NA plates supplemented with 20 mmol l^{-1} of As(V) and 5 mmol l^{-1} of As(III), respectively. Physio-morphological characterization of the isolated bacterial colony was performed following the standard methods of Pelczar et al. [26]. Different biochemical properties of the bacterial isolate, such as enzyme activity (catalase and oxidase), substrate hydrolysis (gelatine, casein, tributyrin, olive oil, starch, cellulose and xylan), substrate utilization (lysine and citrate), ability to produce hydrogen sulphide, utilization of different carbon sources, salt tolerance, temperature, pH, gram characteristic and thermal death point were tested following the standard methods [27]. To study the antibiotic sensitivity of the isolate PFAB1, different standard antibiotic discs (Himedia®) such as, chloramphenicol, amoxicillin, amoxyclav, cefotaxime, ceftriaxone, tetracycline, streptomycin and neomycin were used [10]. Fluorescence microscopy was performed as earlier reported by Banerjee et al. [27].

2.3. 16S rRNA sequencing and phylogenetic study

The genomic DNA of PFAB1 cells was isolated using Zymo Research Fungal/ Bacterial DNA MiniPrep (D6005) and the purity of the DNA was confirmed using Nanodrop 1000 (Thermo Scientific, USA). The ~1.5 kb 16S rRNA was amplified using high fidelity PCR polymerase and the PCR product was sequenced using the universal forward and reverse primers. EzTaxon was used for 16S rRNA identification (http://www.ezbiocloud.net/identify). The phylogenetic tree was prepared following neighbor joining method [28] using MEGA7 [29]. The 16S rRNA gene sequence obtained from this study was further deposited in GenBank.

2.4. Arsenic biotransformation assay

To identify the As (III) oxidation and As (V) reduction, the isolate PFAB1 was supplemented with 5 mM sodium arsenate and 1 mM sodium arsenite, respectively, in yeast mannitol agar media (Himedia®). After 48 h, arsenic biotransformation was verified using 0.1 M silver nitrate solution [10]. A small amount of silver nitrate was added to the media. If the media turned brown, it confirmed the presence of silver arsenate and if turned yellow, the presence of silver arsenite was confirmed.

2.5. Tolerance to As (III) and As (V)

Arsenic tolerance property of the isolate PFAB1 was determined in terms of maximum tolerable concentration (MTC). The MTCs from 1–100 mM sodium arsenite and 10–500 mM sodium arsenate were determined as the highest concentration of As that allows bacterial growth after five days of incubation at 45°C [18]. In order to obtain an appropriate cell density (~10^6 cells/ml), OD_{620} nm (0.05) was considered as the standard growth.

2.6. Structural analysis of the arsenic treated cells

2.6.1. Transmission electron microscopic (TEM) analysis

For TEM analysis, both control PFAB1 cells and those exposed to 1 mM As(III) and 5 mM As(V), respectively, were freshly grown overnight. These were further fixed on carbon-coated grid, which were stained with 0.2% uranyl acetate to observe under JEM-1011 100KV transmission electron microscope.

2.6.2. Scanning electron microscopy (SEM) combined with EDS analysis

The surface morphology of the control PFAB1 cells and those exposed to 1 mM As(III) and 5 mM As(V) was studied with scanning electron microscope (JEOL JSM-6390). For SEM studies, bacterial smear was prepared on a cover glass and heat fixed followed by fixation of the smear with 2.5% glutaraldehyde solution for 30 min. Slides were then dehydrated passing through 50–90% of alcohol solutions and finally through absolute alcohol for 5 min each. The bacteria on the cover glass were platinum coated using JEOL JFC 1600 Auto Fine Coater and observed under accelerating voltage 20 kV.

The elemental arsenic content of both the control and treated samples was analysed using energy dispersive X-ray spectroscopy

(EDS) (INCA 250 EDS) to determine the intracellular presence of arsenic in the arsenic treated cells.

3. Result and discussion

3.1. Sampling, study site and water analysis

The study area Panifala hot spring of West Bengal, India is already reported a few times earlier about its microbial diversity [25,30,31,32,33,34,35,36]. Hot water flows from a borehole (Fig. 1) with a surface water temperature of 66.2°C [25]. The site is not a famous a tourist spot as it is situated in a remote area of coalmine region. Therefore, natural microflora probably is not disturbed with human interference. Previously there have been no reports of ground water arsenic contamination in the Asansol area of West Bengal. However, Panifala has report of high arsenic content of 433 ppb by Banerjee et al. [27]. Previously arsenic-rich hot spring (133 ppb) in India was only reported from Himalayan Manikaran area [37]. Panifala hot spring is alkaline in nature (pH 8.11) with relatively high COD value along with high ammonia and nitrate concentration. Presence of sulphate (5 mg/l) is a characteristic feature of hot springs (Table S1). Presence of arsenic metalloid in sulfur-rich hot springs like our study site Panifala is already reported earlier where elevated arsenic concentration was due to thioarsenic transformation [38].

3.2. Polyphasic characterization of Brevundimonas aurantiaca PFAB1

Isolate PFAB1 was identified as *Brevundimonas aurantiaca* and its 16S rRNA sequence was deposited in GenBank under the accession number KU948293. *B. aurantiaca* PFAB1 is a gram negative bacteria that has the ability to produce catalase and oxidase

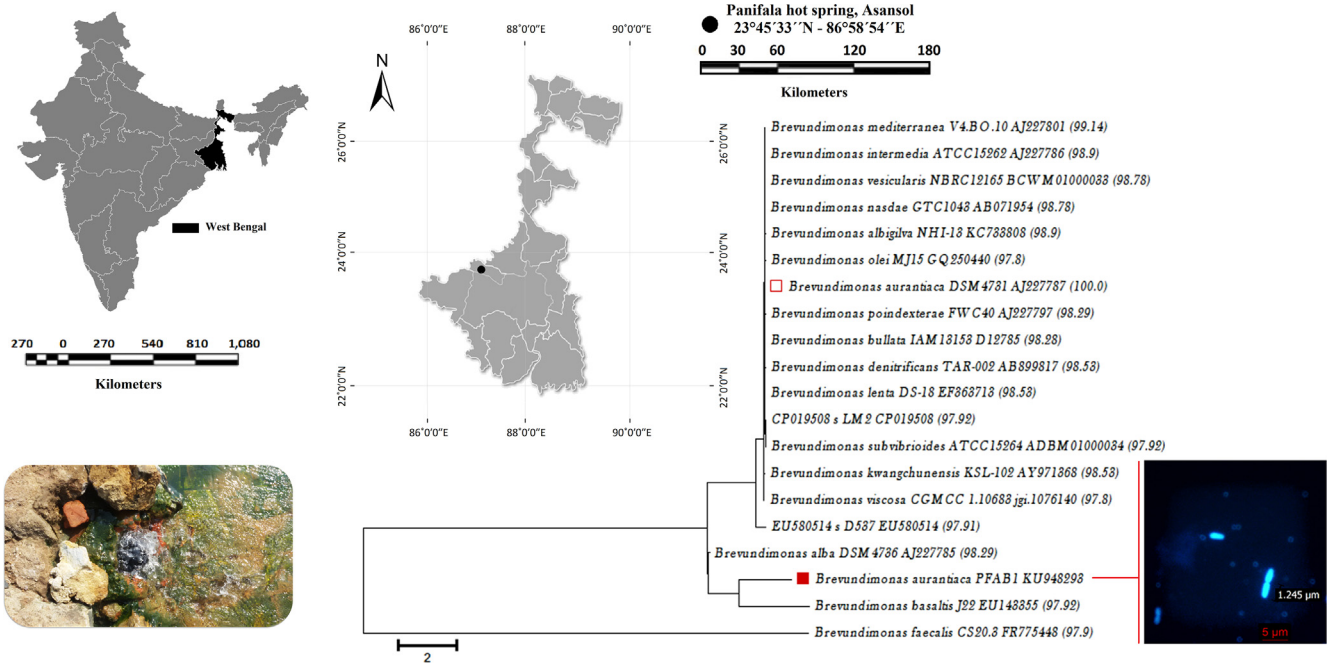


Fig. 1. Phylogenetic tree of *Brevundimonas aurantiaca* PFAB1 (GenBank accession number KU948293) having 100% identity with *B. aurantiaca* type strain DSM 4131(T). Both the water collection site of Panifala hot spring and fluorescence microscopic view of *B. aurantiaca* are presented in the inset.

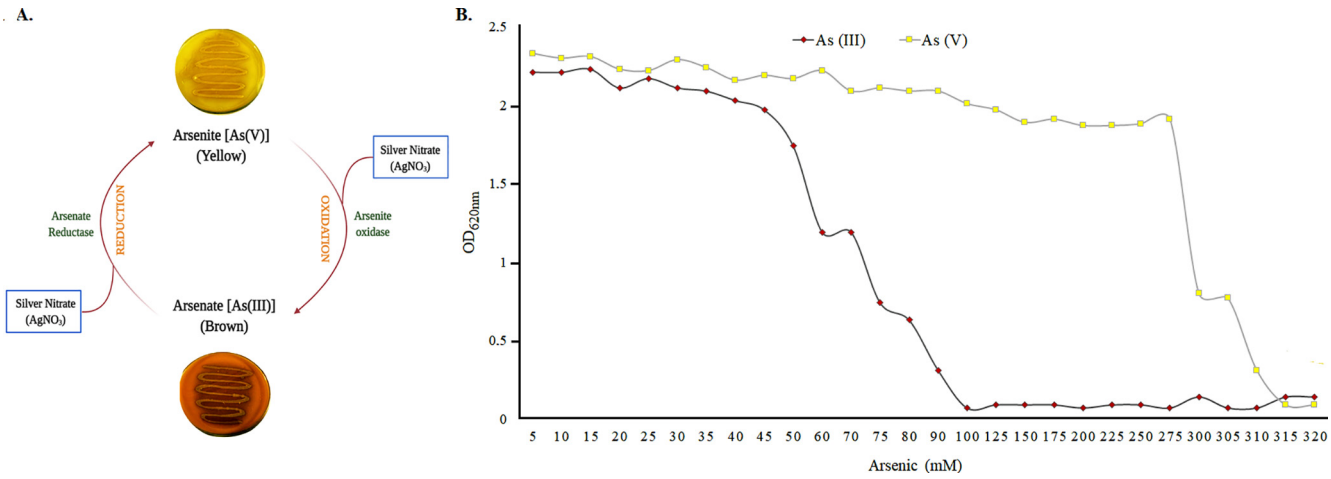


Fig. 2. A. Schematic representation of the results of arsenic biotransformation and B. MTC and MIC of *B. aurantiaca* PFAB1 to As(III) and As(V).

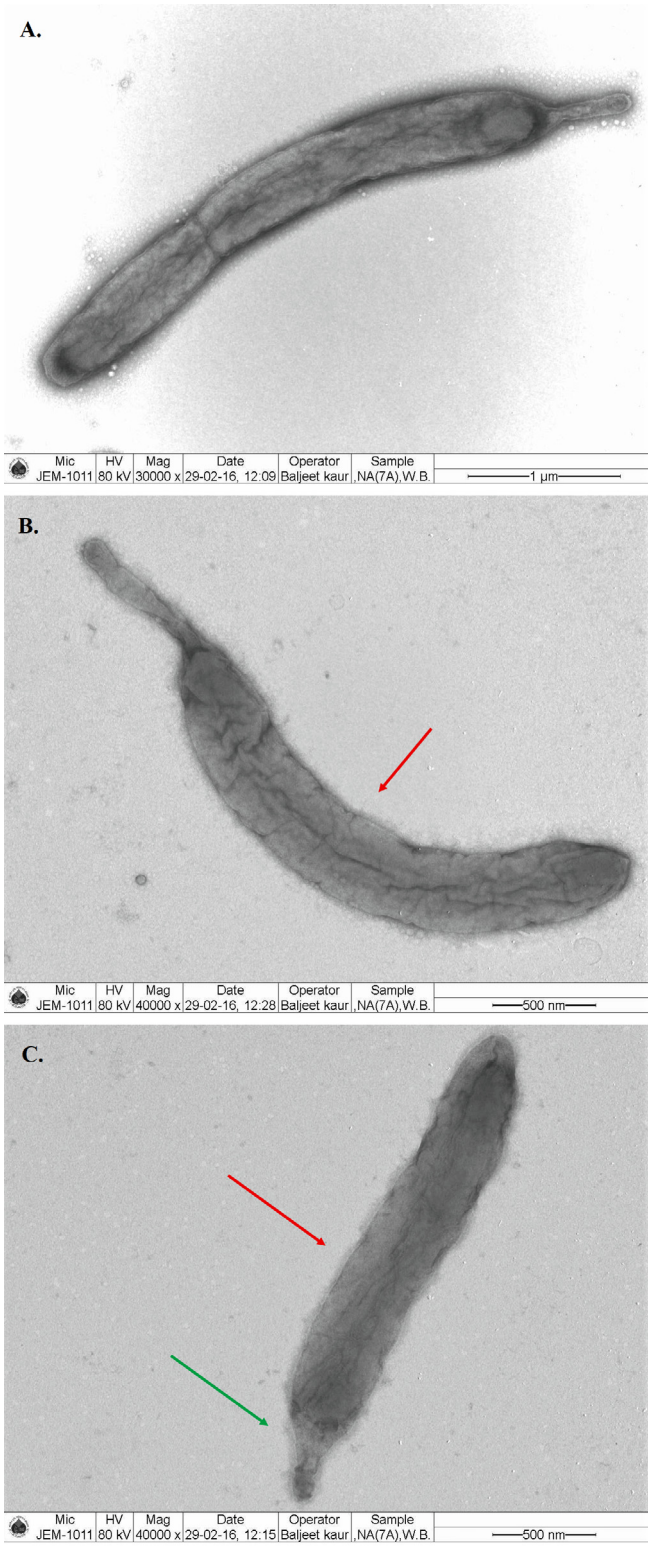


Fig. 3. TEM analysis of the arsenic treated vs. untreated cells; where A. untreated *B. aurantiaca* PFAB1 with smooth surface and well developed stalk in its divisional stage, B. Arsenate treated cells with visible shrinks in the cell wall (red arrow) and C. Arsenite treated cells showing reduction in the stalk size (green arrow) and evident cell wall shrinkage (red arrow).

enzymes in an optimum growth at 45°C (Fig. S1). It was also salt tolerant (up to 4.5%) and exhibited a reasonably variable range of pH for growth with optimum at pH 7.0. Thermal death point of this hot spring origin PFAB1 isolate was as high as 90°C. It was also capable of hydrolysing a large array of compounds, *viz.* gelatine, olive oil, starch and xylan. It utilized citrate for growth. The most attractive feature of this isolate was its carbohydrate fermentation property; more than 10 different carbohydrates including all mono-, di-, and polysaccharides belonged to this list. Other than penicillin, this was sensitive to almost all other antibiotics that includes amoxycillin, amoxyclav, cefotaxime, ceftriaxone, tetracycline, streptomycin, neomycin and chloramphenicol (Table S2). Dividing PFAB1 cells can be observed in Fig. 1 that is ~1.2 µm in diameter.

16S rRNA sequence of isolate PFAB1 shared 100% identity with *B. aurantiaca* type strain DSM 4131(T) obtained from the GenBank database using the BLAST search and the EzTaxon-e EzBioCloud programme. Fig. 1 shows the result of the phylogenetic analysis of *B. aurantiaca* PFAB1.

3.3. Arsenic biotransformation assay

Addition of silver nitrate to 48 h old arsenite containing culture slowly turned the media brown confirming the presence of silver arsenate. The colour change hints the biotransformation of arsenite to arsenate by periplasmic arsenite oxidase enzyme [39] produced by *B. aurantiaca* PFAB1. Bio-transformed arsenate is reported to be effluxed outside of the cell by transporter present in the membrane [39]. Again, when silver nitrate solution was mixed with the *B. aurantiaca* PFAB1 culture containing arsenate, it turned yellow confirming the presence of silver arsenite probably following the action of the intracellular arsenate reductase enzyme [39] (Fig. 2A).

Arsenite oxidizing activity was earlier reported from *Alcaligenes* [40], *Bacillus*, *Aneurinibacillus aneurinilyticus* [10] *Bosea* [1] whereas arsenate reduction has been reported from *Citrobacter* [41] *Desulfuribacillus alkaliarsenatis* [42] *Pseudomonas*, *Bacillus*, *Vibrio* and *Enterobacter* [43]. In this study on arsenic biotransformation, it was observed that the thermotolerant *B. aurantiaca* PFAB1 had both the ability to reduce arsenate to arsenite by the action of arsenate reductase enzyme and oxidize arsenite to arsenate by arsenite oxidase. This isolate may be exploited as a potent biological tool because of its both oxidation and reduction potential, against arsenic toxicity in the future.

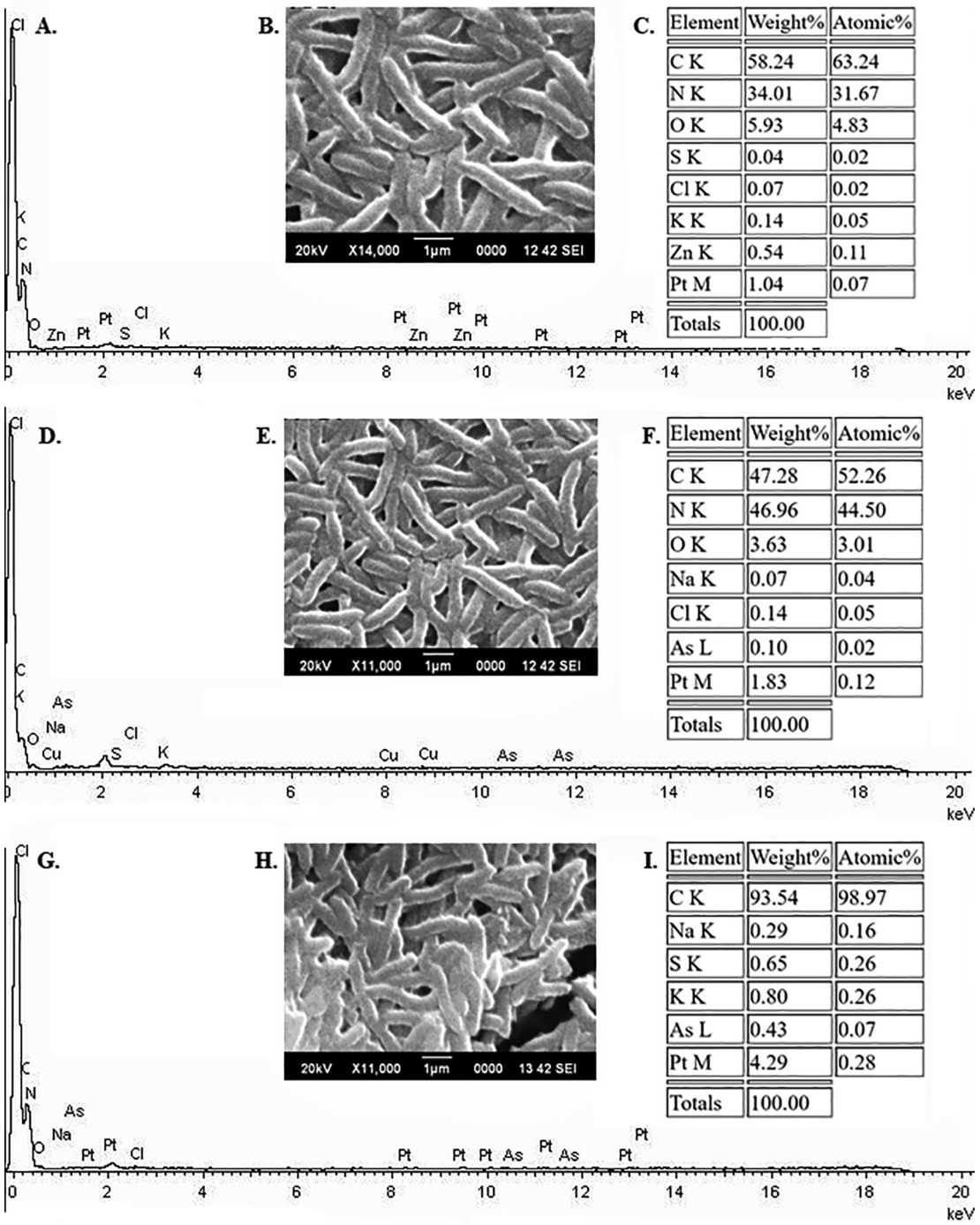
3.4. Tolerance to As (III) and As (V)

Upon treating *B. aurantiaca* PFAB1 with increasing concentrations of both sodium arsenite and arsenate, no momentous change was initially found in the bacterial growth at a minimum tolerance level. For arsenite, the minimum inhibitory concentration (MIC) was 70 mM with a maximum tolerable concentration (MTC) value of 90 mM. On the other hand, for arsenate, the MIC and MTC value were found to be 275 mM and 310 mM respectively (Fig. 2B). Cavalca et al. [44] earlier reported a similar kind of study, where arsenic-tolerant bacteria associated with roots of *Cirsium arvense* (L.) from arsenic polluted soil was grown in the presence of high arsenic concentrations (100 mM arsenate and 10 mM arsenite). The arsenic susceptibility was lower here compared to our study isolate PFAB1. The arsenic-tolerant isolates belonged to thirteen genera, most abundant of which were *Bacillus*, *Achromobacter*, *Brevundimonas*, *Microbacterium*, *Ochrobactrum*, and *Acidithiobacillus* [44,45]. Therefore, compared to the earlier reported instances, this hot spring isolate can be potentially used for bioremediation of the arsenic-contaminated regions in future.

3.5. Structural analysis of the arsenic treated cells

3.5.1. Transmission electron microscopic (TEM) analysis

Transmission electron microscopic (TEM) analysis was performed to understand if any cellular changes were adapted by *B.*



Element	Weight%	Atomic%
C K	58.24	63.24
N K	34.01	31.67
O K	5.93	4.83
S K	0.04	0.02
Cl K	0.07	0.02
K K	0.14	0.05
Zn K	0.54	0.11
Pt M	1.04	0.07
Totals	100.00	

Element	Weight%	Atomic%
C K	47.28	52.26
N K	46.96	44.50
O K	3.63	3.01
Na K	0.07	0.04
Cl K	0.14	0.05
As L	0.10	0.02
Pt M	1.83	0.12
Totals	100.00	

Element	Weight%	Atomic%
C K	93.54	98.97
Na K	0.29	0.16
S K	0.65	0.26
K K	0.80	0.26
As L	0.43	0.07
Pt M	4.29	0.28
Totals	100.00	

Fig. 4. EDS spectra of the A. Untreated control *B. aurantiaca* PFAB1 cells, D. Arsenate treated cells and G. Arsenite treated cells; SEM micrographs of the B. Untreated control *B. aurantiaca*PFAB1 cells, E. sodium arsenate treated cells and H. sodium arsenite treated cells; Elemental compositions of the C. untreated control *B. aurantiaca* PFAB1 cells, F. Arsenate treated cells and I. Arsenite treated cells.

aurantiaca PFAB1 under As (III) and As(V) stress compared to the control condition. Under the control condition without any As treatment, atypical caulobacterial stalk of *Brevundimonas* probably hinted uptake of sufficient nutrients available in the surrounding environment [46]. The cells were observed in the dividing stages indicating the presence of no nutrient stress in the growth medium (Fig. 3A), whereas, under As stress, shrink in bacterial cell wall (Fig. 3B and 3C) and reduction of the stalk size was evident in As (III) which is probably due to the increased toxicity of As (III) compared to As (V) (Fig. 3C). This might be a possible strategy to fight against acute arsenic contamination [47]. The shrinks in bacterial cell wall also hints extreme tolerance to As by *B. aurantiaca* PFAB1. Owing to the arsenic stress, cell shrinking is not a singular peculiarity, abnormalities in stalk also had been noticed [46,47]. These changes are the proof of the growth ability of *B. aurantiaca* PFAB1 in the presence of high As concentration in surrounding environment which make it a more potent candidate for arsenic bioremediation.

3.5.2. Scanning electron microscopy (SEM) combined with EDS analysis

SEM was employed to determine the changes in the surface morphology of *B. aurantiaca* PFAB1 cells before and after exposure to As (III) and As (V) (Fig. 4). The control cells without any arsenic treatment appeared to be normal and revealed more compact structure while visible changes in the surface morphology was observed after interaction with As (III). Smoothness of the cell decreased significantly and the cells appeared little rough with the uptake of As (III) compared to the control [48]. Results of the SEM analysis also supported the findings of the TEM studies. These changes in the cellular micromorphology might be attributed to the interaction/attachment of some As (III) ions with cell wall of *B. aurantiaca* PFAB1 during its arsenic uptake thereby altering surface properties. Further, compact cell structure was observed on As (V) exposure causing minimum surface disturbances. Similar kind of result was earlier reported [47] where surface disturbance was interrelated to diminution in cell diameter compared to the control cells. The result is significant in terms of the rough and wrinkled morphological changes observed in *B. aurantiaca* PFAB1 under As (V) stress, as similar As (V) stress mediated changes in surface morphology was also earlier reported by Singh et al. [48]. This might be attributed to the fact of ion homeostasis under arsenic toxicity by no transient pore formation.

EDS spectroscopy was done to identify significant arsenic molecule accumulation inside the bacterial cells for both As (III) and As (V) treatments compared to the cells grown in control condition (Fig. 4A, 4D and 4G). Presence of no arsenic was observed in the control cells (devoid of As treatment) while, As was found abundant in both As (III) and As (V) treated bacterial cell revealed by sharp peak of As in the EDS spectrum (Fig. 4C, 4F and 4I). Bioaccumulation of arsenic in the As (III) treated *B. aurantiaca* PFAB1 cells was 0.01% of total cell weight and 0.02% of total atomic weight; while in case of As (V) treatment 0.43% of total cell weight and 0.07% of total atomic weight. Probably, increasing arsenic accumulation inside the bacterial cells leaded to the phenotypic changes as earlier exhibited by Singh et al. [48]. The TEM results too reveal electron dense deposition of toxic As heavy metal inside the cell cytoplasm [47]. Beside, negatively charged arsenic ions can be adsorbed by opposite charged amino groups present in the bacterial cell wall by oxidation–reduction of As or sequestration by a range of cysteine-rich peptides which cause morphological changes [46]. This also established the fact of arsenic bioaccumulation by the bacteria that might be particularly beneficial in terms of arsenic removal from arsenic-contaminated water bodies [45,47]. Earlier extreme arsenic resistance is only recounted from archaeal community of hot springs [49]. *B. aurantiaca* PFAB1, in future, may be used as a potent biological tool against arsenic toxicity and play an important role in environmental safety.

4. Conclusions

In this study, we isolated temperature and arsenic tolerant *B. aurantiaca* PFAB1 from an arsenic-rich Indian hot spring. Biotransformation assay has proved its capability in terms of arsenite oxidation and arsenate reduction that is an exclusive feature compared to many other arsenic resistant bacterial strains reported earlier. Electron microscopy has proved important changes in cellular micromorphology and stalk appearance under the presence of arsenic. Considerable reduction in the size of the atypical caulobacterial stalk indicates the toxicity of As (III) compared to As (V) in growth environment under which bacteria was probably absorbing less nutrients. Significantly, all experimental lines of evidence prove the uptake/accumulation of arsenic within the bacterial cell. All these features may help in the exploitation of *B. aurantiaca* PFAB1 as a potent biological defence to fight arsenic toxicity in near future. However, total genome sequencing might help into getting the important insights in its genetic modifications resulting in such unique arsenic tolerance.

Conflict of interest

None.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2021.05.006.

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