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Arsenite-oxidizing bacteria isolated from arsenic contaminated surface and ground water of Uttar Pradesh, India

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Arsenic (As) contamination of surface and ground waters in Uttar Pradesh (UP) is geological in nature, even though some degree of As contamination may be due to anthropogenic activities. Little is known about the arsenite oxidizing bacteria in aquatic environments of UP. In this study we isolated the As resistant bacteria from both surface and ground waters of UP. Seven potential strains were isolated by culture method using chemically defined medium (CDM). Two strains (AOB-GP1 and AOB-MT1) were Gram negative and other five were found to be Gram positive. The presence of arsenite oxidizing bacteria in groundwater is limited if compared to surface waters. 16S rDNA revealed highest homology of 99 - 88%. The strains were similar with *Acinetobacter* sp. *PGSI1*, *Rummeliibacillus stabekisii*, *Cronobacter sakazakii*, *Bacillus tequilensis*, *Brevibacillus agri* strain, *Bacillus thuringiensis* and *Virgibacillus marismortui*. The 16S rDNA gene sequence analysis for the As-resistant strains in surface and ground waters points to prevalence of γ-proteobacteria and Firmicutes, solely isolated γ-Proteobacteria (*Acinetobacter sp.* and *Cronobactersakazakii* sp.) from ground water samples. Based on morphological, cultural, biochemical and physiological characteristics the arsenite oxidizing bacteria in this study were grouped into three orders viz,. pseudomonadales, enterobacteriales and bacillales.

Key words: Arsenite oxidizing bacteria, Pseudomonadales, Firmicuites, surface water, ground water.

INTRODUCTION

India is one of the many countries that have reported the existence of As- contaminated ground waters (Nordstrom, 2002). The two most inorganic arsenic (As) forms are As (III) and As (V) to which human beings can get exposed through drinking water. The two oxidation states of arsenic have toxicological effects on the human body, but the most toxic is the oxidation state of arsenite As (III) (Oremland and Stolz, 2003). The presence of Arsenite As (III) in the surface and ground water system results from the mobility of metalloid Arsenic from solid

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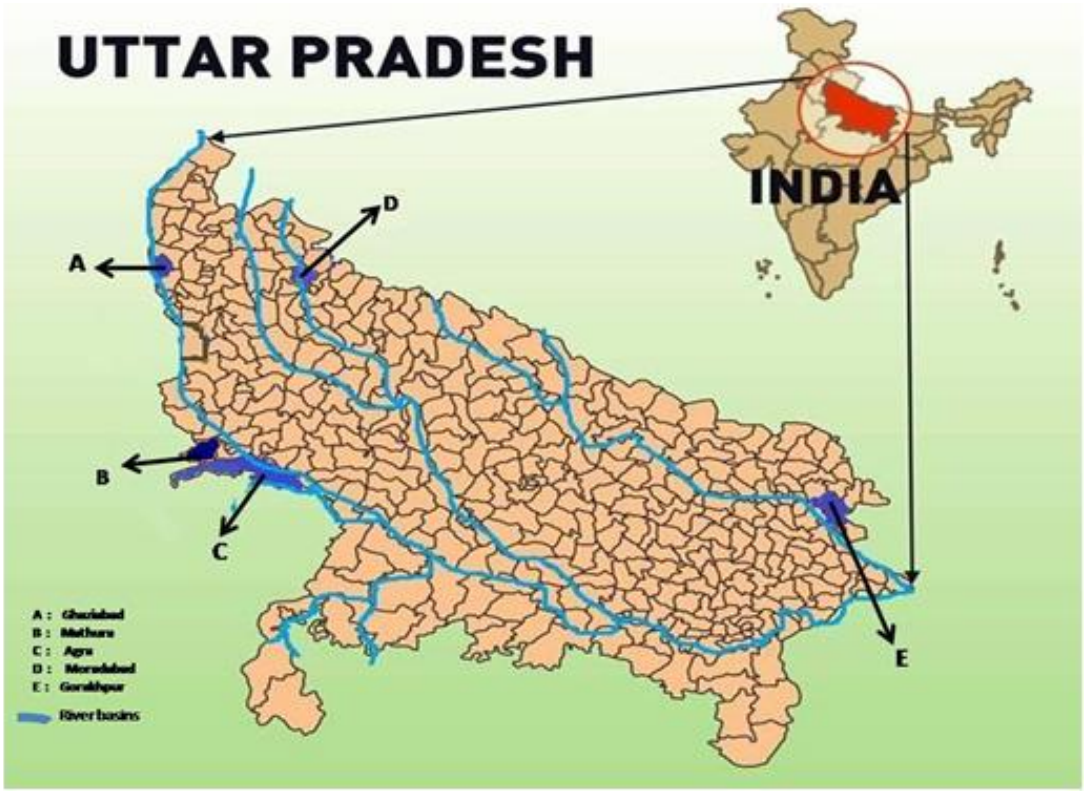

Figure 1. Geographic location of districts of Uttar Pradesh.

state to aquesous state through geologic and anthropogenic activities (Aiuppa et al., 2006). As (III) pollutes fresh waters due to arsenic contaminated waste derived from industrial process as well as use of As compounds in agriculture (Oremland and Stolz, 2003; Donati et al., 2005). As pollution causes a serious health related problems (Ahmed et al., 2010). The world health organization reduced the acceptable threshold for As from 50 to 10 µg/L in drinking water (WHO, 2011). There are certain prokaryotic microorganisms that have the capability to resist As (III). As (III) enters the bacterium cell through membrane associated aqua glycerol-porins to convert the toxic form As (III) to a less toxic form As (V) (Oremland and Stolz, 2005). The presence of ars operon on plasmids or chromosomes of certain bacteria containing aoxA, arsB oxidation and transporter genes causes the oxidation of As (III) (Muller et al., 2007; Tsai et al., 2009). Previous studies have shown that this region of India is characterized by extensive As pollution of rivers and ground waters by both geologic and anthropogenic activities (Srivastava and Sharma, 2013). People living near As-rich polluted surface and ground waters may be exposed to As via drinking water (Ahmed et al., 2006). This study was conducted in five districts of Uttar Pradesh, India to isolate the Arsenite oxidizing bacteria that have a potential to remove As (III) from ground water to a less soluble As (V).

MATERIALS AND METHODS

Study area

The study area covers five districts of Uttar Pradesh (238,000 km^2 area, 166 million populations) in the Upper Plain ganga. The districts are viz,. Ghaziabad (28° 40′ 12″ , 77° 25′ 12″), Moradabad (28° 49′ 48″ , 78° 46′ 48″), Mathura (27° 29′ 33″ , 77° 40′ 25″), Gorakhpur (26° 45′ 31.68″,83° 22′ 10.92') and Agra (27° 10′ 46.35″ , 78° 1′ 15.97) (Figure 1 and Table 1). The main sources of drinking-water for the people in the rural areas are surface water, government tube wells, tap water from shallow aquifers ranging from 50 to 150 ft. The rivers were considered heavily polluted by human activities carried out in the vicinity (Acharyya et al., 1999).

Surface and ground water samples collection

The surface water samples from Hindon River, Yamuna River, and Ganga River, and ground water samples from tube wells were collected during August-September 2013 in polypropylene bottles (500 ml) from Sigma Aldrich (USA). The samples of surface and ground waters were stored at 4°C in dark and were transported to the laboratory. In the laboratory, an amount (15ml) of these water samples was supplemented with sodium arsenite ($NaAsO_2$ Sigma–Aldrich) at final concentrations of 200 µM. After seven days of incubation at 25°C, aliquot of 100 µl of water sample was spread on culture plates. The composition of the chemically defined media (CDM) (g l^{-1}) is as follows: 0.97 g $MgSO_4$, 1.0 g (Sigma-Aldrich USA), NH_4Cl, 1.0 g Na_2SO_4 (Sigma-Aldrich USA), 0.009 g K_2HPO_4 (Sigma-Aldrich USA), 0.05 g $CaCl_2$ (Sigma-Aldrich USA), 5 g

Table 1. Study area of five districts in Uttar Pradesh.

District	Latitude N,E.	Sample type		Area of sample collection
		Ground water	Surface water	
Ghaziabad	28° 40' 12" , 77° 25' 12"	Tube well	Hindon River	Sahibabad, Mohan Nagar GaushalaFatakh,RajNagar and LalKaun.
Moradabad	28° 49' 48" , 78° 46' 48"	Tube well	Ram Ganga River	Harthala, Prem Nagar, Lakri Village, FazalPur and KathGhar.
Mathura	27° 29' 33" , 77° 40' 25"	Tube well	Yamuna River	VisharamGhat,Radhey Sham Ashram ,MubarikPur, GokulBangar and AnandpuriPhokhar.
Gorakhpur	26° 45' 31.68",83° 22'10.92"	Tube well	Lake	Bahrampur, BetuaUrfChanau, BelGhat, Ram GrahTaal, and Sahara Estate.
Agra	27° 10' 46.35" , 78° 1' 15.97"	Tube well	Yamuna River	Agra Fort, Defense Estate, TajGanj, TajMahal Area and Kamala Nagar.

Na-Lactatae (Sigma-Aldrich USA), 0.002 g Fe_2SO_4 (Sigma-Aldrich USA), 0.79 g $NaHCO_3$ (Sigma-Aldrich USA), 0.25 g $NaAsO_2$ (Sigma-Aldrich USA) and 15 g agar. The final pH of CDM was 7.2. The growth of bacterial colonies was observed after 48 h of incubation at 35°C and isolation was done in triplicates.

Isolation of As(III) resistant bacteria

The cultures were sequentially streaked on As (III) supplemented CDM to get the pure colonies of arsenite oxidizing bacteria. The As (III) varied from 0.1 , 0.2, 0.3, 0.4, 0.5 to 0.6% in CDM until the minimum inhibitory concentration (MIC) of the isolate was observed. All culture plates were incubated at 35°C for 48h. All the strains had growth of up to 0.3% of arsentie in R2A agar. However, the MIC for strains AOB-GP2, AOB-MT1, AOB-GZ1 and AOB- MB1 was 0.5% of arsenite, which is the highest. At 0.6% of arsenite, none of the isolate had growth (Table 4).

The ability of the isolates to oxidize arsenite was tested using qualitative potassium permanganate ($KMnO_4$) method (Salmassi et al., 2002). Each isolate resistant to arsenite was inoculated in R2A broth with final concentration of 900 µM sodium arsenite and then shaken on rotatory shaker at 180 rpm for 7 days at 30°C. 1 ml of cultured isolate was added to a 2 ml of glass tube containing 0.01 M $KMnO_4$ and change in color is monitored. A pink color indicates arsenite oxidation (As V). The resulting enriched cultures were placed on the solidified R2A agar media (0.5 g/L glucose, 1 g/L tryptone, 0.3 g/L yeast extract, 0.3 g KH_2PO_4, 0.05 g $MgSO_4$ and 1% agarose). Cultures were incubated for 48 h at 35°C. The pure cultures were preserved in R2A agar medium and kept at 4°C.

Phenotypic characterization

The phenotypic characteristics of all isolates studied were determined and compared to phenotypic data of known organisms described in the Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986). Isolated bacterial cultures grown on R2A agar were examined for morphological characteristics viz., shape and size. Gram staining was done, motility test was done by hanging drop method and observed under light microscope (Leica DMD 108 digital microscope). Oxidase and catalase, the IMVIC (Indole, methyl red, vogues proausker and citrate) test, fermentation of sugars and hydrolysis of gelatin, casein , starch and esculin were done as described by Krieg et al. (2010) and Barrow and Feltham (1993) (Table 2). Growth at different temperatures (10, 25, 35, 45 and 55°C) and pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) was observed (Figures 2 and 3).

DNA isolation and 16S rRNA analysis

DNA was isolated from an overnight broth using the Invitrogen kit USA, according to the instructions mentioned in the protocol. PCR amplification of the 16S rRNA gene sequence was carried out by using primers, 8f 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 926r 5′-CCG TCA ATT CCT TTR AGT TT-3′ (Davolos and Pietrangeli, 2011). PCR amplification was performed in ABI 9700 Thermal cycler (USA) in a 50 µL volume containing 1 µL DNA, 1 µL of each forward and reverse primer, 2 µL of dNTPs, 1.5 µL of $MgCl_2$, 2.5 µL of PCR buffer (tris HCl 100 Mm), 1 µL of DNA polymerase and nuclease free water with a final concentration of 50 µL.

The purified PCR products sequencing, through capillary electrophoresis, was outsourced and the corresponding ab1 files, with the resulting chromatograms, were manually analyzed and validated using Applied Biosystems sequence scanner. Obtained sequences were compared to the GeneBank nucleotide data library using the BLAST software (in order to determine their closest phylo-genetic relatives) (Altschul et al., 1997; Saitou and Nei, 1987. In order to determine visual phylogentic relationship of the isolates with closest species, the phylogenetic tree was prepared using online program phylogentic.fr (Dereeper et al., 2008) (Figure 4).

RESULTS

Seven potential arsenite oxidizing strains AOB-GP1, AOB-GP2, AOB-MT1, AOB-MT2, AOB-MB1, and AOB-GZ1 and AOB-AG1 were identified on the basis of phenotypic and genotypic characteristics. Two strains (AOB-GP1 and AOB-MT1) were Gram negative and the rest five were Gram positive. Colony morphology on R2A

Table 2. Biochemical characterization of the 07 arsenite oxidizing bacterial isolates.

Biochemical test	AOB-GP1	AOB-GP2	AOB-MT1	AOB-MT2	AOB-MB1	AOB-GZ1	AOB-AG1
Gram stain	-	+	+	+	+	+	+
0.8% NaCl	-	+	+	+	+	+	+
Growth at pH (7.0)	+	+	+	+	+	+	+
Growth at (35°C)	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Spore formation	-	+	-	+	+	+	+
Oxidase	-	-	-	+	-	-	-
Catalase	+	+	+	+	+	+	+
Motility	-	+	+	-	+	+	+
Methyl red	-	+	-	+	+	-	+
Voges-Proskauer	-	-	+	-	-	+	-
Indole	+	+	+	+	-	-	-
Urease	+	-	-	-	-	-	-
H_2S Formation	-	-	-	-	-	-	-
Citrate utilization	+	-	+	+	-	-	-
Hydrolysis							
Esculin	+	-	-	-	ND	ND	ND
Casein	-	-	-	+	+	+	-
Starch	ND	+	ND	+	+	+	-
Gelatin	ND	ND	-	+	+	+	+
Acid production							
Sucrose	-	-	-	+	+	+	-
Maltose	+	-	ND	+	+	ND	+
Lactose	+	-	-	+	-	-	-
Glucose	-	+	+	-	+	ND	+
Galactose	+	-	ND	ND	-	-	+

+ Positive, - Negative , ND not done.

agar was quite diverse as shown in Table 2. The 16S rRNA partial sequences of the isolates were uploaded to NCBI website to search for similarity to the known DNA sequences and to confirm the isolate by using BLAST program (Table 3). 16S rRNA gene after BLAST query revealed highest homology of 99 - 88%. Strains AOB-GP1, AOB-GP2, AOB-MT1AOB-MT2, AOB-MB1, AOB-GZ1 and AOB-AG1 had similarity with *Acinetobacter* sp. *PGSI1* (99%), *Rummeliibacillus stabekisii* (98%), *Cronobacter sakazakii* (98%), *Bacillus tequilensis* (99%), *Brevibacillus agri* strain (88%), *Bacillus thuringiensis* (95%), *Virgibacillus marismortui* (95%), respectively (Table 3). AOB-GP1 and AOB-MT1 strains belong to phylum (proteobacteria) and class Gamma Proteobacteria. The rest of the five strains belong to phylum (Firmicutes) and class Bacilli. The phylogentic relationship of AOB-GP1 and AOB-MT1 strains belongs to *Acinetobacter* sp. and *C. sakazakii* sp. respectively and are clinical pathogens. Both strains are Gram negative, belong to Enterobacteriaceae family, are oxidase negative and catalase positive. AOB-GP1 is non-motile while AOB-MT1 is motile. The other five strains are Gram positive; all are catalase positive, rod shaped and belong to family Bacillaceae. Five arsenite oxidizing *Bacillus* sp. strains isolated from surface and ground waters were capable of oxidixing As (Table 3). Arsenic contaminated surface and ground waters revealed culturable arsenite oxidizing bacteria species distributed across γ-Proteobacteria (*Acinetobacter* and *Enterobacter*) and Firmicutes (especially Bacillales).

DISCUSSION

The arsenic contamination in fresh and underground waters examined in Upper Ganga plain is mostly geogenic in nature (Saha and Ali, 2006). The contamination is principally governed by red-ox reactions, organic matter and use of As compounds in agriculture (Mandal and Suzuki, 2002). In this study the isolation of seven potentially arsenite oxidizing strains was attributed to their high As (III) resistance. This may suggest that they would play a role in removing As from arsenic contaminated drinking water. It would be interesting to

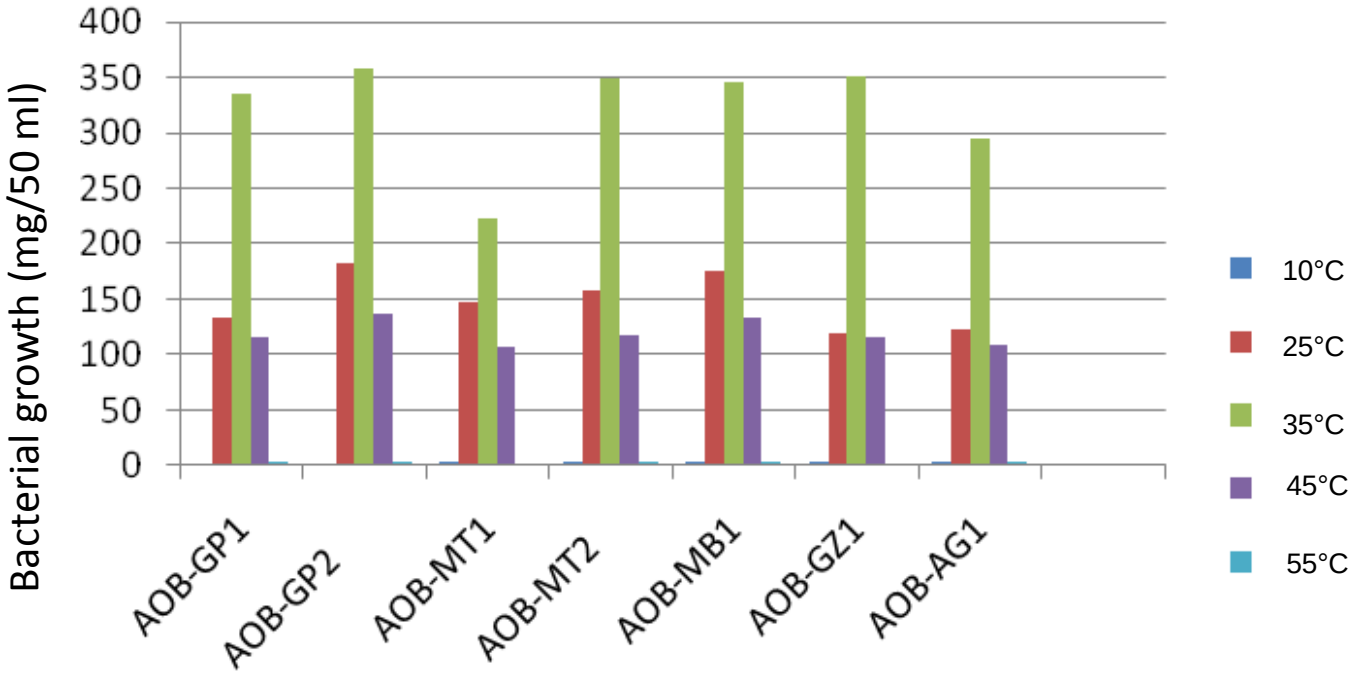

Figure 2. Growth of isolates at different temperatures.

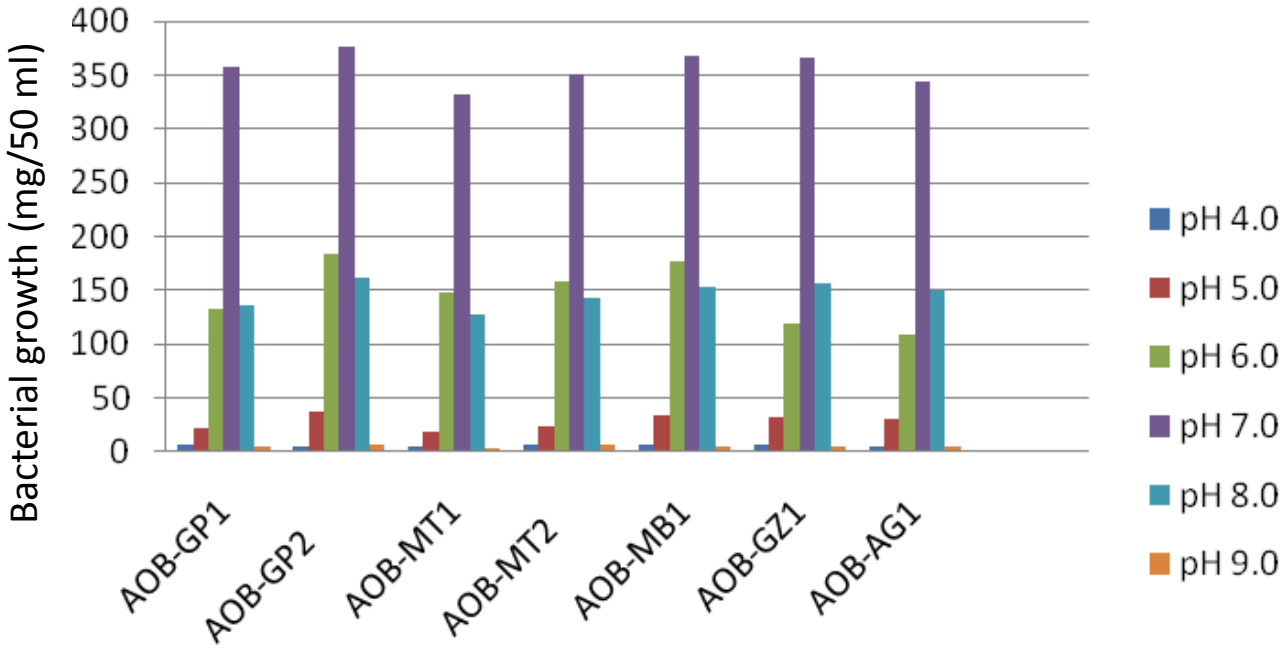

Figure 3. Growth of isolates at different pH.

analyze these isolates in terms of their mechanism of arsenic metabolism. In urban districts like Mathura, Ghaziabad and Agra, the industries effluents have led to the high pollution of arsenic in surface waters. Strains showing 99% similarity could be the novel strains with promising ability to remove As(III) from drinking water. The arsenite oxidizing bacteria in this study were grouped into Pseudomonales and Bacillus based on phylogenetic analysis of 16S rDNA sequence analysis. Previously, arsenic resistant pseudomonales were isolated from

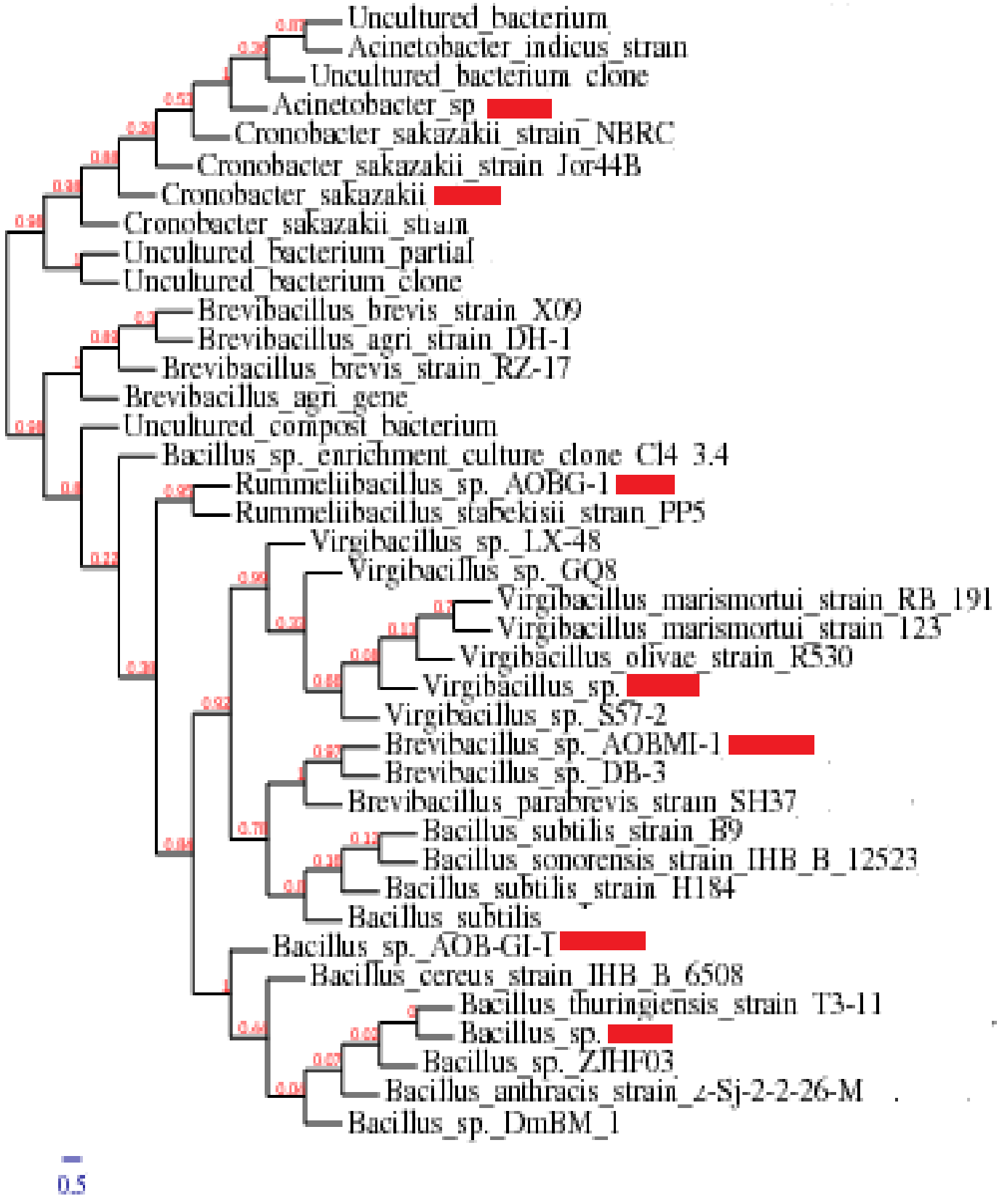

Figure 4. Phylogenetic analysis of 16S rRNA partial gene sequence of 07 Arsenite oxidizing bacterial isolates from ground and surface water(isolates are marked with red line).

Table 3. Phylogenetic analysis of 16S rRNA partial gene sequence of 07 arsenite oxidizing bacterial isolates from ground and surface water.

Isolate	Source	Closest neighbor to known bacteria	Percentage (%) similarity	Identification
AOB-GP1	Underground water	*Acinetobacter* sp. PGSI1	99	*Acinetobacter* sp.
AOB-GP2	Lake	*Rummeliibacillus stabekisii*	98	*Bacillus* sp.
AOB-MT1	Underground water	*Cronobactersakazakii*	98	*Cronobacter* sp.
AOB-MT2	Yammuna River	*Bacillus tequilensis*	99	*Bacillus* sp.
AOB-MB1	Ram Ganga River	*Brevibacillus agri* strain	88	*Bacillus* sp.
AOB-GZ1	Hindon River	*Bacillus thuringiensis*	95	*Bacillus* sp.
AOB-AG1	Hindon River	*Virgibacillus marismortui*	95	*Bacillus* sp.

Table 4. Determination of minimum inhibitory concentration of arsenic.

Strain code	Concentration of As_2O_3 in R2A media					
	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
AOB-GP1	+	+	+	+	-	-
AOB-GP2	+	+	+	+	+	-
AOB-MT1	+	+	+	+	+	-
AOB-MT2	+	+	+	+	-	-
AOB-MB1	+	+	+	+	+	-
AOB-GZ1	+	+	+	+	+	-
AOB-AG1	+	+	+	-	-	-

marine environments (De Vincente, 1990). *Bacillus* arsenic resistant was also reported from West Bengal India from aquifers (Shivaji et al., 2005). Overall, the 16S rDNA sequence data we obtained in our study from ground and surface waters in the five districts of UP (Table 3) are in an agreement with the study conducted by us where we examined ground waters and detected As resistant Proteobacteria, especially from class γ Proteobacteria; while in the surface waters, As resistant bacteria dominated in phylum Firmicutes. A recent study on As-rich contamination in Tiawan reported the number of γ- Proteobacteria (*Enterobacter)* that has the capability to resist AsIII (Liao et al., 2011). It is worth mentioning that some of the bacterial isolates described in this study (Table 3) may be novel species that deserve further examination. For example, *Bacillus* sp. AOB-MI-1, *Bacillus* sp. AOB-GI-1 and *Rummeliibacillus* sp. *AOBG-1* (Table 3) did not conform fully to the phenotypic features of Bacillus arsenicus (Shivaji et al., 2005). Microorganisms that have been isolated which have various resistance mechanisms that allow them to tolerate arsenic concentrations; some of them are associated with genetic determinants; they provide the ability to perform transformations either through oxidation and/or reduction. In the literature there are few related to the isolation of resistant bacteria arsenic from volcanic rocks, where most are oriented to the isolation of a microorganism with the ability to tolerate the presence of metal, without determining the importance and ecological works.

The presence of arsenic in the mineralogical composition of the volcanic rocks, obtained from the Quebrada Camarones, favor the isolation of bacteria resistant to arsenic, mainly species with a high metabolic versatility, which can be based on genetic systems chromosomal level or at plasmidial. Based on this, the microorganisms have developed a variety of mechanisms for survival of arsenic toxicity; among them we can mention arsenic transformations between arsenite (As (III)) and arsenate (As (V)). Furthermore, there are numerous bacterial species capable of oxidizing arsenic and using this compound as an electron donor for growth chemolithotroph, although very few organisms can grow through this mechanism. Among these is *arsenitoxidans Pseudomonas* strain NT-26 arsenopyrite samples isolated from a gold mine.

Among other bacteria capable of oxidizing arsenic described are *Achromobacter* (*Alcaligenes*) *faecalis*, *Agrobacterium albertimagni* AOL15 and *Pseudomonas putida* ULPAs1 (*Cenibacterium* arsenoxidans). Arsenic microbial transformation may indirectly influence the biogeochemical cycling of arsenic. Likewise, the ability of some species, as is the case of *Acidithio bacillus*, arsenic released from minerals, for example, arsenopyrite and arginita takes place through oxidation of arsenic. Arsenite oxidizing bacteria are phylogenetically different but all

bear out the oxidation of arsenite oxidase gene (*aox*), which is a periplasmic enzyme redox having a heterodimeric structure consisting of two subunits; the greater is the catalytic subunit, Molybdenum, and [3Fe-4S] in the smaller subunit formed by a center Rieske [2Fe-2S]. The genes encoding the two subunits were identified and sequenced for the first time in heterotrophic bacteria *Herminiimonas arsenicoxydans*, and demonstrated that both genes are in the same operon called Aox.

Conclusion

The seven isolated strains in our study showed high tolerance to As (III) levels, as they have the potential to oxidize As (III) to its less toxic form As (V), therefore they represent a good source for bioremediation process of arsenic contaminated waters of Uttar Pradesh. However, further work is required to understand the role played by ars operon in these strains.

Conflict of Interests

The authors have not declared any conflict of interests.

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