



Arsenic accumulating bacteria isolated from soil for possible application in bioremediation

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Abstract

Twenty six arsenic (As) resistant bacterial strains were isolated from As contaminated paddy soil of West Bengal, India. Among them, 10 isolates exhibited higher As resistance capacity and could grow in concentration of 12000 mg l⁻¹ of arsenate (AsV) and 2000 mg l⁻¹ of arsenite (AsIII) in growing medium. Maximum growth was observed at 1000 mg l⁻¹ and 100 mg l⁻¹ in case of AsV and AsIII respectively. Results of incubation study carried out in basal salt minimal media (BSMY) containing 25 mg l⁻¹ of AsV and AsIII separately showed that the isolates could accumulate 1.03 - 6.41 mg L⁻¹ of AsV and 2.0 - 7.6 mg l⁻¹ of AsIII from the media. The bacterial isolate AGH-21 showed highest As accumulating capacity both for AsV (25.64%) and AsIII (30.4%) under laboratory conditions. The isolates AGH-21 (NCBI accession no: HQ834295) showed highest sequence similarity (98%) with *Bacillus* sp. and could be used as a potential bioremediator in future to combat with arsenic toxicity.

Key words

Accumulation, Arsenate, Arsenite, *Bacillus*, Bioremediation

Introduction

Arsenic, one of the most prevalent toxic metalloids in the environment, has a long and nefarious history. Its very name has become synonymous with poison (Mandal and Suzuki, 2002). Due to its ability to induce chromosomal aberration during DNA replication, As is considered as a human carcinogen and a potential mutagenic agent (Wang *et al.*, 2001). It also interferes with the DNA repair system, signal transduction pathways and inhibits many enzymatic activities and also damages respiratory, digestive, and circulatory as well as nervous system (Rehman *et al.*, 2010). As intoxication of human through drinking water got the primary and maximum attention; however, a little attention has been paid on As toxicity through food chain (Meharg *et al.*, 2009). Initially soil acts as a major sink, however, continuous addition of As polluted irrigation water made the soil a secondary source and transports it to the edible part of the crops (Mukhopadhyay and Sanyal, 2004), causing serious health hazards among the human (Guha Mazumder,

2008). Thus removal of As is of great importance for human welfare. Considering the metal removal process (chemical precipitation, filtration, reverse osmosis etc) relatively low cost ecofriendly bioremediation techniques using microorganisms can be a promising alternative (Clausen *et al.*, 2000; Singh *et al.*, 2008). Bacteria have evolved several mechanism to tolerate and uptake of heavy metal ions. It mainly involves accumulation and complexation of the metal ion inside the cell, efflux of the metal ion and oxidation–reduction of the metal ion to relatively less toxic forms (Nies *et al.*, 1999). These microbes are present in metal contaminated soil and their exploration is necessary to develop a promising bioremediation options.

In the present study, As resistant bacteria were isolated from As contaminated soil of West Bengal, India. The capability of these bacteria to withstand higher concentration of As and intracellular accumulation of As were determined. The efficient bacterial strain showing highest As accumulation potential was identified with a

view to developed a new strategy of As mitigation.

Materials and Methods

Study site and soil properties : In order to screen out efficient As accumulating bacterial strains, extremely As loaded agricultural soils (0-150mm) were collected from Ghentugachi village in Nadia district, West Bengal. Sampling sites were predominantly in rice growing regions and frequently irrigated with As contaminated ground water (0.146 to 0.573 mg L⁻¹). Total As concentration of that region ranged between 7.6 to 16.25 mg kg⁻¹, with the mean value of 12.67 mg kg⁻¹ (Sarkar *et al.*, 2012). A composite sample of 500 gm was processed and sieved. Representative soil samples were kept at 4°C for microbiological studies. Total As content (Sparks *et al.*, 2006) of the soils were determined by atomic absorption spectrophotometer (model: Perkin Elmer Analyst 200, USA) coupled with FIAS 400.

Isolation of arsenic resistant bacteria : One gram of moist soil sample was dissolved in 10 ml of 0.9% NaCl and shaken for 30 min. 5 ml of soil suspension was inoculated into 50 ml BSMY media. The composition of the media in g l⁻¹ of water was as follows: 1.0 g yeast extract, 0.3 g (NH₄)₂SO₄, 0.14 g MgSO₄·7H₂O, 0.2 g CaCl₂·2H₂O, 0.1 g NaCl, 0.05 g KH₂PO₄, 0.05 g K₂HPO₄, 0.6 mg H₃BO₃, 0.17 mg CoCl₂·6H₂O, 0.09 mg CuCl₂·2H₂O, 0.1 mg MnCl₂·4H₂O, 0.22 mg ZnCl₂, 10 g glucose in 1 liter of Tris-HCl buffer with value of pH 8, containing 150 µg m l⁻¹ AsV and incubated at 30°C on rotary shaker at 120 rpm for 2 days. 5 ml of culture was transferred into fresh BSMY medium containing 250 µg ml⁻¹ AsV, and transferred twice into new medium that was supplemented with 500 µg ml⁻¹ AsV for enrichment. To isolate the As resistant colonies 100 µl of culture was spreaded on BSMY agar plates containing 500 µg ml⁻¹ AsV and incubated at 30 °C for 2 days. Isolates having higher resistance capacity appeared in plate and were selected for further study, purified and preserved. Sodium-arsenate (Na₂HAsO₄·7H₂O) was filter sterilized and used as AsV source in the medium.

As tolerance capacity of the isolates : The extent of As tolerance of the 26 bacterial isolates, the cultures were allowed to grow in 15 ml BSMY broth containing nine different concentration of AsV (1000, 2000, 4000, 6000, 8000, 10000, 12000, 14000 and 16000 mg l⁻¹) and AsIII (500, 750, 1000, 1250, 1500, 1750, 2000, 2250 and 2500 mg l⁻¹) in 50 ml conical flask and were incubated in 30°C for 3 days in a rotary shaker. The growth appearances of the culture were determined by turbidity measurement following spectrophotometric method (optical density at 600 nm). Appearance of growth was denoted by '+' and '-' when there was no growth at all.

Selection of efficient As accumulating bacteria : Bioaccumulation of As by the bacterial strains was assessed

by the methods as suggested by Cernasky *et al.* (2009). Among the 26 isolates under investigation, ten isolates with higher As tolerance were selected for assessing their As accumulation under laboratory conditions in BSMY media containing 25 µg ml⁻¹ of AsV and AsIII separately. The conical flasks containing 20 ml of BSMY media were inoculated with 500 µl bacterial cell (OD value = 0.6) suspension of each isolates and allowed to shake in rotator shaker at 100 rpm at 30°C. After being cultured for three days the experimental media was centrifuged at 10000 rpm for 5 min to separate the cell mass from the media. Microbial biomass separated by centrifugation, washed four times with ultrapure water to completely remove the culture medium. Each experiment was conducted in triplicate. The total As concentration of bacterial biomass and filtered media were then analyzed for total As concentration by atomic adsorption spectroscopy (Majumder *et al.*, 2011). Non-inoculated media containing same AsV and AsIII concentration were used as experimental control.

Phenotypic and biochemical characterization of the isolate:

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 (oxidase, catalase, indole production, nitrate reduction, methyl red test, voges-proskauer test, carbohydrate utilization pattern) of the isolates were studied by following standard procedures of Holtz (1993).

Identification of the bacterial isolates based on 16S rDNA:

The strain having the highest As tolerance capacity was chosen for molecular identification by 16S rDNA sequence analysis. Total genomic DNA was extracted as described by (Sambrook, 2001) and the 16S rRNA gene was amplified by PCR using the forward primer Y1(40) F 5'- TGG CTC AGAAGG AAG GCG GCG GC -3' and the reverse primer Y2(337)R 5'- CCCACT GCT GCC TCC CGTAGG AGT -3' (Chromous Biotech Private Limited, India). Before amplification cycle, DNA was initially denatured for 5 min at 94°C and then 1 min at 94°C. After amplification, a final extension step (10 min at 72°C) was performed. The cycling parameters consisted of 35 cycles at: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, extension at 72°C for 5 min. The PCR product were purified and held at 4°C until analysis by agarose gel electrophoresis (1%). Sequencing was carried out in Chromous Biotech Private Limited, India and 16SrRNA gene sequence was compared with the existing databases in GenBank to identify the most similar species of same sequence.

Phylogenetic analysis of the isolate : Nucleotide sequences were compared with sequences in GenBank database by BLAST-N algorithm (Altschul *et al.*, 1997) to identify

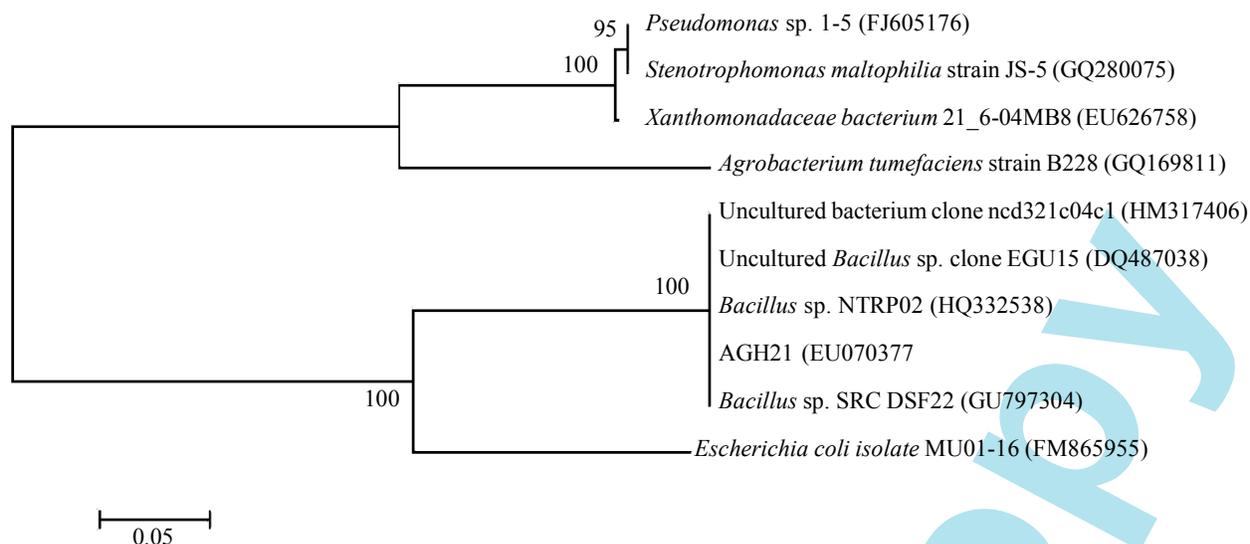


Fig. 1 : Phylogenetic analysis of arsenic accumulating bacterial isolates AGH-21

sequences with a high degree of similarity. Phylogenetic trees of both 16S rDNA gene sequences were generated using the neighbor-joining algorithms using the p-distance model (Saitou and Nei, 1987) in Mega IV (Tamura *et al.*, 2007). The level of support for the phylogenies derived from neighbor-joining analysis was gauged by 500 bootstrap replicates. For 16S rDNA based phylogenetic tree, 52 reference sequences from GenBank were compared with *E. coli* MU01-16 (FM865955) as outgroup. The optimal tree with the sum of branch length of 22.03196114 is shown. The percentage of replicate for the trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 285 positions in the final dataset.

Results and Discussion

Soil containing elevated concentration of metal is potential source of those metal tolerant bacteria. It is because the environmental condition promotes adaptation of those isolates in such environment (Clausen *et al.*, 2000). Soil collected for isolation of *As* accumulating strains contain total *As* concentration of 16.8 mg kg⁻¹, similar to the early reported value of this regions (Sinha *et al.*, 2011).

Twenty six pure bacterial isolates were obtained by enrichment in *As* containing culture medium from contaminated soil. After 3 days of incubation, the *As* tolerance capacity of these isolates were assessed with a series of concentration of *As*V and *As*III (Table 1). It was

observed that all isolates could grow and withstand the *As* toxicity up to 8000 mg l⁻¹ of *As*V and 1250 mg l⁻¹ of *As*III. Ten bacterial isolates viz. AGH-03, AGH-07, AGH-10, AGH-12, AGH-15, AGH-16, AGH-18, AGH-21, AGH-23, AGH-26 sustained and grew in an environment with the concentration of 12000 mg l⁻¹ *As*V of and 2000 mg l⁻¹ of *As*III. The results of the study revealed that all bacterial isolates did not have the same degree of tolerance to *As* toxicity. This might be due to developing of *As* tolerance and resistant ability of the inherent individual soil microorganisms (Smith *et al.*, 1998). Exposure of indigenous bacteria to gradient of *As* concentrations during enrichment for isolation might have developed metal resistance systems for protecting sensitive cellular components (Pattamaporn *et al.*, 2008). Such exposure to *As* might exert a selective pressure on plasmid harboring bacteria to trigger resistant gene on plasmid to encode and express under such stressed condition to combat *As* toxicity. These ten isolates were selected for further study. Out of these ten, a few of the isolates was found capable of resisting 14000 mg l⁻¹ of *As*V (AGH-03, AGH-16, AGH-21, AGH-23) and up to 2250 mg l⁻¹ of *As*III (AGH-03, AGH-21). Above this concentration of both *As*V and *As*III, no growth was observed. Existence of specific mechanism inside the microbial cell along with some environmental factors made the isolates capable to resist and even grow further against the higher metal toxicity (Smith *et al.*, 1998). Bacteria developed resistance mechanisms to *As* by enzymatic transformation of toxic *As* species, precipitation by oxidation/reduction and biosynthesis of metal binding proteins described by Srinath *et al.* (2002) and Zoubilis *et al.* (2004). The bacterial isolates might have similar *As* resistance mechanisms, but with different degrees. Arsenic tolerant capacities of bacterial

Table 1 : Arsenic (AsV and As III) tolerance capacity of the bacterial isolates after 3 days of incubation

Strain	Concentration of AsV in mg l ⁻¹								Concentration of AsIII in mg l ⁻¹									
	1000	2000	4000	6000	8000	10000	12000	14000	16000	500	750	1000	1250	1500	1750	2000	2250	2500
AGH-01	+	*	+	+	+	+	-	*	-	-	-	-	-	-	-	-	-	-
AGH-02	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-03	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-04	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-05	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-06	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-08	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-09	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-11	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-13	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-14	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-17	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-19	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-20	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-22	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AGH-24	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-25	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
AGH-26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*"+"" indicates growth appeared, *"-"" indicates growth not appeared

isolates in the present study were supported by Krumova *et al.* (2008), Chang *et al.* (2010), Ji *et al.* (1992), Hiroki *et al.* (1993) and Omerland *et al.* (2005).

Results from *in-vitro* As bioaccumulation study revealed that different bacterial isolated exhibited different As accumulating capacity (Table 2). Among the 10 selected isolates, AGH-21 was the most efficient one and significantly accumulated highest amount of both AsV (6.4 mg l⁻¹) and AsIII (7.6 mg l⁻¹) from the media and leaving less 18.6 mg l⁻¹ of AsV and 17.4 mg l⁻¹ of AsIII in the medium. It was also noticed that higher As tolerance of AGH-21 was coupled with higher (25.64% of AsV and 30.4% of AsIII) accumulation of both AsV and AsIII. This can be explained by the fact that the bacterium (AGH-21) produce capsule which facilitate As accumulation by binding As with capsular material (Pattamaporn *et al.*, 2008). Similarly the lowest AsV (1.03 mg l⁻¹) and AsIII (2.0 mg l⁻¹) accumulation associated with the highest residue of those As species was recorded by inoculation of bacterial culture of AGH-16 (Table 2). On an average, the accumulation of As ranged

from 1.03 - 6.41 mg l⁻¹ of AsV and 2.0 - 7.6 mg l⁻¹ of AsIII. Influence of bacterial isolates on residual status of both AsV and AsIII in BSMY media followed similar trend The accumulating capacity of the As by the bacterial isolates, in general, was greater for AsIII over AsV. Present study shows that maximum amount of AsV (23.97 mg l⁻¹) and AsIII (23.0 mg l⁻¹) remains in the media when AGH-16 inoculum was added to the media. The reason for the loss or removal of As from the broth may possibly due to microbial cell accumulation (Pornsawan *et al.*, 2001; Qin *et al.*, 2006 and Slavomir *et al.*, 2009). It has been reported that a number of bacterial species, namely *Proteus* sp, *Bacillus* sp, *Escherichia coli*, *Flavobacterium* sp, *Corynebacterium* sp. *Pseudomonas* sp, possess varying degrees of As accumulating abilities (Sanyal *et al.*, 2002). The isolate AGH-21 showed the higher As resistance ability and able to accumulate maximum amount of AsV and AsIII and it was selected for identification.

Phenotypic characteristics of the isolated pure culture of AGH-21 showed white irregular undulating

Table 2 : Accumulation of AsV and AsIII (mg l⁻¹) from BSMYI media initially contains 25mg l⁻¹ AsV and AsIII after 3 day incubation at 30°C

Bacterial isolates	Accumulation of AsV from broth (mg l ⁻¹)	Residual status of AsV in broth (mg l ⁻¹)	Accumulation of AsIII from broth (mg l ⁻¹)	Residual status of AsIII in broth (mg l ⁻¹)
AGH-03	3.21 cde (12.8%)	21.79 cde (87.2%)	3.1 cde (12.4%)	21.9 cde (87.6%)
AGH-07	4.06 bc (16.3%)	20.94 bc (83.8%)	3.9 bc (15.6%)	21.1 bc (84.4%)
AGH-10	3.74 bcd (15.0%)	21.26 bcd (85.0%)	3.2 bcde (12.8%)	21.8 bcde (87.2%)
AGH-12	2.87 de (11.5%)	22.13 de (88.5%)	2.6 def (10.4%)	22.4 def (89.6%)
AGH-15	2.63 e (10.5%)	22.37 e (89.48%)	2.4 ef (9.6%)	22.6 ef (90.4%)
AGH-16	1.03 f (4.1%)	23.97 f (95.8%)	2.0 f (8.0%)	23.0 f (92.0%)
AGH-18	3.62 bcde (12.5%)	21.38 bcde (85.5%)	3.2 bcde (12.8%)	21.8 bcde (87.2%)
AGH-21	6.41 a (25.6%)	18.59 a (74.4%)	7.6 a (30.4%)	17.4 a (69.6%)
AGH-23	3.32 bcde (13.3%)	21.68 bcde (86.7%)	3.4 bcd (13.6%)	21.6 bcd (86.4%)
AGH-26	4.24 b (17.0%)	20.76 b (83.0%)	4.1 b (16.4%)	20.9 b (83.6%)

*Means with same letter are statistically nonsignificant by DMRT

colonies. The isolate was Gram positive, form endospore and capsule, showing long rod shaped morphology in chain under the microscope. Capsule and extracellular polysaccharide substances in *As* accumulating bacteria were also reported by Pattamaporn *et al.* (2008). Biochemical characteristics revealed that the isolate could utilize glucose, fructose, nitrate and could hydrolyse starch. It was catalase, oxidase and gelatinase positive but indole, methyl red and Voges-Proscure negative. The isolate showed alkaline reaction on triple sugar iron agar slant.

Molecular characterization of the strain based on sequencing of 16S rDNA and subsequent comparison with existing databases in GenBank, the isolate AGH-21 was identified as *Bacillus sp.* As resistant microbes isolated and identified worldwide and the tolerance ranged to 40 mM for *Bacillus* (Rehman *et al.*, 2010), 16.66 mM for *Pseudomonas* (Pepi *et al.*, 2007). Several members of the genus *Bacillus* like *Bacillus arsenicus* (Shivaji *et al.*, 2005) and *Bacillus sp.* (Anderson and Cook, 2004) were also reported. Several strains of *Bacillus sp.* have also been recently reported to be able to oxidize AsIII to AsV (Ike *et al.* 2008; Chang *et al.*, 2010). Phylogenetic analysis based on its 16S rDNA gene sequences revealed that the strain AGH21 is classified as firmicutes and clustered with members of the genus *Bacillus* (Fig 1). More specifically, AGH21 showed 99% sequence similarity with members of the genus *Bacillus*. The preliminary characterization of these isolates also supports this placement based on of their Gram reaction and other morphologic and biochemical characters. The Genbank accession numbers for the 16S rDNA sequence is HQ834295.

The strain AGH-21 was found capable of accumulating considerable amount of toxic As from culture media. Biochemical and molecular characterization of the isolate revealed that the isolate was a spore forming rod

shaped *Bacillus sp.* and might be efficiently applied to soil for As detoxification, since its spore forming ability might help in its long term sustainability in soil.

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