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ARSENIC RESISTANT BACTERIA ISOLATED FROM CONTAMINATED SOIL AND SELECTION OF ARSENIC REDUCING STRAINS

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ABSTRACT
Arsenic (As) is one of the most toxic pollutants in the environment. Continuous use of contaminated ground water for irrigation made the soil secondary source of As. Present study was aimed to investigate if bacteria present in contaminated soil can convert immobilized arsenate (AsV) to mobile arsenite (AsIII) and having a potential role to increase As pollution in the environments or not. Thirty five As resistant bacteria were isolated by an enrichment culture method from As contaminated soil in which14 bacterial strains having the resistance capacity of 250-500mgL^{-1} in case of AsIII and 8000-12000mgL^{-1} in case of AsV. Highest resistant capacities showed by the bacterial isolates ArB-15, it could resist upto 12000mgL^{-1} (160 mM) of AsV and 500mgL^{-1} (6.66 mM) of AsIII in culture media. Five arsenic reducing bacterial isolates (ArB-08, ArB-15, ArB -22, ArB-28 and ArB-31) were screened and they were identified by their morphological and biochemical characteristics. This isolates may have potential role in As dissemination in the environments.

INTRODUCTION

Arsenic is a natural toxic element released into the environment by natural phenomenon (geogenic) or by anthropogenic activities (Cullen and Reimer, 1989). Ground water arsenic contamination is a serious problem in many parts of the world especially in West Bengal, India and neighboring Bangladesh. Arsenic exists both in toxic inorganic and comparatively less toxic organic species in the environments. The most common species of inorganic arsenic are trivalent arsenite (AsIII) and pentavalent arsenate (AsV). Mitigation of AsIII from soil and groundwater is of great challenge, due to its higher toxicity and mobility than AsV.

Although arsenic is generally toxic to life, it has been demonstrated that microorganisms can use arsenic compounds as electron donors, electron acceptors, or possess arsenic detoxification mechanisms (Ahmann *et al.,* 1994). The most common oxidation states of arsenic in the environment are the less toxic pentavalent AsV and more toxic trivalent AsIII forms (Cullen and Reimer, 1989). Arsenate ($AsO4^{3-}$) acts as a structural analog of phosphate and inhibits oxidative phosphorylation by producing unstable arsenylated derivatives (Anderson *et al.,* 1992). Despite its toxicity, a number of microorganisms are capable of using either the oxidized form of inorganic arsenic AsV or the reduced form AsIII in their metabolism (Silver and Phung, 2005). Some bacteria use AsIII as the electron donor for chemoautotrophic growth while other bacteria use AsV as the terminal electron acceptor in anaerobic respiration, thermodynamic considerations suggesting that dissimilatory reduction of AsV could provide enough energy for microbial growth (Laverman *et al.,* 1995). Dissimilatory AsV reducing bacteria (DARB) may be involved in the solubilization, fate and transport of arsenic by reducing AsV to AsIII (Ahmann *et al.,* 1997).

Previously, several bacteria of β or γ-proteobacter groups have been reported to reduce AsV (Simeonova *et al.,* 2004). Although the precise mechanism of arsenic mobilization remains to be characterized in detail, respiration of adsorbed As (V) by dissimilatory As (V)-reducing prokaryotes may play a role, resulting in the formation of potentially more mobile As (III) (Oremland *et al.,* 2005). These organisms were stimulated under anaerobic conditions in laboratory microcosms by the addition of acetate as a proxy for organic matter, conditions that have been shown previously to support enhanced rates of arsenic mobilization in analogous sediments from West Bengal (Islam *et al.,* 2004).

In Bengal Delta agricultural fields are frequently irrigated with the contaminated water, mainly in rice (paddy) fields. Rice grains are also containing higher concentration of arsenic (Meharg and Rahman, 2003) and represents as the most common route of arsenic poisioning through food chain (Chowdhury, 2004). Bacterial arsenic transformation of this soil is very important as because rice plants uptake different arsenic species present in soil in different proportion. The aim of the present study was to isolate bacteria from these arsenic-contaminated sites.

MATERIALS AND METHODS

Soil collection and Arsenic determination

Experimental soil was collected from arsenic contaminated

paddy field at the village Gotera (N 23°00′27.4′′ and E 088°36′03.7′′) of the district of Nadia, West Bengal, India during the kharif season of 2009 following the standard soil collection procedure and were kept at 4°C for microbiological studies. Air dried, 2 mm sieved soil samples were analyzed for different physicochemical parameters as described by Jackson (1967). Oxydizable organic carbon (Walkley *et al.,* 1934), available nitrogen content of soils was determined through modified Kjeldahl method. Available phosphorus of the soil was extracted by Olsen reagent (0.5 M $NaHCO_3$; pH 8.5) and estimated by VARIAN CARY-50 UV-VIS spectrophotometer @ 760 nm, available potassium of the soil was extracted by neutral normal ammonium acetate and estimated through systronics microprocessor based flame photometer (model 121). Chloride, Nitrate and sodium content were estimated following the method of APHA, 1992. Total (Sarkar *et al.,* 2012) and Olsen extractable (McLar*en et al.,* 1998) arsenic concentrations were determined by atomic absorption spectrophotometer (AAS, Perkin Elmer AAnalyst 200) coupled with FIAS 400.

Isolation of arsenic resistant bacteria

Arsenic resistant bacteria were isolated and purified through enrichment culture technique in Basal salt minimal media (Pattamaporn *et al.,* 2008). The composition of the media in g $L^{-1}$of water was as follows: 1.0 g yeast extract, 0.3g $(NH_4)_2SO_4$, 0.14 g $MgSO_4$ $7H_2O$, 0.2g $CaCl_2$ $2H_2O$, 0.1g NaCl, 0.05g KH_2PO_4, 0.05g K_2HPO_4, 0.6mg H_3BO_3, 0.17mg $CoCl_2$ $.6H_2O$, 0.09mg $CuCl_2$. $2H_2O$, 0.1mg $MnCl_2$. $4H_2O$, 0.22 mg $ZnCl_2$, 10g glucose in 1L of Tris-HCl buffer with value of pH 8 (Yamamura *et al.,* 2003) containing 150μg mL^{-1} AsV and subsequent enrichments were carried out in media with higher content of AsV. Incubation was carried out at 30°C for 48h. In this way, 35pure cultures were initially isolated.

Arsenic tolerance capacity of the isolates

The degrees of arsenic tolerance shown by the bacterial isolates were determined by comparing their growth in BSMY media containing different concentration of AsV (1000 to 20000mgL^{-1}) and AsIII (100 to and 2000mg mL^{-1}) after incubation at 30°C for 3 days in a rotatory shaker. The growth appearances of the cultures were determined by turbidity measurement following spectrophotometric method (optical density at 600nm). Out of 35, 14 strains were screened based on their higher arsenic tolerance for determination of arsenate reducing ability.

Screening of arsenate-reducing bacteria

The isolates, that exhibited growth in media containing AsV, were screened for arsenate reducing ability. Pure culture of each of the 14 isolates were inoculated in yeast extract mannitol (YEM) broth (Mandal *et al.,* 2002) amended with 2mM arsenate and incubated at 30 $\pm$ 2°C for 48h. Strains were grown up to 0.5–0.6 O.D of cell suspension at 600nm. Then cultures were centrifuged at 10,000 rpm for 5 min and supernatant was taken for further analysis. One mL of the supernatant was added to the 36-well plate followed by addition of 30μL of starch-iodine complex. The plates were incubates in dark for 10 min. AsV reduction to AsIII was detected by blue black colouration by reacting with arsenite (Mandal *et al.,* 2007). Bacterial cultures without arsenate, only YEM, *i.e.* medium without inoculum and AsV and YEM with 2mM AsV were used as organism and reagent control respectively, where the color remained unchanged. Colony characteristics of the isolates on basal salt minimal agar slants and cellular morphology by negative staining, Gram character, spore and capsule formation as well as different biochemical characteristics of the isolates were studied by following standard procedures Holtz (1993).

RESULTS AND DISCUSSION

Agricultural soils in this study showed (Table 1) a moderate to high arsenic concentration (Haq *et al.,* 2003) and the values were close to the levels of other studies of this affected zones (Sarkar *et al.,* 2012; Sinha *et al.,* 2011). Physico-chemical properties (Table 1) of the soil were similar to the previous analyses of the same experimental sites (Banerjee *et al.,* 2010). The pH of the soil is neutral to alkaline and contain higher amount of organic carbon and chloride. Arsenic concentration of soil and presence of different species are generally influenced by pH, redox potential of soil, soil texture, organic matter and cataion exchange capacity of the soil (Mandel *et al.,* 2002). Total arsenic content of the paddy field in the present investigation were similar with the Bangladesh paddy field arsenic concentration (Alam and Sattar, 2000). In rice field, which is reducing and anaerobic in nature, AsIII is the predominant source less retained by soil colloids (Bissen and Frimmel, 2003). Total arsenic concentration of the paddy field does not have any effect on the resistance capacities of the

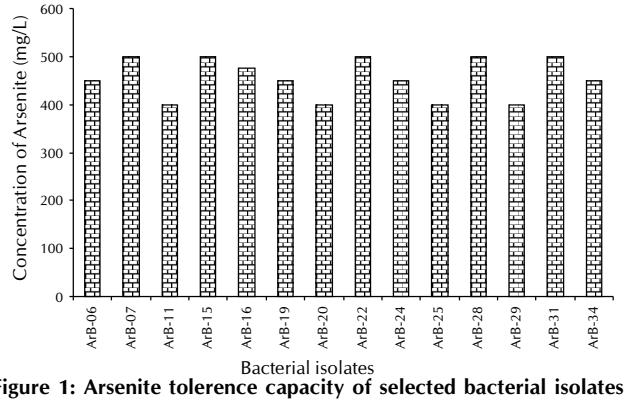

Figure 1: Arsenite tolerence capacity of selected bacterial isolates under laboratory condition

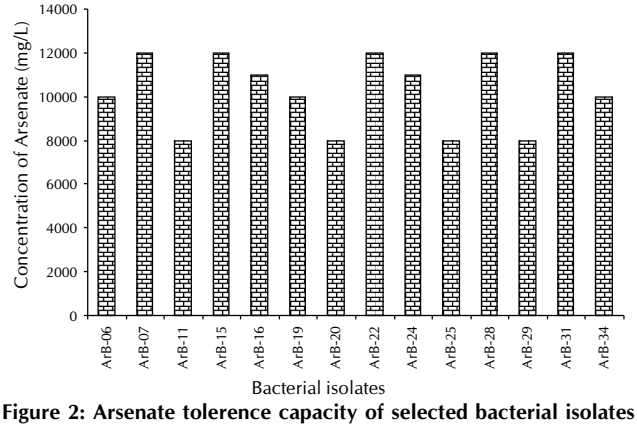

Figure 2: Arsenate tolerence capacity of selected bacterial isolates under laboratory condition

Table 1: Physico-chemical properties of the experimental soils

Soil sample	pH	Organic Carbon (g/ kg)	Chloride (mg kg-1)	Nitrate (mg kg-1)	Phosphate (mg kg-1)	Sodium (mg kg-1)	Potassium (mg kg-1)	Total Nitrogen(%)	Total Arsenic (mg kg-1)	Available Arsenic (mg kg-1)
Site-1	7.46	7.6	7.51	2.6	0.38	6.4	8.2	0.14	16.7	4.5
Site-2	7.21	5.2	8.52	2.4	0.47	7.2	7.9	0.16	19.2	5.1
Site-3	7.68	11.4	5.86	2.7	0.32	6.8	5.6	0.21	16.5	3.9
Site-4	7.71	6.8	7.61	1.9	0.58	6.9	6.7	0.26	18.4	4.8
Site-5	7.44	9.3	6.78	2.2	0.39	7.8	7.4	0.13	20.1	6.2
Site-6	7.92	7.8	9.32	2.6	0.33	8.1	5.5	0.20	13.9	2.8
Site-7	7.31	10.2	7.84	1.8	0.52	6.2	7.5	0.19	15.8	3.6
Site-8	7.54	8.6	6.96	2.1	0.37	7.0	6.2	0.22	18.8	4.2

bacterial isolates; rather the different species of arsenic is related to resistance of arsenic of microbes (Bachet *et al.,* 2009).

The arsenic tolerance capacity of those isolates was estimated with different concentration of AsV and AsIII in BSMYI broth after 3 days of incubation (Fig. 1 and 2). Only one isolate (ArB-15) could grow upto 12000mg L^{-1} (160 mM) of AsV broth and upto 500mgL^{-1} (6.66 mM) of AsIII broth. In general, microbial ability to grow at high metal concentration is found coupled with a variety of specific mechanism of resistance and

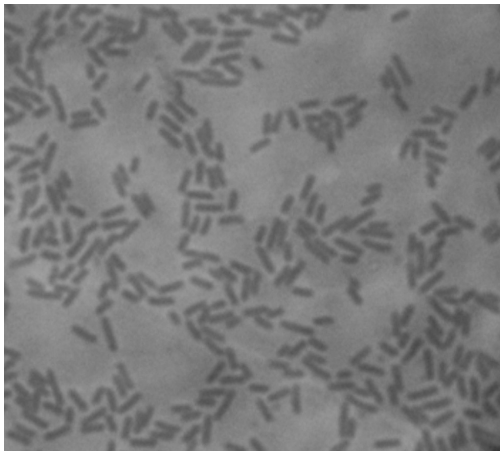

Figure 3: Negative strained cells of arsenic reducing bacteria

environmental factors. Smith *et al.* (1998) observed that many bacterial communities adapt to arsenic contaminated environment by developing resistance and tolerance mechanism. Mechanisms of resistance by microorganism include microbial surface sorption, enzymatic transformation, precipitation by oxidation/reduction reaction, and biosynthesis of metal binding proteins (Srinath *et al.,* 2002; Zoubilis *et al.,* 2004). Isolated microorganisms from the present study might have the similar arsenic resistance mechanisms with different magnitudes. Resistance capacities were similar with the previous observations (Bachet *et al.,* 2009) and three times higher (Duquesne *et al.,* 2008) described for *Thiomonas* sp.

Among these 14 isolates, 5 were found to reduce arsenate in culture media. Bacterial isolates capable of reducing arsenic is probably due to the presence of the enzyme arsenate reductase, coded by *arsC* gene. Researchers through worldwide concentrating more on arsenic oxidizing bacteria, little literature available on arsenate reducing microorganisms. These gene product capable of reducing AsV to AsIII. Detoxification pathway can occur either aerobically or anaerobically and is not governed by previlling redox condition (Jackson *et al.,* 2001). Dissimilatory As (V)-respiring prokaryotes comprise a diverse phylogenetic group, including *Chrysiogenes, Desulfomicrobium, Sulfurospirillum, Shewanella, Citrobacter*, and *Sulfurihydrogenibium* species (Oremland *et al.*, 2005). Arsenate reducing *Bacillus*, *Pseudomonas* and *Escherichia* were isolated from contaminated sites of New Zealand (Anderson *et al.,* 2004).

Table 2: Morphology and biochemical characteristics of the bacterial strain

Characteristics of the Strain	ArB - 08	ArB - 15	ArB - 22	ArB - 28	ArB - 31
Colony colour	White	Cream	Cream	Yellowish	White
Gram character	Negative	Positive	Positive	Negative	Positive
Cell shape	Coccus	Long rod	Long rod	Short rod	Coccus
Indole production	Positive	Negative	Negative	Positive	Negative
Methyle red test	Negative	Negative	Negative	Negative	Negative
Voges-Proskeur test	Negative	Negative	Negative	Negative	Negative
Citrate utilization	Negative	Negative	Negative	Negative	Negative
Gelatin liquification	Negative	Positive	Positive	Negative	Positive
Starch hydrolysis	Negative	Positive	Positive	Negative	Positive
Nitrate reduction	Negative	Negative	Negative	Negative	Negative
Oxidase	Negative	Positive	Positive	Positive	Positive
Catalase	Positive	Positive	Positive	Positive	Positive
Reaction with TSI	Alkaline	Alkaline	Acidic	Alkaline	Acidic
Sugar utilization pattern					
Glucose	+ + +	+ + +	+ + +	+ + +	+ + +
Fructose	+ +	+ +	+ + +	+ +	+ + +
Sucrose	+ +	+ +	+ +	+ +	+ +
Mannitol	+	+ +	+	+	+ +

"+" indicated slow growth, "+ + " indicates medium growth and "+ + +"indicates fast growth rates.

Different genes and enzymes involved in arsenic reduction were described by Silver and Phung, 2005. Biochemical (Table 2) and morphological (Fig. 3) characterization of these five isolates were observed. Molecular characterization of the arsenate reducing isolates studied herein would provide much more detailed information about the phylogenetic affirmation of the isolates. Bacterial strains isolated from paddy field could transform immobilized arsenic to its mobilized arsenite forms and having a large contribution in arsenic contaminations soil environments.

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