Molecular Identification of Arsenite Oxidizing and Arsenite Oxidase Gene in Two Native γ- Proteobacteria Isolated from Arsenic Contaminated Ground Water.

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Abstract

Metalloid arsenic (As) is a proven human carcinogen. The As (III), As (V), and inorganic oxyanions are the most prevalent As species observed in natural waters. While sodium arsenate (Na3 AsO4) and sodium arsenite (NaAsO 2) have higher solubilities in water than arsenic trioxide and arsenic acid (H3 AsO 4), respectively. In this investigation, materials were collected from Mathura and Ghaziabad in north India and streaked on a chemically defined medium containing sodium arsenite shows the growth of two γ-Proteobacteria. The two strains (AOB-GPI and AOB-MTI) were identified Gram Negative. Based on 16S rRNA gene sequence analysis strains AOB-GPI and AOB-MTI revealed highest homology of 98% - 99% to Cronobacter sakazakii and Acinetobacter sp. respectively. To the best of our knowledge the native bacterial isolates have potential to oxidize arsenite and are reported for the first time for the presence of arsenite oxidase (AroA, AroB) and arsenite transporter (arsB,ACR3) gene. Acinetobacter sp. strain (AOB-GPI) and Cronobacter sp. strain (AOB-MTI) sequences were determined, and they were deposited in the Gene-Bank under accession numbers KR069103 and KR069104, respectively. Under aerobic circumstances, the stain Acinetobacter sp. oxidised the arsenite at a concentration of 10 mM and 15 mM for Cronobacter sp. in 24 hours. Our research demonstrates that these two isolated strains can be employed as a bioremediation alternative to remove arsenic from arsenic-contaminated water.

Key words: Arsenite oxidizing bacteria; Cronobacter sp. strain AOB-MT1; Acinetobacter sp. strain AOB-GP1.

1. Introduction

A metalloid with a high prevalence in aquatic settings is arsenic (As) (1). As (III)-, As (0), As (III)+, and As (V) are the four oxidation states in which arsenic can be found in nature. Inorganic arsenate As (V) and arsenite As (III) also coexist there. More poisonous than arsenate is arsenite (2) As (III) commonly predominates as 74–98% of total arsenic. Arsenic (III) is more difficult to remove than As (V). In arsenic-contaminated aquifers, the presence of dissolved organic matter (DOM) affects the activity of bacterial communities (3). The World Health Organization determined an acceptable level for arsenic in drinking water at 10ug/L. (4). To make groundwater safe to drink, practical technologies to remove arsenic from it must be developed (2). Previous studies have shown that anthropogenic and geologic sources both contribute to the widespread river and groundwater arsenic contamination. Due to the Ganga River's flood plain, other states, such as Uttar Pradesh, Chandigarh, Jharkhand, and Bihar, are most at risk (5,6). A small amount of arsenic (1ug-1) included in numerous meals is necessary for optimal health. However, exceeding the allowed limit of this element may induce digestive and respiratory problems as well as damage to the cells of biological systems. bladder, skin, and liver cancer (7). Using conventional methods including coagulation, layer filtration, particle trading, and adsorption, arsenic was removed from contaminated waterways (8). All of these methods cause pollution and are intrusive. Higher amounts of As (III) and the detoxifying process of arsenic As (III) oxidation may not be toxic to microorganisms (9).As (III) may serve as an electron donor for microbial respiration in combination with O_2 under anoxic and oxic environments (10,11). Microorganisms, *Acinetobacter,& Pseudomonas* (12), *Acinetobacter calcoaceticus* NCCB 22016T(13)and *Enterobacter ludwigii* EN-119T (14) are known for arsenite oxidation.

2. Material and methods

2.1 Site and Sampling

The study was done in Ghaziabad (N.E 28° 40′ 12″, 77° 25′ 12″) and Mathura (N.E 27° 29′ 33″, 77° 40′ 25), districts of Uttar Pradesh India. The dug-wells were the primary source for water needs followed by surface waters (Rivers and lakes).The pumping rate for each tube-well is approximately 3L min^{-1} and is done manuallyThe ground water samples were taken from tube wells between August and September 2019 and placed in 1000 ml (Sigma Aldrich) polypropylene bottles. The Samples of ground waters were immediately placed in a light proof insulated box containing ice-packs and are kept in the dark so that cooling is rapid and were transported to laboratory. In the laboratory 1000 ml of each water sample was supplemented with 0.002g of sodium arsenite (15). Portable pH meter (Hanna HI 9814, Italy) was used for measurement of pH.

2.2 Isolation of Arsenite Oxidizing Bacteria

On plates with chemically modified medium (CDM), an aliquot of 100µl of each sample that was supplemented with a ground water sample of sodium arsenite was disseminated. $MgSO_4$ (8.12 Mm), NH_4Cl (8.12 Mm), Na_2SO_4 (18.7 mM), K_2HPO_4 (0.075 mM), $CaCl_2$(0.457 mM), ($Na_3H_5O_3$) (44.6 mM), Fe_2SO_4 (0.012 mM), $NaHCO_3$ (9.5 mM), ($NaAsO_2$) (15 mM), pH (7.2) and Agar (15g) (16). The plates were inverted and heated to 37°C for 48 hours. Duplicate samples were examined three times. Purified colonies were chosen for additional research.

2.3 Phenotypic and Biochemical characterization

Colony shape, Gram stain reaction, motility, fermentation of carbohydrates, and enzyme reactions were among the phenotypic traits of the isolates of bacteria that were determined and compared to phenotypic information of recognized species reported in Bergey's Manual of Systematic Bacteriology (17).

2.4 Physiological characterization:

Determination of optimum pH and Temperature

The pH of the Arsenite R2A broth As (III) concentration 15 mM was varied to pH 4, 5, 7, 8, and 9 to find the pH where all the isolates grow best. A 24-hour-old culture in an equal amount was added to the arsenite R2A broth for inoculation. All the flasks were incubated at 35°C . After 24h growth of bacteria was determined by calculating the wet mass of bacteria in mg/50ml sample. A graph was plotted between pH along X-axis and wet mass along Y-axis and for the optimum temperature, each isolate was incubated at various temperatures ranging from (10, 25, 35, 45,55) at pH 7.2. R2A broth supplemented with 15mM

of arsenite was inoculated equal volume of 24h old culture and wet mass of bacteria in mg/50ml was noted after 24h of incubation. Wet mass was taken on Y-axis and temperature was taken on X-axis.

2.5 Sliver Nitrate ($AgNO_3$) test

Sliver Nitrate ($AgNO_3$) method was used to confirm the ability of bacterial isolates to oxidize arsenite [18].Bacterial Isolates were streaked on the Reasoner's 2Agar (R2A) Glucose (0.5/L), Tryptone (1g/L), Yeast extract (0.3g/L), KH_2PO_4 (0,3g), MgSO4 (0.05g/l), Agarose (12g), $NaAsO_2$ (1.95g) plate supplemented with 15mM of sodium arsenite and incubated at 37°C for 48 h. After the growth of isolates, 0.1 M Sliver nitrate solution was added to the culture plates and change in color was monitored. The brownish precipitate (presence of arsenate) observed in culture plate indicates the isolates are arsenite oxidizing bacteria.

2.6 Oxidation of arsenic by whole cells using batch culture system.

The isolates were cultured in R2A broth at 35 °C for 24 and 48 hours with constant shaking, and the cells were then harvested by centrifuging (10,000 g) for 10 min. at 4 °C. After being carefully cleaned with sterile tris HCl buffer (pH 7.0), the cell pellet was re-suspended in the same buffer. In a 250 ml flask of R2A broth that had been treated with 15 mM of arsenic, suspended whole cells were used to oxidise the As. The cell density was set at 109 cells per ml. the flasks were continuously shaken at 35 degrees while being incubated (150 rpm). Samples were taken aseptically, and a standard procedure was used to check for residual arsenic. After the 24 hr the arsenite oxidation was highest found in AOB-GP1 (123 mg/l) and AOB-MT1 (118 mg/l). While as after 48 hrs. arsenite oxidation has been almost doubled AOB-GP1(306 mg/l) and AOB-MTI (342 mg/l).

2.7 Genomic DNA extraction and PCR amplification:

Using an Invitrogen kit, total DNA was extracted from broth that had been cultured overnight (USA). The bacteria's 16S rDNA was amplified by PCR using universal primers 8f 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 926r 5′-CCG TCA ATT CCT TTR AGT TT-3′. (19). PCR amplification was carried out in an ABI 9700 Thermal Cycler using a 50ul volume comprising 2ul of DNA, 1ul of each primer, 2 ul of dNTPs, 1.5 ul of $MgCl_2$, 2.5ul of PCR buffer (tris HCl 100Mm), and 1ul of DNA polymerase (USA). With the addition of nuclease-free water, the final volume was increased to 50 µl. Initial denaturation (94ºC, 5 min), denaturation (30 cycles at 90 ºC, 1 min), primer annealing (54ºC, 1 min),, and extension (72ºC, 1 min). comprised the reaction protocol.

2.8 DNA sequencing of the samples

At Bioserve Biotechnologies Pvt. Ltd. in Hyderabad, India, the DNA sequencing analysis was carried out utilising an ABI 3730XLS DNA Analyzer and an ABI large dye terminator kit. The unincorporated dye terminators were removed by performing post-processing step (ethanol precipitation). To 5µl of PCR product 2µl of 3M Sodium acetate (pH-5.2) and 80µl of absolute ethanol was added and centrifuged at 4500rpm in Beckman 25R Allegra machine for 20min. Then supernatant was discarded and 120µl of 80% ethanol was added. The plate was again centrifuged at 4500rpm (Beckman Allegra 25R) for 20min. The DNA pellet was air dried and resuspended in 10µl of loading solution and loaded onto the machine to generate the sequences.

2.9 16S rDNA Sequence Analysis

To identify their closest evolutionary relatives, the Sequences were matched to the Gene Bank nucleotide data pool using the BLAST program [20]. Sequences were initially visually inspected, moved to allow for maximum alignment, and aligned using the CLUSTAL X algorithm [21]. Phylogenetic trees were constructed by the neighbor-joining method using the phylogenetic.fr online program [22].

2.10 Amplification of arsenite oxidase aroA and aroB and arsenite transporter genes *arsB, ACR3 (1) and ACR3 (2)* gene.

The amplification of arsenite transporter genes using the primers for arsenite oxidase gene are (aroA) F-GTA TG TCAC GTT GTC AAA AC /R-TTATAGAACGTTGGACAGAC and (aroB) F-ACTCTTCACCTATATCGCCGA/R- TTC TCG TAA CCG AAC ATG ACA (23). The arsenite transporter genes were amplified by using degenerate primers. The primers used for *ars*B were F-GGT GTGG AACAT CGTCT GGAA YGCNA C/R CAG GCC GTA CACC ACCA GR TA CA TN CC. For ACR3(1) and ACR3(2), the primers used were F-TCGCGTAATACG CTGGAGAT/R-ACTTTCTCGCCGTCTTCCTT and F-TG AT CT GG GT CA TG AT CT TC CC VA TGMTGVT /R-CG GC CAC GCC AGYTCRAARAARTT respectively (24). In an ABI 9700 Thermal Cycler (USA), the arsenite oxidase and transporter genes were amplified by first denaturing them at 94°C for 4 minutes, followed by denaturing them at 90°C for 45 seconds, annealing them at 50° for 45 seconds, and extending them at 72° for 50 seconds. The final step was a 72°C for 5min step to make sure the PCR products were successfully extended. Negative control was performed simultaneously to detect possible

DNA contamination and DNA from the bacterium *Ochrobactrum tritici* was used as a positive control to confirm that the reaction was successful and to establish a standard size for amplified fragments.

2.11 Accession number and base pair of the isolates

The accession numbers for Acinetobacter sp. strain AOB-GP1 and Cronobacter sp. strain AOB-MT1 are KR069103 and KR069104, respectively, in the NCBI GenBank, where the sequences have been submitted.

3 Results

Acinetobacter sp. strain AOB-GP1 and Cronobacter sp. strain AOB-MT1 were recovered from arsenic-contaminated ground water as two possible arsenite oxidising bacteria. The biochemical and phenotypic characteristics were completed. Gram-negative, rod-shaped, catalase-positive, and oxidase-negative bacteria were present. Strain *Acinetobacter sp. strain* AOB-GP1 was nonmotile while *Cronobacter sp. strain* AOB-MT1 was motile. *Acinetobacter sp. strain* AOB-GP1 was the only isolate that produce urease enzyme. None of the isolates produced H_2S gas (Table 1). The growth of isolates was determined at different pH ranging from 4.0-9.0. The maximum growth was observed at pH 7.0, with increase in pH (8.0-9.0) growth of isolates decreased (Fig 1). The optimum temperature of the isolates at the constant pH (7.0) was observed at different temperatures and 37^{o}C was found the most favourable temperature for the growth of isolates (Fig 2).

3.1 Arsenic resistance of the isolates:

The potential of the isolates to grow in presence of arsenite was observed after 24hr growth in R2A agar supplemented with different concentration of arsenite (2Mm -15mM). $AgNO_3$ test demonstrate the ability of the isolates to oxidize arsenite, Presence of brownish precipitates (formation of arsenate) in medium (Fig 3). *Acinetobacter sp. strain* AOB-GP1 showed resistance to arsenic upto 10mM of arsenite and *Cronobacter sp. strain* AOB-MT1 was able to grow in 15mM of arsenite (Fig 4).

3.2 Genotypic characteristics and arsenite oxidizing genes of arsenic resistant isolates.

The isolates' 16S rRNA partial sequences were uploaded to NCBI in order to confirm the isolate by utilising the BLAST software and looking for similarities with other known rDNA sequences. The BLAST search found that the 16S RNA genes' homology ranged from 98% to 99%. Acinetobacter sp. PGSI1 and Cronobacter sakazakii were comparable to each other (99% and 98%) in the strains AOB-

GP1 and AOB-MT1, respectively. The phylogenetic tree which reflects the evolutionary relationships among prokaryotes shows a distinct phylogenetic Cluster-I AOB-GP1 and AOB-MT1 strains are members of the class Gamma Proteobacteria and phylum Proteobacteria (Fig 5). The *Cronobacter sp. strain AOB-MT1* shows the similarity with *Cronobacter sakazakii strain* ZJN392B1 (98%, accession no : JX307658), *Cronobacter malonaticus strain* WJ1639 (98%, accession no KC818194) and *Cronobacter sakazakii strain* ATCC 29544 (98%, accession no: NR_118449) and the *Acinetobacter sp. strain AOB-GP1*shows the similarity with *Acinetobacter sp. PGSI1* (99%,accession no: KF318036), *Acinetobacter sp. NIO-S7* (99%, accession no : KC462735) and *Acinetobacter indicus* (99%, accession no: AB859733). The arsenite transporter gene (arsB) was found in γ Proteobacteria, *Acinetobacter sp. strain AOB-GP1* and *Cronobacter sp. strain AOB-MT1* while as, ACR3 (1) and ACR3 (2) gene in PCR was not found positive. The arsenite oxidase (aroB) was observed in *Acinetobacter sp. strain AOB-GP1* and *Cronobacter sp. strain AOB-MT1*.

4 Discussion

Microorganisms having ability to oxidize As (III) to As (V) are either chemolithoautotrophic or chemoheteroprophs (25,26). Strains in our study showing the 99% similarity could be the new novel strains with promising removal of As (III) from underground water. Our study is one of the first endeavours to find the presence arsenite oxidase in bacteria isolated from contaminated waters of Uttar Pradesh. Phenotypic AgNO3 method confirms that the isolates have potential to oxidize arsenite. To the best of our knowledge, the presence of arsenite oxidase (aroA, aroB) and arsenite transporter (arsB) genes in Cronobacter sp. strain AOB-MT1 and Acinetobacter sp. strain AOB-GP1 was reported in the current investigation for the first time. It has been reported that proteobacteria possess the arsenite transporter in *Escherichia coli* (27), *Pseudomonas lubricans (28), Pseudomonas aeruginosa (29), Acinetobacter (30),* and *beta proteobacteria* (31). Henceforth, we propose our strains could be a novel strains and can be used effectively to oxidize the As (III) to less toxic As (V). The fact that so many people live in Asia and are susceptible to fatal diseases linked to arsenic toxicity can serve as an example. According to the current study, treating As (III)-contaminated groundwater by using native isolated

strains of bacteria in combination with bacterial biomass has the potential to be both effective and economical.

In the present study two potential isolates were able to survive at high arsenic As (III) concentration (15Mm) in ground waters indicating their arsenic resistance. The 16S rDNA sequence data and phylogenetic relationship that we obtained from the isolates confirms that in ground water the arsenic resistant bacteria dominates in *Gammaproteobacteria (Acinetobacter sp. strain AOB-GP1, Cronobacter sp. strain AOB-MT1).* The *γ Proteobacteria two* gram negative *Cronobacter sp. strain AOB-MT1and Acinetobacter sp. strain AOB*-GP1 were isolated from the tube wells located in Mathura and Ghaziabad. The possible explanation for isolation of these strains from wells is microbial contamination. Previous studies have shown the contamination of shallow wells by *Escherichia coli (32To the best of our knowledge, the bacterial isolates Acinetobacter sp. strain AOB-GP1 and Cronobacter sp. strain AOB-MT1 are unique strains that have been identified as having the ability to oxidise the As for the first time (III).* Previous studies have reported arsenic resistant bacteria from aquifers *Achromobacter, Acinetobacter calcoaceticus NCCB 22016T* (13), *Pseudomonas alcaligenes ATCC 14909T* [33], *Enterobacter ludwigii EN-119T* (14), *Acinetobacter, and Pseudomonas* (12). Acinetobacter sp. strain AOB-GP1 and Cronobacter sp. strain AOB-MT1 had the highest levels of arsenite oxidation in our investigation. It is proposed that additional research is needed to fully comprehend the function of the ars operon in these strains.

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Informed Consent Statement: Not Required

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Institutional Review Board Statement:

Ethical approval: NA

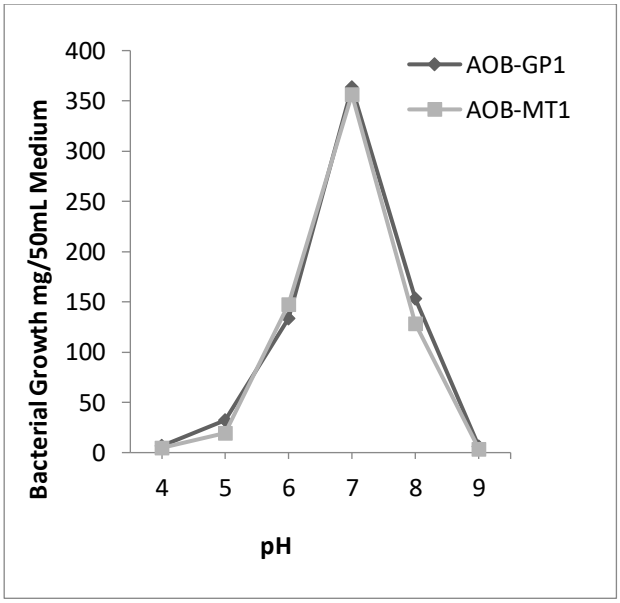

Fig.1 Growth pattern of isolates at pH ranging from 4-9.

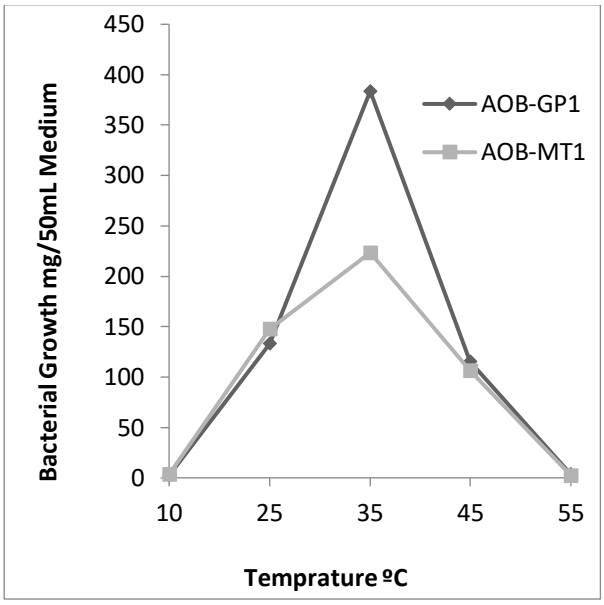

Fig.2 Growth pattern of isolates at temperature ranging from 10-55^{o}C

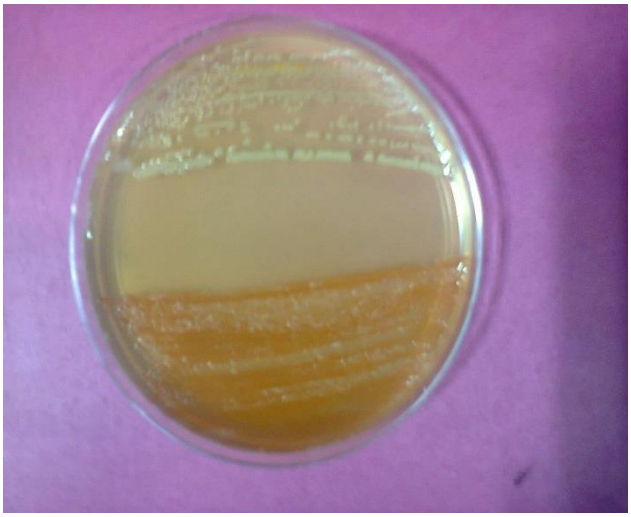

Fig 3. $AgNO_3$ test Presence of brownish precipitates (formation of arsenate) in medium.

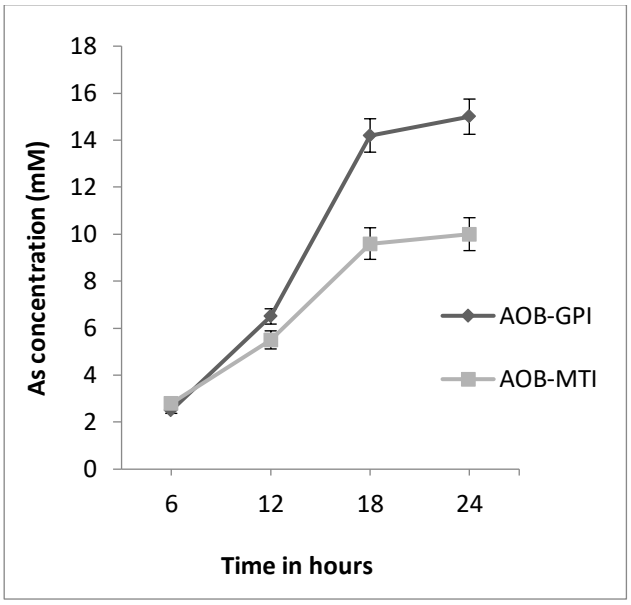

Fig.4 Arsenite Oxidation pattern of AOB-GPI and AOB-MTI in presence of 10mM and 15mM.

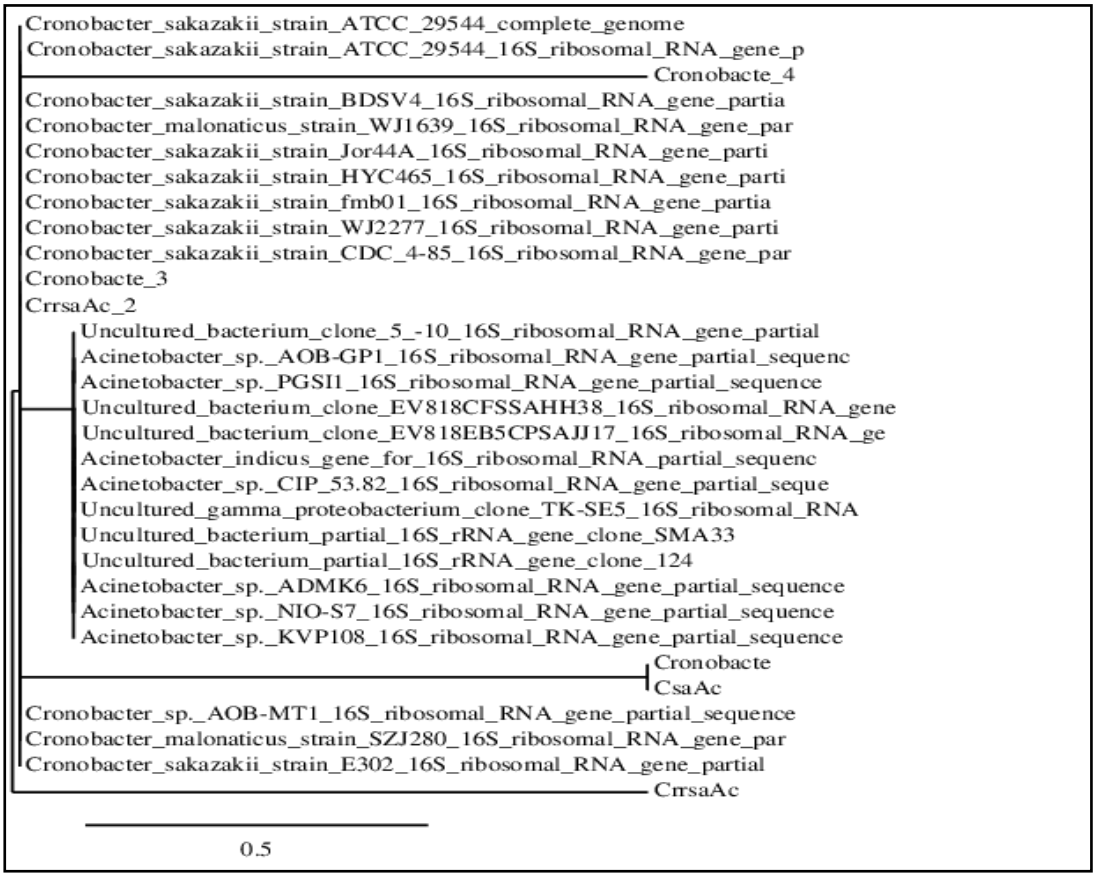

Fig. 5. Phylogenetic tree of the two new Gram negative arsenite oxidizing bacteria based on 16S rRNA sequences.

Table 1: Phenotypic and biochemical characterization of the seven Arsenite oxidizing bacterial isolates.

Biochemical Test	AOB-GP1	AOB-MT1
Gram Stain	-	-
0.8 % NaCl	-	+
Growth at pH (7.0)	+	+
Growth at (35^0C)	+	+
Cell shape	Rod	Rod
Spore formation	-	-
Oxidase	-	-
Catalase	+	+
Motility	-	+
Methyl red	-	-
Voges-Proskauer	-	+
Indole	+	+
Urease	+	-
H_2S Formation	-	-
Citrate utilization	+	+
Hydrolysis of		
Esculin	+	-
Casein	-	-
Starch	ND	-
Gelatin	ND	ND
Acid Production		
Sucrose	-	-
Maltose	+	ND
Lactose	+	-
Glucose	-	+
Galactose	+	ND

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