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EXPERIMENTAL ARTICLES

Isolation and Characterization of Halophilic Bacteria from Urmia Lake in Iran[1]

Sepideh Zununi Vahed[a, b, *], Haleh Forouhandeh[a, *], Salar Hassanzadeh[a, c, *], Hans-Peter Klenk[d], Mohammad Amin Hejazi[e], and Mohammad Saeid Hejazi[a, 2]

[a] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*

[b] *Department of Medical Biotechnology, School of Advanced Biomedical Sciences (SABS), Tabriz University of Medical Sciences, Tabriz, Iran*

[c] *Faculty of Agriculture, Zanjan University, Zanjan, Iran*

[d] *DSMZ, German Collection of Microorfanisms and Cell Cultures, Braunschweig, Germany*

[e] *Branch for the Northwest and West Region, Agriculture Biotechnology Research Institute of Iran (ABRII), Tabriz, Iran*

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Abstract—Urmia Lake is one of the most permanent hypersaline lakes in the world which is threatened by hypersalinity and serious dryness. In spite of its importance no paper has been published regarding bacterial community of this lake. Accordingly, the present study aimed to investigate the halophilic bacteria in the aforementioned lake. In so doing, thirty seven strains were isolated on six different culture media. The isolated strains were characterized using phenotypic and genotypic methods. Growth of the strains occurred at 25–35°C, pH 6–9 and 7 to 20% (w/v) NaCl indicating that most of the isolates were moderately halophiles. Catalase, oxidase and urease activities were found to be positive for the majority of the isolates. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolated bacteria belonged to two major taxa: *Gammaproteobacteria* (92%, including *Salicola* [46%], *Pseudomonas* [13.5%], *Marinobacter* [11%], *Idiomarina* [11%], and *Halomonas* [8%]) and *Firmicutes* (8%, including *Bacillus* [5%] and *Halobacillus* [3%]). In addition, a novel bacterium whose 16S rRNA gene sequence showed almost 98% sequence identity with the taxonomically troubled DSM 3050^T, *Halovibrio denitrificans* HGD 3^T and *Halospina denitriflcans* HGD 1–3, each, was isolated. 16S rRNA gene similarity levels along with phenotypic characteristics suggest that some of the isolated strains could be regarded as potential type strain for novel species, on which further studies are recommended.

Keywords: halophilic bacteria, Urmia Lake, moderately halophilic bacteria.

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Halophiles are distributed in a broad range of hypersaline environments, all over the phyla and the orders of bacterial domain [1–3]. Upon their response to NaCl, they can be classified into three groups: slightly halophiles, moderately halophiles and extremely halophiles with optimal growth rates at 2–5, 5–20 and 20–30% NaCl, respectively [1, 4, 5]. In order to adapt to high saline conditions, two principal mechanisms have been evolved by halophilic microorganisms: (i) salt-in-cytoplasm, and (ii) compatible solute strategies [6–8]. Bacteria employing second mechanism, accumulate organic compounds such as sugars, amino acids and/or amino acid derivatives (e.g., ectoine, hydroxyectoine and glycine betaine) in response to an osmotic stress [3, 6]. The production of compatible solutes as well as other compounds such as bacteriorhodopsins, exopolysaccharides, hydrolases and biosurfactants has remarkable potentials in industry [9–12].

It was reported that moderately halophilic bacteria, *Marinobacter*, *Idiomarina* and *Halomonas* strains are able to degrade organic pollutants, organic nitrogen compounds (found in foods, organic materials, fertilizers, poisons, and explosives) [13–15], Moreover, since they are metabolically different and able to adapt to extreme salinity, they could be considered suitable candidates for the bioremediation of hypersaline environments [3, 13, 16].

Urmia Lake, in northwest of Iran at Azarbayjan region, is the largest saline lake in the Middle East and it's ranked as the second largest salt water lake on the Earth. Like the Dead Sea, Urmia Lake is precious for the extreme salinity of its water. Traditionally, it is believed that the water of the lake is full of healing properties, e.g. as a major source to cure rheumatism [17].

In 1915, Urmia Lake NaCl concentration was measured about 34 g per liter which has risen to more than 300 g per liter due to drought, evaporation pro-

[1] The article is published in the original.

[2] Corresponding author; e-mail: msaeidhejazi@yahoo.com; saeidhejazi@tbzmed.ac.ir

* Sepideh Zununi Vahed, Haleh Forouhandeh, and Salar Hassanzadeh contributed equally to this work and should be considered as co-first authors.

Fig. 1. Lake Urmia is the second largest salt water lake on earth and is threatened by dryness. The white shadows (shown by arrows) demonstrate the salt layers left on the ground as the lake dries. The light blue areas illustrate parts of the lake where the depth of the water has decreased resulting in appearance of increasing amounts of salt precipitants under the water. The depth of water in these areas is mainly less than 1 m.

cess and increased demands for agricultural water in the lake's basin [18, 19]. This continuing development became meanwhile the source of great concerns and serious doubts about the survival of the lake for the near future (Fig. 1).

Urmia Lake has, unfortunately, so far been neglected for diversity studies. According to our knowledge no systematic analysis for potential novel types of halophilic bacteria has been reported for this lake, which clearly justifies the aim of the here reported exploration.

MATERIALS AND METHODS

Water sample collections. The studied strains were isolated from the water and soil specimens of Urmia Lake. Samples were taken in December 2006 and July 2009 from different wharfs of Saray Coast of Urmia Lake, they were delivered to the laboratory in sterilized containers and they were used immediately.

Halophile isolation and purification. In order to maximize the chances of recovering abundant moderately halophilic bacteria, isolation and enrichment procedures were performed in different media including: MH medium [20], Halomonas medium [21], Marine agar (Difco), MGM medium with 2, 7, 15, 25% total salt concentrations, Luria-Bertani (LB) with 7.5% NaCl and Nutrient agar with 10% NaCl. For this purpose, 400 μl of water samples were inoculated on the mentioned solid media and incubated at 30°C. Colony growth was first observed after a couple of days. The growth continued for more than 20 days after initial plating. To obtain pure cultures, single colonies were picked from all of plates and were used for Gram-staining and stock preparation, by growing in liquid media in

an orbital shaker. Axenic microbial cultures were stored for further use at −70°C in the isolation medium supplemented with 30% glycerol.

Characterization and identification of isolates. A selection of phenotypic characters including morphological, physiological and biochemical tests were determined for each strain. Colonies on respective media plates were examined using their characteristics, such as color, form, catalase and oxidase tests. For physiological tests, growth at various salt concentrations was carried out by spreading 40 µl of stock onto the respective agar media with sea-salt concentrations of 0, 7.5, 10, 20 and 30% (w/v). The pH growth range was determined in the similar way on before mentioned media between 5–10.

Various biochemical tests were carried out with the isolates. Acid production from carbohydrates test was performed with eighteen carbohydrates including *D*-glucose, *D*-mannose, *D*-fructose, maltose, *D*-mannitol, *L*-rhamnose, sucrose, *D*-terhalose, *D*-galactose, *D*-cellobiose, *D*-raffinose, *D*-xylose, lactose, *D*-ribose, *L*-arabinose, *D*-melezitose, *D*-salicin, and aesculin. H_2S production was tested in liquid media supplemented with 0.01% (w/v) of *L*-cysteine. The indicator used in this experiment was a band of paper impregnated with lead-acetate placed in the neck of the tube. To examine nitrate reduction, 0.2% (w/v) KNO_3 was added to the liquid media. Urea hydrolysis and arginine decarboxylase were tested according to Mac Faddin [22]. Gelatin and starch hydrolysis were tested by flooding cultures on solid media containing 1% (w/v) gelatin and starch, respectively. Casein hydrolysis was indicated by a clear zone around bacterial growth on double-strength solid media plus an equal quantity of skimmed milk [23]. Hydrolysis of Tween 20 and Tween 80 were tested on solid media supplemented with 1% (w/v) of separately autoclaved Tween 20 and Tween 80 [23]. Tyrosine hydrolysis was revealed by clear zones in cultures on solid media containing 5 g/l tyrosine [23].

PCR amplification of the 16S rRNA genes. The 16S rRNA gene amplification of the isolated strains was conducted directly, without genomic DNA extraction step. PCR amplifications were carried out on a gradient master cycler (Eppendorf). The PCR reaction mixture (50 µl) contained 50 pmol each of forward (16F27: 5'-AGA GTT TGA TC(AC) TGG CTC AG-3') [24] and reverse (16R: 5'-TAC CTT GTT AGG ACT TCA CC-3') primers [25], four dNTPs with 0.2 mM of each, 2.0 mM $MgCl_2$, 0.3 µl bacterial stock as the template DNA and 1.5 U *Taq* DNA polymerase was used to obtain a PCR product of about 1.5 kb corresponding to 16S rRNA gene base positions 8-1488 based on *Escherichia coli* numbering [26]. The 16S rRNA gene amplification was performed according to Pournaghi-Azar et al. [25].

Amplified PCR products directly were sent to a commercial facility for sequencing (Macrogen Inc., Seoul, South Korea). In order to read 16S rRNA gene sequence; sequencing was carried out using forward, reverse and 3 other primers called H400 (5'–GGG TTG TAA AGC ACT TTC AG–3'), H550 (5'–CCA GTA ATT CCG ATT AAC GC–3') and H900 (5'–ACT CAA ATG AAT TGA CGG GG–3') designed in this study. Data obtained from sequencing were analyzed by bioinformatic softwares such as Edit seq, Blast-2-sequences (NCBI) and Read Seq. (Bioinformatics & Molecular Analysis) in order to obtain the complete sequences of 16S rRNA genes.

Phylogenetic analysis on the basis of the 16S rRNA gene sequences. Obtained results were compared to reference 16S rRNA gene sequences available in Eztaxon. Phylogenetic analysis was carried out using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 [27], after multiple alignments of data by CLUSTAL-X. Distances and clustering with the neighbor joining method were carried out using bootstrap values based on 1000 replications.

The 16S rRNA gene sequences reported in this study were submitted to the GenBank under accession numbers EU305725–EU305729, EU251075 and HQ190037–HQ190041.

RESULTS

Thirty seven strains were isolated from water samples of Urmia Lake, thirty four of them stained Gram-negative and three were Gram-positive. Colony pigmentation of these samples was orange to white, most of which was cream. All isolates were catalase positive and most of them were not able to grow in the absence and at 30% of NaCl. For most of the isolates, growth occurred at 25–35°C, pH 6–9 and 7 to 20% (w/v) NaCl. Furthermore, more than half of the isolated strains could not produce acid from sugars. Hydrolysis of gelatin and starch were found to be negative whereas urease activity and oxidase were found to be positive for the majority of the isolates. Some of the strains reduced nitrate to nitrite while the remaining ones continued the reduction process to NO, N_2O or N_2. Characteristics of the novel halophiles were listed in Table 1.

In order to evaluate their phylogenetic positions, the 16S rRNA gene sequence of each strain was analyzed and a phylogenetic tree was constructed (Fig. 2a). 16S rRNA gene sequence analysis revealed that the isolates belong to two major groups, *Gammaproteobacteria* and *Firmicutes*.

Gammaproteobacteria, the first group, comprised of five genera including *Halomonas*, *Salicola*, *Pseudomonas*, *Idiomarina* and *Marinobacter*. Eight percent of the isolated bacteria belonged to genus *Halomonas* and showed 98–99.4% similarity to *Halomonas saccharevitans*, *H. fontilapidosi* and *H. taeanensis*. Similarity between isolated bacteria belonging to this genus was 95.5–97.2%. The second genus, *Salicola*, covered 46% of isolates and exhibited >99% similarity with its closest phylogenetic relatives; *Salicola* marasensis 7Sm5^T and *Salicola salis*. Based on 16S rRNA gene sequences, these bacteria clustered into three groups according to possession of C/T in positions 86 and 861 (according to *E. coli* numbering). Genus *Pseudomonas* represented 13.5% of strains and indicated >99% similarity. The

Table1. Phenotypic features of the isolated strains

Characteristics	Strain TBZ3	Strain TBZ21	Strain TBZ26	Strain TBZ6	Strain TBZ24	Strain TBZ39
Morphology	Coccoid	Long rods	Rods	Rods	Rods	Rods
Pigmentation	Cream	White	Light cream	Light cream	Light cream	Light cream
Oxidase	+	+	+	–	+	+
20% NaCl	+	–	+	–	+	+
pH 9	+	+	+	–	+	+
H_2S formation	–	–	+	–	+	–
Phenylalanin deaminase	+	+	–	–	–	–
Hydrolysis of:						
L-tyrosine	–	–	+	–	–	–
Tween 20	+	+	–	+	+	+
Tween 80	+	–	–	+	+	+
Acid production						
D-glucose	–	–	+	–	–	–
D-mannose	–	–	+	–	–	–
Isolation media	Halomonas medium	MH medium	LB with 7% NaCl	Halomonas medium	Halomonas medium	Halomonas medium

Characteristics	Strain TBZ19	Strain TBZ126	Strain TBZ1	Strain TBZ4	Strain TBZ29
Morphology	Short rods	Rods	Short Rods	Short rods	Short rods
Pigmentation	Orange	Red	Dark cream	Cream	Cream
Oxidase	+	–	+	+	+
Salt range (%, w/v)					
7.5%	+	–	+	+	+
20%	–	+	+	–	–
pH range					
8	–	–	+	+	+
9	–	–	+	+	+
H_2S formation	–	+	+	+	+
Indol production	–	+	–	–	–
Tween 20	+	–	+	+	+
Tween 80	–	–	+	+	+
Casein	–	–	+	–	+
Starch	–	+	–	–	–
Gelatin	–	+	–	–	–
Nitrate reduction	–	–	+	+	+
Isolation media	MH medium	Marine agar	Halomonas medium	Halomonas medium	LB with 7% NaCl

Table 1. (Contd.)

Characteristics	Strain TBZ118	Strain TBZ104	Strain TBZ2	Starin TBZ33
Morphology	Rods	Rods	Rods	Rods
Pigmentation	Orange	Cream	White/Cream	Glassy
Oxidase	–	+	+	+
Salt range (%, w/v)				
0%	+	+	–	–
7.5%	+	+	+	–
10%	–	+	+	+
20%	–	–	+	+
pH range				
5	–	+	–	–
6	–	+	+	+
7	–	+	+	+
8	–	+	+	+
9	–	+	+	+
H_2S formation	+	+	–	–
Indol production	+	–	–	–
Tween 20	+	+	–	+
Tween 80	–	–	–	+
Casein	+	+	+	–
Starch	–	–	+	–
Gelatin	+	–	–	–
Nitrate reduction	–	–	–	+
Acid production				
D-glucose	–	–	+	–
D-mannose	–	+	+	–
D-fructose	–	+	+	–
Maltose	–	–	+	–
D-mannitol	–	+	+	–
L-rhamnose	–	–	+	–
Sucrose	–	–	+	–
D-terhalose	–	–	+	–
D-galactose	–	–	+	–
D-cellobiose	–	–	+	–
D-raffinose	–	–	+	–
D-xylose	–	–	+	–
Lactose	–	–	+	–
D-ribose	–	+	+	–
L-arabinose	–	+	+	–
Melezitose	–	–	+	–
D-salicin	–	–	+	–
Aesculin	–	–	+	–
Isolation media	MGM medium	LB with 7% NaCl	Halomonas medium	Nutrient agar with 10% NaCl

Fig. 2. (a)—phylogenetic relationships of the isolated strains based on partial 16S rRNA gene sequences. The sequence alignment was performed using the CLUSTALX program and the tree was generated using the neighbor joining method with Kimura 2 parameter distances in MEGA 4. Numbers at nodes indicate percent bootstrap value above 50 (1000 replicates); (b)—the phylogenetic position of strain TBZ33.

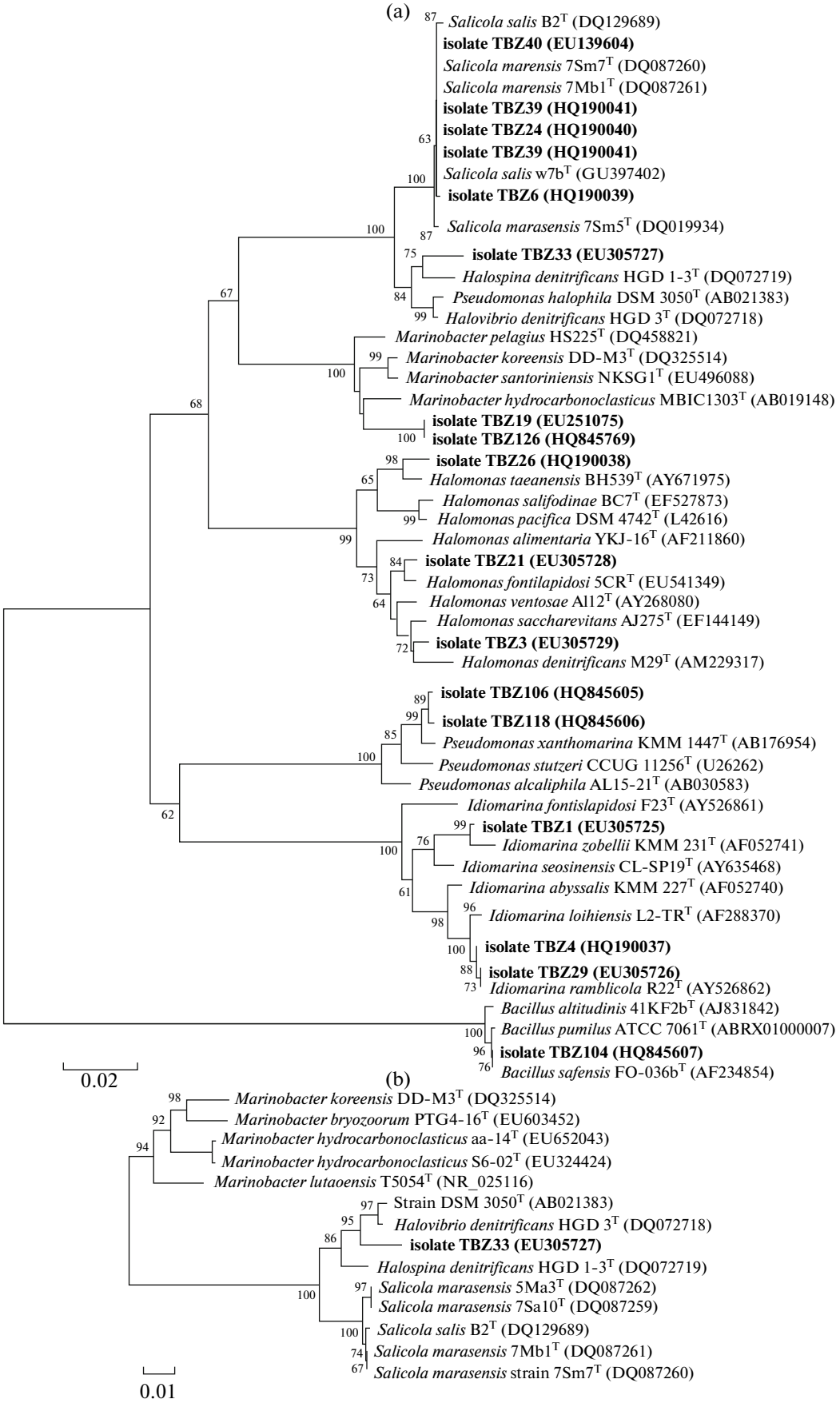
(a)
Salicola salis B2T (DQ129689)
isolate TBZ40 (EU139604)
Salicola marensis 7Sm7T (DQ087260)
Salicola marensis 7Mb1T (DQ087261)
isolate TBZ39 (HQ190041)
isolate TBZ24 (HQ190040)
isolate TBZ39 (HQ190041)
Salicola salis w7bT (GU397402)
isolate TBZ6 (HQ190039)
Salicola marasensis 7Sm5T (DQ019934)
isolate TBZ33 (EU305727)
Halospina denitrificans HGD 1-3T (DQ072719)
Pseudomonas halophila DSM 3050T (AB021383)
Halovibrio denitrificans HGD 3T (DQ072718)
Marinobacter pelagius HS225T (DQ458821)
Marinobacter koreensis DD-M3T (DQ325514)
Marinobacter santoriniensis NKSG1T (EU496088)
Marinobacter hydrocarbonoclasticus MBIC1303T (AB019148)
isolate TBZ19 (EU251075)
isolate TBZ126 (HQ845769)
isolate TBZ26 (HQ190038)
Halomonas taeanensis BH539T (AY671975)
Halomonas salifodinae BC7T (EF527873)
Halomonas pacifica DSM 4742T (L42616)
Halomonas alimentaria YKJ-16T (AF211860)
isolate TBZ21 (EU305728)
Halomonas fontilapidosi 5CRT (EU541349)
Halomonas ventosae Al12T (AY268080)
Halomonas saccharevitans AJ275T (EF144149)
isolate TBZ3 (EU305729)
Halomonas denitrificans M29T (AM229317)
isolate TBZ106 (HQ845605)
isolate TBZ118 (HQ845606)
Pseudomonas xanthomarina KMM 1447T (AB176954)
Pseudomonas stutzeri CCUG 11256T (U26262)
Pseudomonas alcaliphila AL15-21T (AB030583)
Idiomarina fontislapidosi F23T (AY526861)
isolate TBZ1 (EU305725)
Idiomarina zobellii KMM 231T (AF052741)
Idiomarina seosinensis CL-SP19T (AY635468)
Idiomarina abyssalis KMM 227T (AF052740)
Idiomarina loihiensis L2-TRT (AF288370)
isolate TBZ4 (HQ190037)
isolate TBZ29 (EU305726)
Idiomarina ramblicola R22T (AY526862)
Bacillus altitudinis 41KF2bT (AJ831842)
Bacillus pumilus ATCC 7061T (ABRX01000007)
isolate TBZ104 (HQ845607)
Bacillus safensis FO-036bT (AF234854)
0.02
(b)
Marinobacter koreensis DD-M3T (DQ325514)
Marinobacter bryozoorum PTG4-16T (EU603452)
Marinobacter hydrocarbonoclasticus aa-14T (EU652043)
Marinobacter hydrocarbonoclasticus S6-02T (EU324424)
Marinobacter lutaoensis T5054T (NR_025116)
Strain DSM 3050T (AB021383)
Halovibrio denitrificans HGD 3T (DQ072718)
isolate TBZ33 (EU305727)
Halospina denitrificans HGD 1-3T (DQ072719)
Salicola marasensis 5Ma3T (DQ087262)
Salicola marasensis 7Sa10T (DQ087259)
Salicola salis B2T (DQ129689)
Salicola marasensis 7Mb1T (DQ087261)
Salicola marasensis strain 7Sm7T (DQ087260)
0.01

Table 2. Halophilic bacteria isolated from saline environments

Isolated from	Gram-negative halophiles	Gram-positive halophiles	Reference
Saltern ponds in Taean-Gun, Korea	*Vibrio* *Pseudoalteromonas* *Halomonas* *Marinobacter* *Idiomarina*	*Bacillus* *Halobacillus* *Jeotgalibacillu* *Pontibacillus*	[32]
Saline environments in Alexandria, Egypt	*Pseudoalteromonas* *Flavobacterium* *Chromohalobacter* *Halomonas* *Salegentibacter*	*Halobacillus* *Salinicoccus* *Staphylococcus* *Tetragenococcus*	[33]
El-Djerid salt lake in Tunisia	*Salicola* *Halomonas*	*Halobacillus* *Pontibacillus* *Marinococcus*	[1]
Sal saltworks in Guerrero Negro, Baja California (Mexico)	*Halorubrum* *Haloarcula* *Halomonas* *Salicola* *Salinibacter*	*Halovibrio*	[34]
Urmia Lake in Iran	*Idiomarina* *Salicola* *Halomonas* *Pseudomonas* *Marinobacter*	*Bacillus* *Halobacillus*	Present study

group of the *Idiomarina* was represented by 11% of the strains and displayed about 99.5% similarity with *Idiomarina zobellii*, *I. loihiensis* and *I. ramblicola* and also 97.7–100% similarity with each other. 11% of strains affiliated to the genus *Marinobacter*, exhibited about 97% pairwise similarity with their closest phylogenetic relative, *Marinobacter hydrocarbonoclasticus* ATCC 49840^T. The *Firmicutes* group consisted of two genera; *Bacillus* was represented by 5% and *Halobacillus* was represented by 3% of the isolated strains, respectively. Furthermore, another bacterium was isolated that had high similarity to DSM 3050, a phylogentically troubled strain [28–30]. The phylogenetic analysis of this isolate, called TBZ33, indicated that the isolated strain also had about 97.5% similarity to *Halovibrio denitrificans* HGD 3^T and *Halospina denitrificans* HGD 1–3^T (Fig. 2b).

DISCUSSION

In the past few years, much attention has been paid to halophilic bacteria especially to moderately halophilic bacteria. Several studies have been conducted on their biotechnological applications such as production of bioactive compounds (antibiotics) as well as their ecologic and phylogenetic characteristics [3, 16]. Urmia Lake is one of the most permanent hypersaline lakes in the world and it is an oligotrophic lake with thalassohaline origin an ionic strength between 5.5–7.5, located in Azarbayjan region at northwest of Iran [31]. The present study made a first attempt to investigate halophilic bacteria in Urmia Lake. Apart from lack of any previously published article on its bacterial community, subjection of the lake to high salinity and dryness were the other reasons doing this study.

Throughout the course of this work, thirty seven strains were isolated which were predominately Gram-negatives (92%). For most of the isolates, growth occurred at 25–35°C, pH 6–9 and 7 to 20% (w/v) NaCl, but not at 30% NaCl, suggesting that most of these isolates considered moderately halophilic according to the definition by Ventosa et al. [3]. Most of the isolates were also able to reduce nitrate, implying that they may be involved in the global nitrogen cycle within the lake.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that most of the isolates were members of five genera within the *Gammaproteobacteria*, including *Idiomarina*, *Salicola*, *Halomonas*, *Pseudomonas* and *Marinobacter*. Furthermore, another bacterium; TBZ33; was isolated that was most closely affiliated with DSM 3050^T, which was recently proposed for renaming [30]. The community of the isolated bacteria in this study is compared with those of recently studies on halophilic bacteria isolated from saline environments in Table 2.

Urmia Lake has a phylogenetically diverse population of moderately halophilic bacteria. The presented study has offered a first insight into the bacterial genetic pool of Urmia Lake. Follow up studies also considering the archaeal diversity of the lake, as well as using state of the art metagenomics techniques will be required to understand the microbial diversity as well as applications of halophilic bacteria in Urmia Lake. 16S rRNA gene similarity levels along with phenotypic characteristics suggest that some of the isolated strains could after more detailed analysis become representatives of novel species.

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