RESEARCH ARTICLE

Isolation and Characterization of Arsenic Tolerant Bacteria Collected from Arsenic Contaminated Site of West Bengal, India

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Abstract

In the present study, arsenic tolerant bacteria were isolated and characterized from rhizospheric soil of rice plant growing in the arsenic contaminated area of West Bengal, India. Among 31 bacterial isolates, nine isolates were showed tolerance at higher concentration of arsenate (As V; 20000 μg mL^{-1}). Selected arsenic tolerance bacterial isolates were characterized based on biochemical analysis, partial 16S rDNA gene sequencing and phylogenetic analysis. Results revealed that selected arsenic tolerant bacterial strains were lavished at 37^0C and neutral pH 7 and showed a positive response for catalase and oxidase enzymes. Using partial 16S rDNA based identification and phylogenetic analysis, selected bacterial isolates were identified as *Jeotgalicoccus nanhaiensis*, *Alcaligenes faecalis, Paenalcaligenes* sp., *Providencia* sp., *Kocuria* sp. and *Pseudomonas stutzeri*. Further, the isolated As tolerant bacterial strains may be tested for their possible utilization in arsenic mobilization in the rhizosphere for enhancing phytoremediation potential to remediate arsenic from contaminated sites. However, efficient arsenic accumulator strain like *Pseudomonas stutzeri* can be employed as bioresource to develop low-cost bioremediation technology for arsenic removal.

Keywords: Arsenic tolerance, Bacterial characterization, Bioremediation, Detoxification.

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Introduction

Arsenic (As) is a toxic metalloid, widely distributed in the earth's crust and naturally found in soil, water and air (Jang *et al*., 2016; Patel *et al*., 2007). Due to the anthropogenic activities, global cycle of the non-essential elements such as arsenic has been modified and placed at a higher rank in the list of hazardous substances by the Agency for Toxic Substances and Disease Registry and the United States Environmental Protection Agency (Goering *et al*., 1999). Arsenic (As) contamination in soil and water is a worldwide problem and threatened the health risk of millions of people. Arsenic is toxic to human life (Chitpirom *et al*., 2009), and significantly causes deadlier diseases like skin problem, genetic damage, cancer of the skin, bladder, lung, and Kidney, etc. (Kaiser, 2001; Sarkar and Paul, 2016). Arsenic contaminated groundwater used for domestic water supplies in Southeast Asian nations affects the millions of human population across the world (Ravenscroft *et al*., 2009). The condition is more severe in the West Bengal in India where As becomes a potent environmental toxic metalloid (Chatterjee *et al*., 2018; Mukherjee *et al*., 2006) due to the way of crop cultivation and geographical location may this region more prone to As contamination. In addition, using As contaminated groundwater for irrigation, resulted in accumulation of approx 1000 tons of As per year to the agricultural fields in Bangladesh (Ali *et al*., 2003). Inorganic species of As, particularly the arsenate (AsV) and arsenite (AsIII), are more dominant chemical species in the water and soil and often create more toxicity as compared to its organic forms (Bentley and Chasteen, 2002).

To mitigate poisoning, various techniques have been used from time to time but could not get the desired level. In this context, phytoremediation, for removal of arsenic by the plants is cost effective and ecofriendly technology (Zhu *et al*., 2014; Chen *et al*., 2010). However, various limiting factors such as soil condition, metal availability, plants, types of microorganism and pH of the soil, etc. affecting its effectiveness (Shin *et al*., 2012). To enhance phytoremediation efficiency, advance researches have taken to explore plant-microbe interactions, which influence the metal uptake and sequestration (Ma *et al*., 2009; Weyens *et al*., 2009). Microbial resistance to As and other toxic elements are quite common in natural environments and affects metal availability in the rhizosphere of the plants (Valenzuela *et al*., 2009). Therefore, the use of tolerant and solubilizing bacteria for metal remediation could be a promising approach. Different microorganisms have evolved different mechanisms which either effects arsenic uptake into the cells or rapidly returns in the environment (Welch *et al*., 2006). Similarly, bacteria have different defense mechanisms for As-induced toxicity (Bhattacharjee and Rosen, 2007) and mostly depends on the redox changes between the AsIII and AsV. Bacterial-induced metal solubilization and transformation may affect the mobility of arsenic in the rhizosphere which has not yet been explored. Many bacteria have been isolated which exhibit tolerance to high As concentrations (Jackon *et al*., 2005; Zhu *et al*., 2014). Different bacterial strains have been reported to efficiently remove metals and metalloids from water through accumulation inside the cell (Ozdemir *et al*., 2004).

The present study was undertaken to screen, isolate, identify and characterize tolerant bacteria collected from As affected rhizospheric soil of rice (*Oryza sativa*) in a different area of West Bengal for their possible exploitation in bioremediation of arsenic.

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Materials and methods

Collection of Soil Samples

Total nine sites (Beliaghat, Pokharpara, Ambikapur, Vijaynagar, Barasat Kanchrapara, Kalyani Mode, Sonadanga and Ranaghat) were selected from the arsenic contaminated area of the North-24-Parganas and Nadia districts of West Bengal, India to collect soil from rice rhizosphere. The soil samples were collected following the protocol of Mason (1992). Rice rhizosphere soil was collected from the root zone and kept in sterilized plastic bags and brought to the laboratory in refrigerated condition (at 4°C) for analysis of arsenic and bacterial stains.

Total Arsenic Estimation

For arsenic estimation, oven dried soil samples at 105°C were grind into a fine powder and passed through 1 mm sieve and stored at room temperature. A weighed amount of soil samples (100 mg) were taken in 250 mL glass beaker and digested with nitric acid and perchloric acid (3:1 ratio), filtered and diluted to 15 mL with double distilled water. Filtrates of soil samples were estimated for determination of total As concentration by Inductively-Coupled Plasma Spectrometer ICP-MS 7500 cx (Agilent Technologies, USA).

Isolation of Arsenic-tolerant Bacteria

To isolate arsenate tolerant bacteria, rhizospheric soils (1 g) were suspended into the nutrient broth (NB) medium (beef extract 1 g L^{-1}; yeast extract 2 g L^{-1}; peptone 5g L^{-1}; sodium chloride 5g L^{-1}, pH-7.0), supplemented with 100 µg L^{-1} arsenate (AsV, Merck, Germany) in the form of sodium arsenate and incubated at 28°C with shaking (200rpm) for 48 hours (Kinegam *et al*., 2008). The culture was selectively enriched by subculturing twice, each time transferring at 2 mL from the primary culture to the fresh medium including the same concentration of AsV and incubating temperature. The resulting potential arsenate resistant enriched culture was used for isolating the As tolerant bacteria by serial dilutions method on a nutrient agar plate (Hi-Media Laboratories Pvt. Ltd. India).

Determination of Relative Arsenate Tolerant

Relative arsenate tolerance of the purified isolates was determined by growing on a nutrient agar plate containing (100 to 20000 µg mL^{-1}) concentration of arsenate in the form of sodium arsenate and incubated at 28 ± 2°C as described (Singh *et al*., 2010). Most efficient Arsenic tolerant bacterial isolates showed tolerance up to 20000 µg mL^{-1} were used for biochemical and molecular characterization. Purified bacterial cultures were stored at –80°C in nutrient broth supplemented with 15% glycerol for further study.

Characterization of Arsenic-tolerant Bacteria

Biochemical Identification

The potentially arsenic resistant purified bacteria culture were revived on nutrient agar plate supplemented with 20000 µg mL^{-1} As. The plates were incubated at 28°C for 48 hours and various biochemical test viz., Gram reaction, cell morphology, colony appearance, motility were also analyzed. Furthermore, catalase, oxidase, hydrolysis of casein, gelatin, starch, indole test, nitrate reduction, hydrogen sulfide production, utilization of carbohydrates and oxidation or fermentation of glucose were performed as described by Barrow and Feltham (1993).

Molecular Characterization of the Arsenic-tolerant Bacteria

16S rDNA Amplification

For molecular identification, total genomic DNA from all the purified bacteria was isolated from freshly grown broth culture according to the protocol of Marmur, (1961) with minor modifications. In brief, the cells were pelleted and resuspended in an equal volume of TES buffer (50 mM Tris buffer, 1 mM EDTA, 8.56% wt/vol sucrose) pH 8.0 and sodium dodecyl sulfate was added to the mixture. The solution was treated once with chloroform-isoamyl alcohol (24/1; v/v) and with a mixture of phenol, chloroform and isoamyl alcohol (25/24/1; v/v/v). The DNA was precipitated by an equal volume of isopropanol and dissolved in 1x Tris-EDTA buffer and stored at –20°C for further use.

Amplification of 16S rDNA was performed by using universal primers 27F and 1492R (Weisburg et al., 1991). PCR amplification was carried in a 50 µl reaction mixture containing: 100 ng of purified DNA as template, 1xTaq DNA polymerase buffer, 10 mM dNTPs, 1.5 mM $MgCl_2$, and 0.4 µL of Taq DNA polymerase (Fermentas Life Sciences, Vilnius, Lithuania) in gradient mastercycler (Eppendorf, Hamburg, Germany). The amplification reaction was performed with an initial denaturation at 95°C for 2 min; followed by 35 cycles of denaturation at 95°C for the 30s, annealing at 50°C for 60s and extension at 72°C for 60s; and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel containing (0.5µg mL^{-1}) ethidium bromide. PCR products were gel purified using the QIA quick purification kit (Qiagen, Limburg, Netherlands) according to the manufacturer's protocol. Purified amplicons were sequenced bi-directionally with 27F and 1492R primers. The resulting reads were assembled and the consensus sequence has been submitted to the GenBank database.

Phylogenetic Analysis of Arsenic-tolerant Bacteria

The preliminary identification based on 16S rRNA gene sequence was done using BLAST-n (Altschul *et al*., 1990). Thereafter, sequences of 16S rRNA gene of all the bacteria species and outgroup taxa from GenBank were aligned from CLUSTAL W and trimmed for an equal length. The phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987). Pair-wise sequence similarity search was performed to determine the similarity of the sequence with their reference strains available in the GenBank database in MEGA7.0 software (Kumar *et al*., 2016). The level of support for the tree, derived from NJ analysis, was determined from 1000 bootstrap replicates. The tree is drawn to scale with branch lengths shown in the same units as for inferred evolutionary distances.

Nucleotide Sequence Accession Number

The partial 16S rDNA gene sequence was deposited in the GenBank database and appear in the DDBJ, EMBL, and NCBI nucleotide sequence databases under (KP057589-KP057597) accession numbers (Table 3). The accession numbers of reference organisms used in phylogenetic analysis are shown in Figure 1.

Results

Isolation and Biochemical Characteristics of Arsenic-tolerant Bacteria

Soil samples collected from the rice rhizosphere of nine selected sites of the North-24-Parganas and Nadia districts of West Bengal were analyzed for arsenic concentration and isolation of arsenic-tolerant

bacteria. The maximum concentration of arsenic (117.23 mg Kg^{-1}) was recorded in the soil collected from Barasat followed by Beliaghat (111.19 mg Kg^{-1}) of the district North-24-Parganas, West Bengal (Table 1). Out of thirty one highly arsenic tolerant bacteria were isolated from the rhizospheric soil of selected sites, nine isolates showed tolerance at high concentration of AsV (20000 mg L^{-1}) were selected for biochemical identification (Table 2). Colonies of arsenate tolerant bacteria were found circular and smooth on a nutrient agar plate after 24–48 hours of incubation. Most of the isolates were Gram-negative rods or short rod in morphology and grew well in aerobic condition at temperature range 28–37°C, with optimal growth at approximately 30°C, while no growth was observed at temperature >45°C. More variable characteristics in physiological and biochemical properties of each isolate were summarized in Table 3. All As tolerant rhizobacteria showed positive reaction for catalase and oxidase, however, negative reaction for indole production and casein hydrolysis. Toxicity and chemical behavior of As compounds are largely influenced by the form and speciation of As (Table 2).

Molecular Characterization and Identification of Arsenic-tolerant Bacteria

The 16S rDNA sequences of all nine representative isolates were analyzed by BLAST-n search to obtain its closest reference sequence available in the GenBank database. Results revealed that all the strains shared closed homology with the nine reference strains (Table 4). Pair-wise sequence similarity between selected reference strains and isolates ranged from 97.6–100% to six formally described species. The constructed phylogenetic tree confirmed the phylogenetic positions of these strains in their group (Fig. 1). The 16S rDNA based phylogenetic analysis grouped these strains into six different clades. Three strains (NK-4, NK-6, and NK-9) grouped with *Alcaligene facecalis,* two strains (NK-2 and NK-10) with *Jeotgalicoccus nanhaiensis* and the rest four strains (NK-7, NK-8, NK-11, and NK-12) clustered with *Paenalcaligenes suwonensis*, *Providencia rettgeri*, *Kocuriarosea*, and *Pseudomonas stutzeri,* respectively.

West Bengal is the state of the Indian subcontinent where arsenic poisoning is highly prevalent in the districts like Malda, Murshidabad, Nadia, North-24-Parganas, South-24-Parganas, Bardhaman, Howrah, Hooghly and Kolkata (Roy *et al*., 2014). The high As content in these areas may be due to geogenic reasons like the location of the low lying area, natural erosion, groundwater contamination and irrigation (Goswami *et al*., 2015; Gupta and Gupta, 2013). Globally, various authors reported the high As contamination the in paddy soils due to continuous irrigation with As-contaminated groundwater (Meharg, 2004; Liao *et al*., 2005). In present study, different As-tolerant bacteria have been isolated from groundwater and sediments in West Bengal and reported the presence of eight different arsenic-resistant bacteria from the rhizosphere soil of the different sites of the West Bengal areas, includes *Jeotgalicoccus nanhaiensis, Alcaligene facecalis* sub sp. *parafaecalis, Alcaligene facecalis* sub sp. *faecalis, Paenalcaligenes suwonensis, Providencia rettgeri, Jeotgalicoccus aerolatus, Kocuria rosea, Pseudomonas stutzeri.* These bacteria were found to be a novel occurrence in rhizospheric soil of West Bengal, India. Among the eight isolated strains, six strains were first time reported from the As contamination soil. High tolerance for As in selected bacterial strains may be either due a common mechanism of reduction of AsV to AsIII, and then exclusion of toxic AsIII oxyanions from the cell by transporters (Mukhopadhyay and Rosen, 2002; Rosen, 2002) or detoxification through converting inorganic As to less toxic volatile organic compounds (Qin *et al*., 2006). Similarly, high tolerance for arsenic has also been reported in the bacterium *Corynebacterium* glutamicum (Ordonez *et al*., 2005) and *Bacillus* sp. strain DJ-1 (Joshi *et al*., 2009). In another study, *Staphylococcus hominis* has also been reported from arsenic contamination area of West Bengal for high As tolerance up to 30000 mg L^{-1} (Srivastava *et al*., 2012). This study is

Table 1: Arsenic concentration in rhizospheric soil collected from different sites of arsenic contaminated of West Bengal, India

S. No.	*Selected Site (District)*	*As concentration (mg Kg^{-1})*
1.	Beliaghat (North-24-Pargana)	111.19 ± 12.18
2.	Pokharpara, (North-24-Pargana)	77.14 ± 8.31
3.	Ambikapur (North-24-Pargana)	99.48 ± 13.42
4.	Vijaynagar (North-24-Pargana)	62.96 ± 7.88
5.	Barasat (North-24-Pargana)	117.26 ± 12.81
6.	Kanchrapara (North-24-Pargana)	84.75 ± 9.48
7.	Kalyani mode (Nandia)	52.62 ± 6.65
8.	Sonadaunga (Nandia)	47.24 ± 5.10
9.	Ranaghat (Nandia)	46.48 ± 4.14

Values are mean ± SD (n = 3)

Table 2: Relative arsenic tolerance in different bacterial strains isolated from rhizospheric soil of rice growing in the arsenic contaminated area of West Bengal, India

		Concentration of As V(mg L^{-1})					
S. No.	*Isolate*	*250*	*500*	*1000*	*5000*	*10000*	*20000*
1.	NK2	+++	+++	+++	+++	++	+
2.	NK4	+++	+++	+++	+++	+++	+++
3.	NK6	+++	+++	+++	+++	+++	+++
4.	NK7	+++	+++	+++	+++	++	+
5.	NK8	+++	+++	+++	+++	++	+
6.	NK9	+++	+++	+++	+++	+++	+++
7.	NK10	+++	+++	+++	+++	+++	+++
8.	NK11	+++	+++	+++	+++	+++	+++
9.	NK12	+++	+++	+++	+++	+++	+++

+ ++, luxuriant growth; ++, moderate growth; +, retard growth

Table 3: Differential biochemical characteristic of the arsenic-tolerant bacteria isolated from rhizospheric soil of rice growing in the arsenic contaminated area of West Bengal, India

	Isolate								
Test	*NK2*	*NK4*	*NK6*	*NK7*	*NK8*	*NK9*	*NK10*	*NK11*	*NK12*
Motility	–	+	+	+	+	+	–	–	+
Nitrate reduction	–	+	+	+	+	+	–	+	–
Gram reaction	+	–	–	–	–	–	+	+	–
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Optimum growth (°C)	32	37	37	28	37	37	37	37	37
Indole production	–	–	–	–	–	–	–	–	–
Acid production from Glucose	+	+	+	+	–	+	–	+	+
Mannitol	+	+	+	+	+	+	–	+	+
Casein hydrolysis	–	–	–	–	–	–	–	–	–
H_2S production	–	–	–	–	–	–	–	–	+
Citrate utilization	+	+	+	+	–	+	–	–	–
Gelatinase production	+	+	+	–	–	+	–	–	–
Starch hydrolysis	–	+	+	–	+	+	–	+	–

+, positive test; –, negative test

Table 4: Arsenic tolerant bacteria isolated from rhizospheric soil of Rice growing in different site of West Bengal, India.

S. No.	*Strain*	*GenBank accession number*	*Closest related species in the GenBank database*	*% similarity with EZ-taxon*
1.	NK2	KP057589	Jeotgalicoccus nanhaiensis	99.17
2.	NK4	KP057590	Alcaligene facecalis sub sp. parafaecalis	98.39
3.	NK6	KP057591	Alcaligene facecalis sub sp. faecalis	99.36
4.	NK7	KP057592	Paenalcaligenes suwonensis	97.62
5.	NK8	KP057593	Providencia rettgeri	100
6.	NK9	KP057594	Alcaligene facecalis sub sp. faecalis	99.16
7.	NK10	KP057595	Jeotgalicoccus aerolatus	98.77
8.	NK11	KP057596	Kocuria rosea	99.42
9.	NK12	KP057597	Pseudomonas stutzeri	100

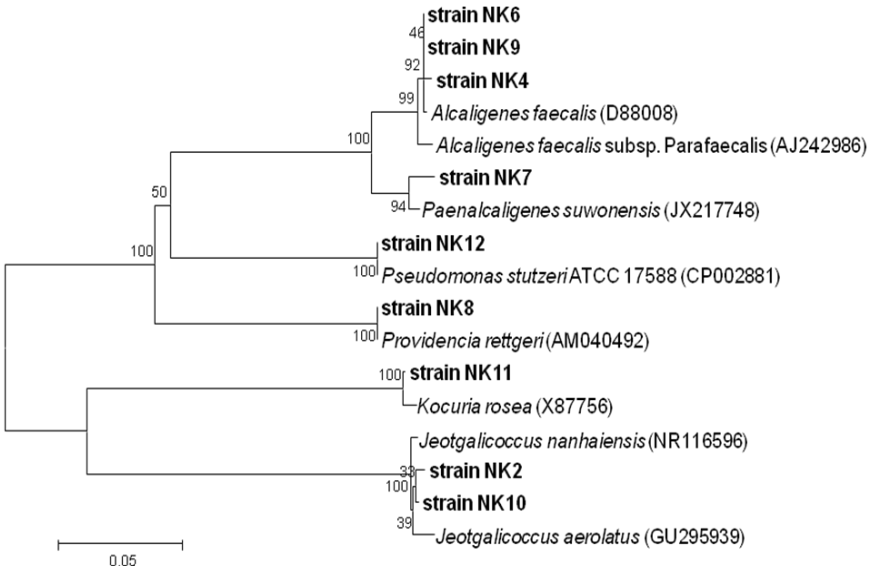

Fig. 1: Neighbor-joining phylogenetic tree on the basis of partial 16S rRNA gene, sequences of nine screened arsenic oxidizing bacterial isolates (**boldface**), showing the relationship to their representatives in the NCBI database. Numbers at nodes are bootstrap percentages based on 1000 re-sampled datasets. Bar represents 0.05 substitution per nucleotide position.

accordance with the other previous studies in which the presence of arsenic-resistant genera likes *Alcaligenes* and *Pseudomonas* have been reported (Raja *et al*., 2006; Shakya *et al*., 2012; Sarkar *et al*., 2013). The bacterium *Pseudomonas stutzeri* has also been reported as an efficient arsenic accumulator (Joshi *et al*., 2009). In addition, enhanced arsenic mobilization in the rhizosphere and uptake in the sunflower plant has also been reported in the presence of strain *Alcaligene*s sp. Dhal-L (Joshi *et al*., 2008). Furthermore, we have first time reported the presence of arsenic resistance in the members of the genus *Jeotgalicoccus, Providencia* and *Paenalcaligenes* which are still not reported as the extent of our knowledge.

Conclusion

Arsenic tolerant bacteria found in the rhizospheric soil of the rice plant growing in the arsenic contaminated agricultural field showed tolerance and well adapted to grow at high concentration of arsenic. The bacterial strains; *Alcaligene facecalis, Paenalcaligenes suwonensi, Kocuriarosea* which showed positive responses for acid production, catalase and oxidase may be tested for their possible utilization in mobilization of arsenic and used for enhancing phytoremediation efficiency to remediate arsenic contaminated soil; further, efficient arsenic accumulator strain like *Pseudomonas stutzeri* could be employed as bioresource entities and give the insight to develop low-cost bioremediation technology for arsenic reduction. Therefore, arsenic tolerant bacteria influencing As mobilization in the rhizosphere and uptake in plants may be optimized and exploit for enhancing phytoremediation efficiency.

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