

***Rhizobium Leguminosarum*: A Model Arsenic Resistant, Arsenite Oxidizing Bacterium Possessing Plant Growth Promoting Attributes**

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Abstract

The threat of arsenic (As) pollution has become serious leading to opting for low-cost microbial remediation strategies. Some bacteria have the ability to resist As. A group of rhizosphere bacteria have the ability to absorb arsenic. So these bacteria may be a good candidate for arsenic bioremediation from contaminated environment. Our present study of identifying suitable rhizobacterial strains led to the isolation of As-tolerant strains from arsenic polluted rhizospheric soils of lentil in West Bengal, India. The isolated rhizobacterial strain LAR-7 had a high MIC (minimum inhibitory concentration) towards arsenate (260 mM) and arsenite (27.5 mM) and transformed 39% of arsenite to arsenate under laboratory condition. Further, the strain LAR-7 had enormous plant growth-promoting characteristics (PGP), as categorized by efficient ability to solubilize phosphate, siderophore production, production of indole acetic acid-like molecules, ACC deaminase production, and nodule formation under As stressed condition. Based on 16S rRNA homology the LAR-7 was identified as *Rhizobium leguminosarum* and emerged as the most potent strain for As decontamination and plant growth promoter under the stress environment of As.



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Introduction


The human populations of eastern India (West Bengal mainly) and Bangladesh are severely

suffered by arsenic (As) contamination of water and food.^{1,2} The concurrent application of As enriched irrigation seems to be the major reason for soil

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build-up,^{3,4} and subsequent accumulation in standing crops.⁵ Generally, As residues are found in the top layer of soil or surface soil and enter the plants easily because of their low volatility and low solubility.⁶ As has both organic and inorganic forms⁷ that are found as an oxyanion in the environment.⁸ The high-risk involvement and high cost of remediation⁹ emphasize the scope of bioremediation through novel As-tolerant microbes.¹⁰

In As polluted area, there are several soil-based microbes¹¹ with adaptive strategies for surviving in a contaminated atmosphere mostly by utilizing the toxic arsenic as a nutrient for their metabolisms and proliferation.⁶ Some bacteria such as *Burkholderia*,¹² *Agrobacterium*, *Bacillus*, *Rhizobium* are best suitable candidate for arsenic bioremediation and plant growth promotion (PGP).¹³ These microorganisms can have a dual purpose environmental beneficial impacts by metal decontamination and being plant growth promoters.^{14, 15} These PGP bacteria are able to enrich soil nutrient and mitigate soil arsenic also.¹³ The physical and chemical process for arsenic mitigation are very costly so these rhizobium-legume symbiosis may be an alternative new tool for As mitigation.¹⁶

The unique attributes of As resistance and plant growth promotion (PGP) in rhizospheric microbes is quite noteworthy. These adopted indigenous soil microbes tend to manifest several PGP traits through secretion of 1-aminocyclopropane-1-carboxylate (ACC), deaminase, indole-3-acetic acid (IAA), phosphate solubilizers, producing siderophores that reduce metal toxicity and encourage plant-assisted bioremediation.^{17, 18, 19} Most remarkably, such PGP characters remain active even under intense arsenic stress conditions.^{20, 21}

In this backdrop, we have categorized the study and identified efficient As-resistant bacterium from contaminated rhizospheric soil, and investigated the PGP attributes of identified bacteria with a view to harvest their capacity to develop plant's resistance to stress conditions, encourage plant growth, and give a path to contribute accelerated remediation of arsenic polluted soils.

Materials and Methods

Soil Sample Analysis

Soil samples (2 cm diameter, 10 cm depth) were collected in sterilized zipper bags to maintain an

aseptic condition from arsenic (As) contaminated rhizospheric zone of lentil in Chakdaha, West Bengal (23°05' N latitude and 88°54' E longitude), India, noted for As concentrations in the groundwater above World Health Organization (WHO)-defined safe limit.²² Atomic Absorption Spectrophotometer (AAS, Model-Perkin Elmer A Analyst 200) was used to measure total²³ and available As²⁴ of the soil samples.

Isolation of As-tolerant bacteria

2 g of soil (taken from lentil rhizosphere separately) was suspended in 2 mL sterile distilled water, 1 mL of each was re-suspended in Yeast Extract Mannitol (YEM) liquid medium separately in two conical flasks, spiked with 1 mM arsenate and 1 mM arsenite. The total experimental set up was incubated for 48 hours at 30°C.²⁵ After incubation, 2 ml bacterial culture was further inoculated in a YEM liquid medium. The procedure was repeated twice. Around 0.1 mL of As spiked culture was spread on a YEMA (Yeast Extract Mannitol Agar) solid medium plate to isolate the As tolerant bacteria. To test the *Rhizobium* strain, the congo red YEMA test was categorized.²⁶

MIC (Minimum Inhibitory Concentration) Value of the Bacteria

The MIC is the lowest concentration of arsenate or arsenite which entirely inhibits microbial growth and activity.^{27, 28} 1.0 mL of overnight bacterial culture was inoculated in two conical flasks containing 99.0 mL of YEM liquid medium, containing either arsenite (NaAsO_2 1-50 mM) or arsenate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$; 1-500 mM) separately and incubated at 30°C with 48 hrs of shaking. The OD (optical density, measurement of microbial growth) of the bacterial cultures was detected using a UV-Vis spectrophotometer (Model: Varian CARY-50) at 600 nm wavelength.

Identification of the As Tolerant Bacteria

The As tolerant bacterial genomic DNA was isolated and PCR amplification was done for molecular identification by 16S rRNA sequence analysis.²⁷ The bacterial isolate was studied for Gram reaction, colony morphology and characterized for catalase, urease, and oxidase activities by standard protocols.²⁹

Scanning Electron Microscopic (SEM) Study

For the SEM study, first the bacterial cells were collected and allowed to wash properly with sodium phosphate buffer (pH 7.4). Then a thin layer of

bacterial cells were kept on a glass cover slip and prepared a smear, after that it was allowed to heat-fix over a flame for 1–2 sec followed by fixation with 2.5% glutaraldehyde for 45 min.³⁰ The slides were then dehydrated with 50–90% alcohol solutions and finally through absolute alcohol for 5 min each and observed under a 15 kV scanning electron microscope (HITACHI, S-530, SEM and ELKO Engineering).

Arsenite Transformation and Species Detection

The arsenite transformation ability of the bacterial isolate was determined by modified laboratory-based standard protocol.²⁷ As concentrations were determined by using a spectrophotometric method.³¹ The recoveries of the As species from the liquid culture medium by the efficient bacterial strains were determined by HPLC-ICP-MS.³² All chemicals used for speciation study were reagent grade. All of the experimental solutions were prepared with Milli-Q (Millipore, Bedford, MA, USA) water. A Perkin Elmer Series 200 Micro Pump was used instead of the quaternary pump for the isocratic method. The isocratic mobile phase was 30 mM $\text{NH}_4\text{H}_2\text{PO}_4$ at pH 5.6. The flow rate was 1.0 mL min^{-1} with 100 μL sample injections. The LC column effluent was directly attached to the nebulizer with PEEK tubing (1.59 mm OD) and a low dead volume PEEK connector (Part No.:WE024375).

Plant Growth Promoting (PGP) Characteristics of The As Tolerant Bacterium

The isolated arsenic resistant bacterial PGP attributes (IAA-production, ACC Deaminase activity, phosphate solubilization, nodulation, and siderophore production) were determined *in vitro* in culture medium under arsenic stress conditions (spiking levels being 0 mg/L, 15 mg/L, and 30 mg/L As(III)/As(V)).

Ability to Solubilize Phosphate

This encompasses the growth of the bacterial strain in Pikovskaya's medium³³ (containing 0.5% of tricalcium phosphate (TCP) spiked with three levels of arsenate and arsenite 0, 15, 30 mg/kg) at 30°C for 5–6 days and 170 rev/min. Then the culture was allowed to centrifuge at 6500 times gravity ($\times g$) and supernatant was collected. The solubilized phosphate in supernatant of culture medium was estimated.³⁴

Screening of Indole Acetic Acid (IAA)

The As resistant isolates were determined by growing them in an L-tryptophan (0.5 mg/ml) supplemented minimal medium in presence of different concentrations of As (0, 15, 30 mg/L) and incubated for 5 days in the dark at 30°C. 2 ml bacterial suspension was transferred in 100 μL 10 mM orthophosphoric acid and 4 ml Salkowski's reagent (2% solution of 0.5 M FeCl_3 in 35% perchloric acid) in a test tube. The entire mixture was shaken vigorously before incubation for 45 min until a pink colour develops. Absorbance of the resultant solution was determined at 530 nm for obtaining the content of IAA-like molecules in liquid culture medium.³⁵

ACC Deaminase Activity

The ACC Deaminase enzyme activity is the quantity of α -ketobutyric acid production by the breakdown of ACC³⁶ by the bacterial strain. To determine this, a minimal medium was prepared using 1-aminocyclopropane-1-carboxylic acid or ACC (3 g L^{-1}) as a source of nitrogen, spiked with three different concentrations of As (V) and As(III) (0, 15, 30 mg/L) separately and the bacterial cells were grown. The quantity of ketobutyrate (KB) formed per mg of protein per hour is the total value of the specific enzyme activity.

Nodulation Efficiency

The nodulation efficiency of bacterial strain³⁷ was assessed through a pot study. Soils were sterilized and the seeds of lentil were sown in the sterilized soil spiked with As (0, 15, 30 mg/kg) and the nodule counts were taken after 30 days.

Screening for Siderophore Production

The siderophores production was qualitatively assayed by using the Chrome Azural S method of Schwyn and Neilands.³⁵ The MM9 (Tris buffer, casamino acids (0.3%), L-glutamic acid (0.05%), (+) - biotin (0.5 ppm), and sucrose (0.2%)) liquid medium with the absence of Fe was used for bacterial growth. The bacterial culture medium was inoculated in this medium and allowed to incubate for 5 days at 30°C temperature. For control 0.2 μM of iron (freshly prepared, filter-sterilized $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ stock solution) was also inoculated. The stationary phase of bacterial culture was collected and pelleted by centrifugation (6,500 $\times g$ for 15 minutes).

In supernatant solution, the most important qualitative conformation of the presence of siderophore is simply the color change from blue to orange.

Statistical Analysis

Statistical computations were performed using Microsoft Excel 2016 and SPSS version 23.0.

Results

Characterization of the Experimental Site

The total As concentration and available As concentration of experimental rhizospheric soil of lentil were measured. Results revealed a considerable load of the total and available arsenic i.e. 17.2 ± 1.72 and 1.50 ± 0.27 mg kg⁻¹ respectively (presented as the mean of three observations \pm SD).

Isolation and Identification of the As Tolerant Bacteria

For isolation of Rhizobium, the YEMA medium with congo red was used. A few distinct colonies were found (Fig 1). From these, a distinct colony was picked and allowed to further study.

The isolated strains of rhizobacteria were found to be gram-negative and rod-shaped. These isolated strains were studied for phenotypic and biochemical studies have been represented in Table-1. The bacterial isolate was positive for catalase, glucose, sucrose, sorbitol, lactose, mannitol, maltose, xylose, and fructose; while oxidase, indole, citrate, MR, VP, urease activity were found to be negative.

Further employing 16S rRNA gene sequencing a phylogenetic tree was prepared (Fig-2). These identified bacterial isolates are thus assumed to be *Rhizobium leguminosarum* (LAR-7, accession number MK696942).

Further to confirm the results, the SEM study of the bacterial isolates (Fig- 3) was also categorized.

MIC of The Arsenic Resistant Bacterial Isolates

The arsenic tolerant strains were tested for their minimum inhibitory concentration of As. The result showed that LAR-7 (*Rhizobium leguminosarum*) had a high MIC value. The bacterial isolate had a high MIC towards arsenate (260 mM) and arsenite (27.5 mM).

Arsenite transformation and species detection

The As accumulation and volatilization potential of the isolate was experimented through a quantitative estimation by incubating for 24 hr in a liquid culture medium spiked with 30mM As(III). The As content in liquid medium was analyzed to attain the quantity of As decontamination. The bacterial strain LAR-7 has shown a significant level of arsenic decontamination ability. 35% of total arsenic was decreased from the liquid culture medium containing 30 mM of arsenite. The bacterial transformation of arsenite to arsenate was $437.5 \mu\text{M h}^{-1}$, which is considered 35% of the total arsenite of media (Table 2). LAR-7 could oxidize 35% of 30 mM arsenite within 24 hours.



Fig. 1: Rhizobium isolates in congo red YEMA medium

Table 1: Morphological and biochemical properties of the bacteria

Biochemical Tests	Performance
Catalase	+
Oxidase	-
Urease	-
Indole	-
Citrate utilization	-
MR	-
VP	-
Fructose	++
Mannitol	++
Sorbitol	++
Lactose	++
Sucrose	++
Xylose	++
Maltose	++
Glucose	++

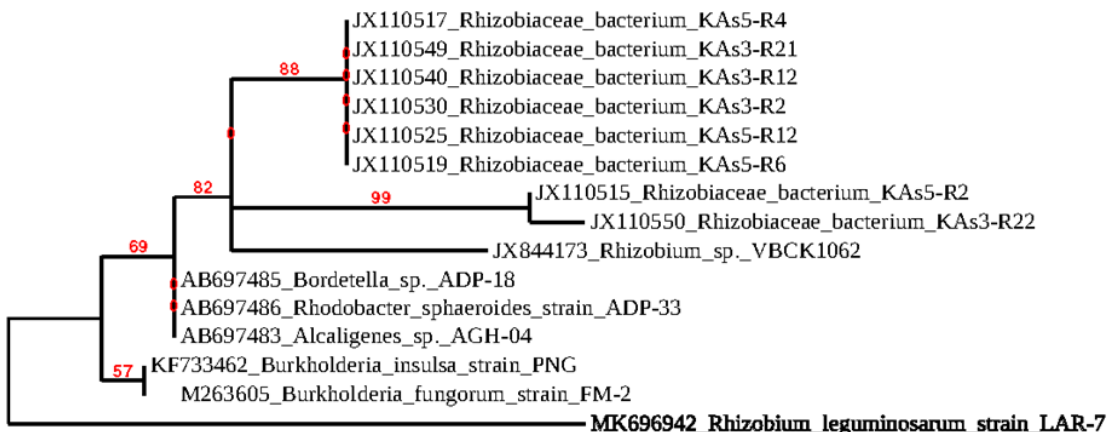


Fig. 2: Phylogenetic tree based on partial 16S rRNA gene sequences of arsenic and heavy metal tolerant bacterial isolates from arsenic contaminated soil and other arsenic oxidising bacterial isolates from the database with accession numbers

Table 2: Arsenic transformation and species detection of the bacterial isolates under 10mg/L As (III) application

Isolate	Total As	Arsenite As(III)	Arsenate As(V)	% transformation As(III) to As(V)	Species recovery
LAR-7	9.32±2.21	5.69±1.96	3.63±1.63	39%	61%
Control	9.37±2.38	9.30±2.64	0.07±0.02	0%	0%

Each value is a mean of three replicates. (mean ± standard deviation)



Fig. 3: SEM image of the bacterial strain

In this study, about 61% of the total As was recovered in As species. The transformation of arsenite to arsenate in liquid culture broth varied from 0 (uninoculated control) to 39% (medium inoculated with LAR-7) by the selected arsenic resistant microbial strains.

Potential Plant Growth Promoting Properties of As Tolerant Bacteria

The plant growth promoting traits of the As tolerant isolate was categorized. The strain LAR-7 was able to solubilize phosphate, produce IAA and ACC Deaminase under As (V) and As (III) stressed conditions (Table-3).

LAR-7 was observed to solubilize a good amount of phosphate (440.8, 440.5, 337.5µg/L when the medium was spiked with 0,15,30 mg/kg arsenate respectively). Similarly, the same results were also found under arsenite stress conditions. This bacterial isolate also solubilizes phosphate 440.8, 140.0, 137.2 µg/L when the medium was spiked with 0,15,30 mg/kg arsenite respectively. Under As stress condition, the amount of phosphate solubilization decreased but still the bacteria was able to solubilize phosphate.

Table 3: Plant Growth promoting properties of the bacterium LAR-7

Arsenic species	As spike (mg/kg)	Phosphate solubilization ($\mu\text{g L}^{-1}$)	IAA Production ($\mu\text{M IAA ml}^{-1}$)	Acc deaminase activity (nM α -ketobutyrate mg protein $^{-1}\text{h}^{-1}$)	Number of nodule production	Siderophore production
As(III)	0	440.8 \pm 53.01	16.4 \pm 8.62	1.99 \pm 0.56	112 \pm 22.3	+
	15	140.0 \pm 26.35	10.0 \pm 5.32	1.09 \pm 0.62	90 \pm 21.3	+
	30	137.2 \pm 23.69	10.0 \pm 5.26	1.00 \pm 0.29	90 \pm 13.5	+
As(V)	0	440.8 \pm 51.06	16.4 \pm 7.69	1.99 \pm 0.45	112 \pm 22.9	+
	15	440.5 \pm 22.35	12.8 \pm 6.23	1.99 \pm 0.68	110 \pm 12.6	+
	30	337.5 \pm 21.05	12.8 \pm 6.12	1.87 \pm 0.64	100 \pm 11.6	+

Each value is a mean of three replicates. (mean \pm standard deviation.)

LAR-7 also was able to produce Indole acetic acid (IAA) and had a good ACC Deaminase activity. The quantity of IAA and ACC Deaminase activity were 16.4, 12.8, 12.8 $\mu\text{M IAA ml}^{-1}$ (medium containing 0, 15, 30 mg/kg arsenate) and 1.99, 1.99, 1.87 nM α -ketobutyrate mg protein $^{-1}\text{h}^{-1}$ (medium containing 0, 15, 30 mg/kg arsenite) respectively. Under arsenite (medium containing 0, 15, 30 mg/kg arsenite) stress condition the quantity of IAA and ACC Deaminase activity were 16.4, 10.0, 10.0 $\mu\text{M IAA ml}^{-1}$ and

1.99, 1.09, 1.00 nM α -ketobutyrate mg protein $^{-1}\text{h}^{-1}$ respectively. Under arsenite and arsenate stress condition arsenic resistant bacterial isolate LAR-7 was also able to produce the root nodule and also had the ability to produce siderophore. The siderophore production was qualitatively tested. So LAR-7 (*Rhizobium leguminosarum*) emerged as an arsenic resistant bacterium also able to carry a bunch of plant growth promoting properties (Fig-4).

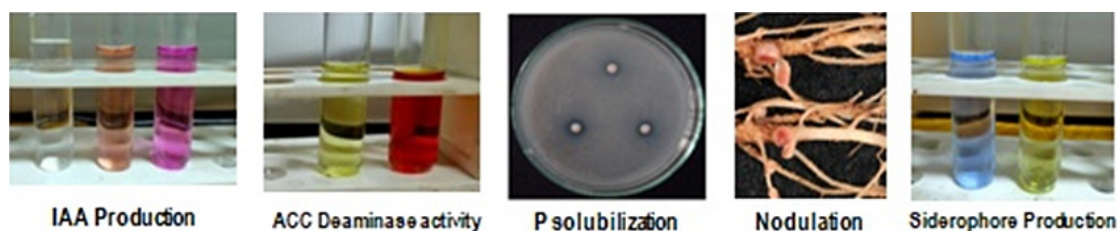


Fig. 7: PGP attributes of the bacterial strain LAR-7

Discussion

Identification and Isolation of As-Resistant Bacterial Strains from Contaminated Soils

In the course of identification and characterization of arsenic resistant PGP bacteria from the As polluted area in the present investigation LAR-7 (*Rhizobium leguminosarum*) bacterial isolate had emerged with a high MIC towards arsenate (260 mM) and arsenite (27.5 mM) which is higher than the previously reported MIC for arsenate (260 mM) and arsenite (27.5 mM) for *Rhizobium radiobacter* of 10 mM of As(V) in agricultural soils³⁸ and also greater than other As-oxidizing, As-resistant bacteria in soil (183 mM arsenate and 6 mM of arsenite),¹⁵ in ground water (200 mM arsenate and 5 mM arsenite),³⁹

in mines (10 mM As (V)).⁴⁰ LAR-7 also showed a greater MIC value than the earlier bacterium which showed the highest MIC of >1,500 mg/L of arsenic.⁴¹ The isolate LAR-7 in our present investigation was identified as *Rhizobium leguminosarum* (based on 16S rRNA gene sequence homology) which can oxidize at the rate of 437.5 $\mu\text{M h}^{-1}$ (35%) in presence of an arsenite concentration (30 mM) within 24 h under laboratory condition. The behaviour is somewhat similar to *Alcaligenes* sp. which completely oxidizes arsenite within 40 h when exposed to relatively reduced arsenite concentrations (1 mM).⁴² The enhanced oxidation efficiencies of isolates in the present research have been quantitatively corroborated via speciation

analysis. It was previously reported that some beta and gamma proteobacteria are also able to oxidise arsenic.⁴³

Plant Growth Promoting Attributes In As Resistant, Arsenite Oxidizing Bacterial Strains

Recent studies have thrown some light on the PGP traits shown by the As oxidizing bacteria. The isolated bacterial strains of *Acinetobacter sp.*, *Klebsiella sp.*, *Pseudomonas sp.*, *Enterobacter sp.* and *Comamonas sp.* from As- polluted tannery wastes under agricultural lands in Thailand³⁵ possess both As tolerance and siderophore production capacity.¹⁰ *Arthrobacter globiformis*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus pumilus*, *Staphylococcus lentus*, *Enterobacter asburiae*, *Sphingomonas paucimobilis*, *Pantoea sp.*, *Rhizobium rhizogenes* and *Rhizobium radiobacter* are some arsenic resistant bacteria. *Rhizobium rhizogenes* has a high MIC Value.¹³ *Rhizobium leguminosarum* are also capable to mitigate As.⁴⁴

Rhizobium community can survive the heavy metal pollutant in soil and are effective to enrich the soil nutrient and enhance the growth of the plant in contaminated field.¹⁶ *Bradyrhizobium japonicum* has potential as a plant growth-promoting rhizobacterium and carries a bunch of PGPR attributes. *Rhizobium meliloti* also produce IAA-like molecules and able to solubilize a significant amount of phosphate⁴⁵ under stress conditions. *Rhizobium japonicum*, *Rhizobium sp.*, *Rhizobium leguminosarum* are the three plant growth promoting bacteria which are showed their PGPR activities under heavy metal stress conditions. The LAR-7 also could solubilize a significant amount of phosphate and produce IAA. Most bacteria under *Pseudomonas sp.*, *Acinetobacter sp.* and *Paenibacillus sp.* were reported to be potential plant growth promoters³⁵ along with *Bacillus aryabhatai* which behaves similar to our studied strain for being an As resistant plant growth promoter.²⁰

Similar observations were also obtained with As-resistant bacteria of Alpha proteobacteria, Beta proteobacteria, and Gamma proteobacteria manifesting potential PGP attributes.^{46, 10} *Pseudomonas sp.* has been reported to possess

high As (III) oxidizing capacity while at the same time found to solubilize a significant amount of phosphate, indulge in siderophores, IAA-like molecules, and ACC Deaminase production.³⁵

The present investigation has indisputably established the manifestation of PGP traits of As tolerant, As oxidizing bacterial isolate *Rhizobium leguminosarum*, in solubilizing phosphate, producing siderophores, root nodule, IAA-like molecules, and ACC deaminase under As stress. *Rhizobium leguminosarum* (LAR-7) had emerged as best performing candidate isolates with regard to As resistance and PGP traits.

Conclusion

In pursuit of providing environmental safeguard to restore food safety and sustaining food security to the burgeoning population and combat abiotic pollution, a low-cost alternative to exorbitant pollution control strategies remained an absolute priority. The outcome of the present investigation revealed the candidate bacterial isolate *Rhizobium leguminosarum* as a potent, novel bacterial strain for As mitigation and may be useful, through fulfillment of mass production and field validation protocols, in As decontamination and plant growth promotion.

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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

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