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Research Article

Arsenic Methylating Microorganisms in Waters, Health Treating Features and Biotechnological Importance

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Abstract

The methylated form of arsenite is more toxic than inorganic arsenic, which can be seen as a risk factor in health, but it is also a new study topic for some new biotechnological applications. The isolated bacteria from Balçova Ilıca Creek were identified by phenotypic and genotypic methods and S-adenosylmethionine transferase, the arsenic methylating enzyme, was detected by polymerase chain reaction.

From seven bacteria, six of them were identified as *Pseudomonas sp.* and one of was identified as *Pannonibacter phragmitetus*. We detected Arsenic S-adenosylmethionine transferase gene (arsM) in three of bacterial isolates.

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INTRODUCTION

Arsenic has valances of -3, 0, +3 and +5 oxidation stages. Its +3 and +5 stages, As(III) and As(V), are abundant in environment of humans. While arsenite, arsenic trioxide, monomethylarsonous acid, dimethylarsinous acid are trivalent oxidation state; arsenate, arsenic pentaoxide, monomethylarsonic acid, dimethylarsenic acid, trimethylarsine oxide, arsenilic acid, arsenobetaine are pentavalent oxidation state [1].

Microorganisms can oxidize arsenite to arsenate or they can reduce arsenate to arsenite by their enzymes. Thus they play a role in the arsenic cycle in nature. As (III) oxidation may be aerobic or anaerobic depending on the enzymes of the microorganisms. As (V) reduction is carried out with two types of metabolic activity. The first one is arsenate reductase (ArsC) which plays a role in detoxification mechanism of As (V) resistant bacteria and the second is anaerobic arsenate reductase (Arr) which is used in As (V) as the last electron acceptor in anaerobic respiration [2]. Mono-, di- and trimethyl arsines can be formed by chemically or biologically methylating of inorganic As(V). The higher toxicity to lower toxicity of soluble inorganic and organic arsenic species are respectively; dimethylarsenite (DMAs(III)), monomethylarsenite (MMAs(III)), As(III), As(V), dimethylearsenate (DMAs(V)), monomethylarsenate (MMAs(V)), trimethylarsine (TMAs), trimethylarsine oxide (TMAsO). When arsenic is methylated three times, it becomes volatile. This method is another method that microorganisms use to deal with arsenic toxicity [3].

The role of microorganisms in the metal cycles in nature, as well as its role in the arsenic cycle, has gained importance in both biotechnological applications and in attracting attention to arsenic toxicity. In this study, it was aimed to isolate the aerobic mesophilic bacteria with arsenic metabolism from Balçova Ilıca Creek where thermal water is involved. Than it was aimed to screen for S-adenosylmethionine transferase (arsM) gene of isolated bacteria. Considering the fact that three times methylated arsenic has become gaseous, new studies can be carried out for biotechnological use in light of the findings. The acquisition of such new information is important not only in terms of engineering use for the benefit of the environment, but also in terms of human health when taking new decisions and practices.

MATERIAL AND METHOD

Selection of Sampling Point and Detection of Chemical and Physical Parameters

Balçova Thermal Waters and Ilıca Creek where thermal waters infiltrate in İzmir, contain plenty of arsenic and other heavy metals [4]. Therefore, it is contemplated that arsenic resistant bacteria may be isolated from there. In the coordinate 38º 23' 7" N, 27º 2' 1" E, thermal water flowing from the bottom of the concrete to Ilıca Creek was noticed. 1000mL Nalgene® 215-(PPCO) bottle which sterilized by autoclave is used for water sampling (Fig. 1). Temperature, pH and electrical conductivity are measured by Eutech Instruments Cyberscan pH110.

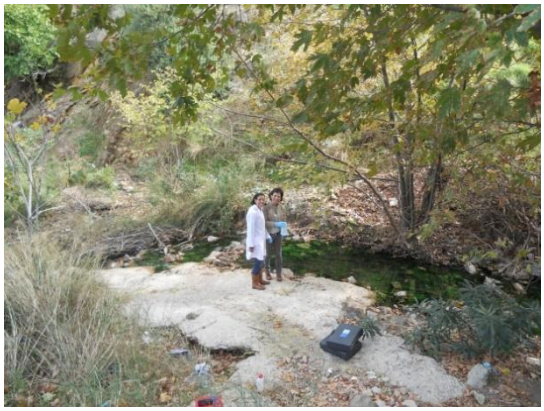

Fig ***1.*** Sampling area

Isolation and Identification of Arsenic Resistant Bacteria

Water sample inoculated to each plate count agar which include 0 mg/L, 100 mg/L, 200 mg/L, 400 mg/L, 800 mg/L and 1600 mg/L sodium arsenate heptahydrate by pour plate method than incubated in 30º C [5]. Second selection is made from isolated pure bacteria by suspending them in physiological saline as Macfarland 1 and inoculating 10µL on plate count agar which include 500 mM As(V) (156005 mg/L sodium arsenate heptahydrate). The isolates which can grow in 30 ºC for 3 days are chosen for genotypic and phenotypic identification.

Gram staining, KOH test, cytochrome oxidase test, nitrate reduction, mobility test, endospore test are performed [6]. Other phenotypic properties are determined by API 50 CH and API 20 E [7].

Bacterial DNAs were isolated by PureLink Genomic DNA mini kit [8]. 8-27F 5'-AGA GTT TGA TTC TGG CTC AG-3' and 1476-1495R 5'-CTA CGG CTA CCT TGT TAC GA-3' universal primers are used for 16S rDNA synthesis. Polymerase chain reaction (PCR) adjusted 5 minutes at 95ºC for initial denaturation and 1 minute at 95ºC for denaturation, 1 minute at 56ºC for annealing, 1 minute at 72ºC for extension in 40 cycle and 10 min at 72ºC for final extension [9]. The replicated sequence was sent to a bi-directional sequence analysis. The sequences were corrected using Finch TV and ApE programs. It was compared to the bacteria that showed similarity in NCBI and recorded in GenBank.

Screening of S-Adenosylmethionine Methytransferase Gene (*arsM*)

5'-AGG ACG AGG TGC TGT ATG G-3' forward ve 5'-GGT AGG TGG CCG AGT AGA A-3' reverse primers are used [10]. "One TaqTM 2X Master Mix with Standard Buffer" is used in this study. Total reaction volume is prepared 20µL according to ratios in the product manual. PCR adjusted 30 seconds at 94ºC for initial denaturation and 20 seconds at 94ºC for denaturation, 1 minute at 56ºC for annealing, 1 minute at 68ºC for extension in 30 cycle and 5 min at 68ºC for final extension [11].

Determination of Minimum Inhibitory Concetrations (MIC) for Arsenate and Arsenite

We used 1000 mM, 900 mM, 800 mM, 700 mM, 600 mM, 500 mM, 400 mM and 300 mM sodium arsenate heptahydrate ($Na_2HAsO_4.7H_2O$ Alfa Aesar A18275) containing Luria Bertani broth (LB, Merck

1.10285.0500) and 100 mM, 50 mM, 25 mM, 12.50 mM, 6.25 mM, 3. 13 mM, 1.56 mM, 0.78 mM, 0.39 mM, 0.20 mM, 0,10 mM, 0,05 mM sodium meta arsenite ($AsNaNO_2$ Sigma-Aldrich S7400) containing LB broth for determination of MICs [11]. We used triphenyl tetrazolium chloride (TTC) to show microbial activity and vitality in wells of microplate [9].

RESULT AND DISCUSSION

Chemical and Physical Parameters of Sampling Water

Table 1. Chemical and physical parameters of water sample

Temperature (ºC)	44,2
pH	8,47
Electrical Conductivity (µS/cm)	10460

Phenotypic and Genotypic Identification of Bacterial Isolates

We identified six isolates as *Pseudomonas sp.* and an isolate as *Pannonibacter phragmitetus*. Their GenBank accession numbers in National Center for Biotechnology Information (NCBI) are shown in table-2. All bacterial isolates are Gram negative bacilli. All of them are positive for KOH test, cytochrome oxidase test and mobility test. They grow on MacConcey Agar. Their colony colour is cream. Api 50 CH and Api 20 E findings are shown in table 3 and table 4.

Table 2. Partial 16S rRNA similarities of isolates

Isolates	Species	Most similarity ratio	Base counts of partial 16S rDNA	GenBank Accession Number
Pseudomonas sp. B	*Pseudomonas mendocia* *Pseudomonas pseudoalcaligenes* *Pseudomonas sp*.	%99	782	-
Pseudomonas sp. C	*Pseudomonas mendocia* *Pseudomonas pseudoalcaligenes* *Pseudomonas sp.*	%99	1385	MF000784
Pannonibacter phragmitetus D	*Pannonibacter phragmitetus* *Pannonibacter indicus* *Achromobacter sp.*	%99	1358	MF000785

Pseudomonas sp. E	*Pseudomonas mendocia* *Pseudomonas pseudoalcaligenes* *Pseudomonas oleovorans* *Pseudomonas sp.*	%99	1398	MF000786
Pseudomonas sp. F	*Pseudomonas pseudoalcaligenes*	%100	1404	MF000787
Pseudomonas sp. G	*Pseudomonas pseudoalcaligenes* *Pseudomonas oleovorans* *Pseudomonas sp.*	%99	1393	MF000788
Pseudomonas sp. H	*Pseudomonas mendocina* *Pseudomonas pseudoalcaligenes*	%99	891	-

According to sequencing of partial 16S rRNA, *Pseudomonas sp.* G shows %99 similarity with *Pseudomonas pseudoalcaligenes* and *Pseudomonas oleovorans*. However, *Pseudomonas oleovarans* ATCC 8062T and *Pseudomonas pseudoalcaligenes* ATCC 17440T produced acid from D-fructose [12], *Pseudomonas sp.* G does not produce acid. DNA-DNA hybridization is required for further identification.

Table 3. API 50 CH findings of isolates

	B	C	D	E	F	G	H
0) Control	-	-	-	-	-	-	-
1) Glycerol	-	-	-	-	-	-	-
2) Erythritol	-	-	-	-	-	-	-
3) D-Arabinose	-	-	-	-	-	-	-
4) L-Arabinose	-	-	+	-	-	-	-
5) D-Ribose	-	-	-	-	-	-	-
6) D-Xylose	-	-	+	-	-	-	-
7) L-Xylose	-	-	-	-	-	-	-
8) D-Adonitol	-	-	-	-	-	-	-
9) Methyl-βD-Xylopyranoside	-	-	-	-	-	-	-
10) D-Galactose	-	-	-	-	-	-	-
11) D-Glucose	-	-	+	-	-	-	-
12) D-Fructose	-	-	-	-	+	-	+
13) D-Mannose	-	-	-	-	-	-	-
14) L-Sorbose	-	-	-	-	-	-	-
15) L-Rhamnose	-	-	-	-	-	-	-
16) Dulcitol	-	-	-	-	-	-	-

17) Inositol	-	-	-	-	-	-	-
18) D-Mannitol	-	-	-	-	-	-	-
19) D-Sorbitol	-	-	-	-	-	-	-
20) Methyl-αD-Mannopyranoside	-	-	-	-	-	-	-
21) Methyl-αD-Glucopyranoside	-	-	-	-	-	-	-
22) N-AcetylGlucosamin	-	-	-	-	-	-	-
23) Amygdalin	-	-	-	-	-	-	-
24) Arbutin	-	-	-	-	-	-	-
25) Esculin ferric citrate	-	-	+	-	-	-	-
26) Salicin	-	-	-	-	-	-	-
27) D-Cellobiose	-	-	-	-	-	-	-
28) D-Maltose	+	-	-	-	+	-	-
29) D-Lactose	-	-	-	-	-	-	-
30) D-Melibiose	-	-	-	-	-	-	-
31) D-Saccharose (Sucrose)	-	-	-	-	-	-	-
32) D-Trehalose	-	-	-	-	-	-	-
33) Inulin	-	-	-	-	-	-	-
34) D-Melezitose	-	-	-	-	-	-	-
35) D-Raffinose	-	-	-	-	-	-	-
36) Amidon (Starch)	-	-	-	-	+	-	-
37) Glycogen	-	-	-	-	-	-	-
38) Xylitol	-	-	-	-	-	-	-
39) Gentiobiose	-	-	-	-	-	-	-
40) D-Turanose	-	-	-	-	-	-	-
41) D-Lyxose	-	-	-	-	-	-	-
42) D-Tagatose	-	-	-	-	-	-	-
43) D-Fucose	-	-	+	-	-	-	-
44) L-Fucose	-	-	+	-	-	-	-
45) D-Arabitol	-	-	-	-	-	-	-
46) L-Arabitol	-	-	-	-	-	-	-
47) Potassium Gluconate	-	-	-	-	-	-	-
48) Potassium 2-KetoGlukonate	-	-	-	-	-	-	-
49) Potassium 5- KetoGlukonate	-	-	-	-	-	-	-

Table 4. API 20 E findings of isolates

	B	C	D	E	F	G	H
ONPG	-	-	+	-	-	-	-
ADH	+	-	+	+	-	+	-
LDC	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-
CIT	+	+	+	+	+	-	+
H_2S	-	-	-	-	-	-	-
URE	-	-	+	-	-	-	-
TDA	-	-	-	+	-	-	-
IND	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-
GEL	-	-	-	-	-	-	-
GLU	-	-	+	-	-	-	

NO_2	+	+	+	+	+	+	+
N_2	-	-	+	-	-	-	-
MAN	-	-	+	-	-	-	-
INO	-	-	+	-	-	-	-
SOR	-	-	+	-	-	-	-
RHA	-	-	+	-	-	-	-
SAC	-	-	+	-	-	-	-
MEL	-	-	+	-	-	-	-
AMY	-	-	-	-	-	-	-
ARA	-	-	+	-	-	-	-

S-Adenosylmethionine Methytransferase Gene (arsM)

Pure ArsM protein of *Pseudomonas alcaligenes* NBRC14159 which called PaarsM can methylate As(III) between pH6 and pH10. Inorganic As(III) returned to organic monomethyl arsenate (MAs(V)), dimethyl arsenate (DMAs(V)) and volatile trimethyl arsenate (TMAs(V)) by PaarsM with adenosylmethionine (SAM) as methyl source and reductive glutatione (GSH) in pH 7.5 phosphate buffer in 37ºC [10]. In this study, arsenic resistant bacteria isolated and they are screened for *paarsM* by polymerase chain reaction. With agarose gel electrophoresis, while there is no band observed in *Pseudomonas sp.* B, *Pannonibacter phragmitetus* D, *Pseudomonas sp.* E, *Pseudomonas sp.* H isolates, bands bigger than 1517 bp are observed in *Pseudomonas sp.* C ve *Pseudomonas sp.* F. On the other hand the expected 180 bp band which shows *arsM* is observed in *Pseudomonas sp.* G (Fig 2.)

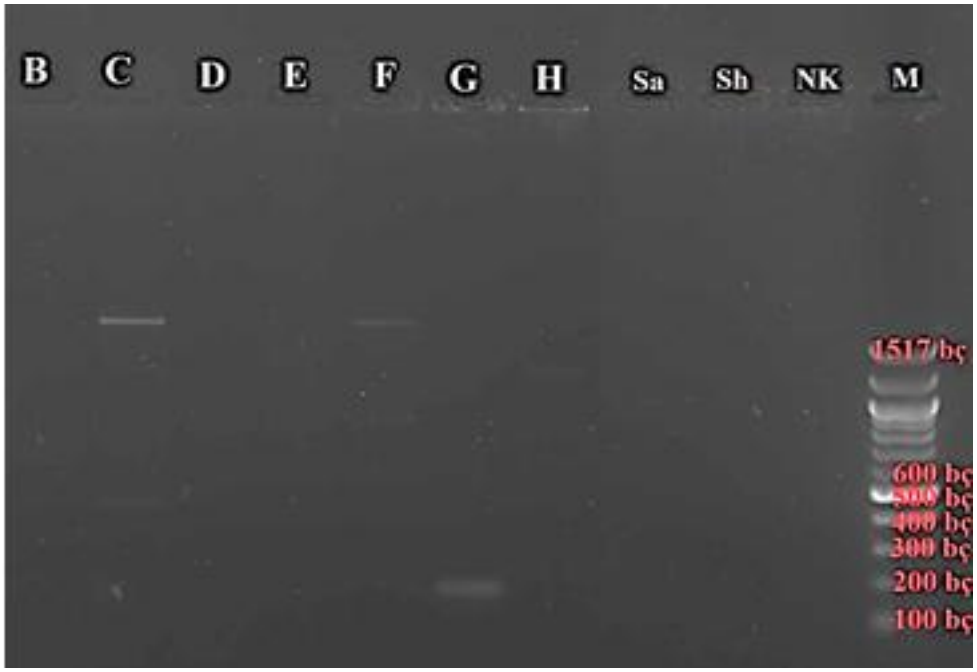

Fig 2. Gel electrophoresis for *arsM* gene. B*: Pseudomonas sp*. B, C: *Pseudomonas sp*. C, D: *Pannonibacter phragmitetus* D, E: *Pseudomonas sp*. E, F: *Pseudomonas sp*. F, G: *Pseudomonas sp*. G, H: *Pseudomonas sp.* H, Sa: *Staphylococcus aureus* ATCC 25923, Sh: *Shewanella sp.* ANA-3

Minimum Inhibitory Concetrations (MIC)

We determined high minumum inhibitory concentrations for arsenite and arsenate (Table 5).

Tablo 5: Minimum inhibitory concetration for arsenite and arsenate

Isolates	**As(V) MIC (mM)**	**As(III) MIC (mM)**
Pseudomonas sp. B	700	25
Pseudomonas sp. C	700	25
Pannonibacter phragmitetus D	600	12,5
Pseudomonas sp. E	700	25
Pseudomonas sp. F	600	25
Pseudomonas sp. G	600	12,5
Pseudomonas sp. H	700	25

CONCLUSION

Pseudomonas species can be used in the production of various biotechnological products in the industry [13], as well as in the treatment of polycyclic aromatic hydrocarbon (PAH) and arsenic [14]. Despite biotechnological importance of *Pseudomonas* species, they may also be opportunistic pathogens [15] and have a negative effect on antibiotic resistance in aquatic systems [16]. *Pseudomonas* species can be chosen for designing new bioremediation techniques to eliminate arsenic by making gas form of it. However, pathogenicity of microorganisms, arsenic metabolism of microorganisms and the toxicity of arsenic species should be considered.

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