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Diversity of Halophilic Archaea From Six Hypersaline Environments in Turkey

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Abstract The diversity of archaeal strains from six hypersaline environments in Turkey was analyzed by comparing their phenotypic characteristics and 16S rDNA sequences. Thirty-three isolates were characterized in terms of their phenotypic properties including morphological and biochemical characteristics, susceptibility to different antibiotics, and total lipid and plasmid contents, and finally compared by 16S rDNA gene sequences. The results showed that all isolates belong to the family *Halobacteriaceae*. Phylogenetic analyses using approximately 1,388 bp comparisions of 16S rDNA sequences demonstrated that all isolates clustered closely to species belonging to 9 genera, namely *Halorubrum* (8 isolates), *Natrinema* (5 isolates), *Haloarcula* (4 isolates), *Natronococcus* (4 isolates), *Natrialba* (4 isolates), *Haloferax* (3 isolates), *Haloterrigena* (3 isolates), *Halalkalicoccus* (1 isolate), and *Halomicrobium* (1 isolate). The results revealed a high diversity among the isolated halophilic strains and indicated that some of these strains constitute new taxa of extremely halophilic archaea.

Keywords: Halophilic archaea, 16S rDNA, hypersaline, phylogeny

The halobacteria comprise a well-defined, monophyletic group of aerobic or facultatively anaerobic microorganisms that demand high salt concentrations for growth, and are recognized as a division of the Archaea. Members of *Halobacteriaceae* are chemoorganotrophic organisms that use amino acids or carbohydrates to grow and need at least 1.5 M NaCl for growth, most exhibit optimal growth at 3.5–4.5 M NaCl, and are red pigmented because of the presence of carotenoid pigments [9]. Extremely halophilic archaea belonging to the family *Halobacteriaceae*, order *Halobacteriales*, are found extensively in hypersaline environments worldwide. To date, 64 species and 20 genera of *Halobacteriaceae* have been described [5, 8, 9, 26, 27], and new taxa in the *Halobacteriales* are still being described.

Halophilic archaea have been isolated from various hypersaline environments such as aquatic environments including salt lakes and saltern crystalizer ponds, and saline soils [9]. Turkey, especially central Anatolia, accommodates extensive hypersaline environments. To the best of our knowledge, there is quite limited information on extremely halophilic archaea of Turkey origin. Previously, extremely halophilic communities of the Salt Lake-Ankara region were partially characterized by comparing their biochemical and morphological characteristics [2]. In another study, Elevi *et al.* [7] isolated halophilic archaea from the Ayvalik saltern and characterized them by determining their morphology and polar lipid patterns. Finally, the phenotypic, characterization, and partial 16S rDNA gene analyses of halophilic archaeal isolates from Tuzkoy Salt Mine have been reported [3].

The main purpose of this research was to isolate halophilic archaeal strains from different salt lakes of Turkey and characterize them with respect to some phenotypic characteristics and 16S rDNA sequences.

Materials and Methods

Archaeal Strains

Archaeal strains were isolated from water and soil samples collected from Salt Lake (Ankara), Bolluk Lake (Konya), Seyfe Lake (Kirsehir), Aci Lake (Denizli), Tuzla Lake (Kayseri), and Salda Lake (Burdur) using Sehgal-Gibbons (SG) medium [17] (Fig. 1). After 2 weeks of incubation, representative colonies were transferred to fresh SG medium and grown at 37°C, and pure cultures were obtained. The strains of *Halobacterium salinarum*, *Haloferax mediterranei*, *Haloferax volcanii*, *Haloarcula marismortui*, and *Natrialba asiatica* were kindly provided by Prof. Dr.

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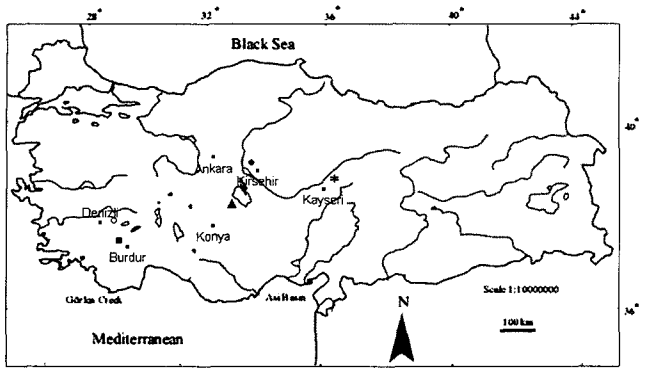

Fig. 1. The sampling areas in Turkey.
●, Seyfe Lake; ◆, Salt Lake; *, Tuzla Lake; ▲, Bolluk Lake; ■, Salda Lake; ○, Aci Lake.

Aharon Oren, Jerusalem, Israel. *Haloarcula vallismortis* DSM 3756 and *Halococcus morrhuae* CCM 537 were used as the reference strains. Some available aquatic parameters for 4 of the 6 salt lakes are given in Table 1 [29].

Phenotypic Characterization

All phenotypic characterization tests were carried out as specified by the proposed minimal standards for the description of new taxa in the order *Halobacteriales* [18]. Cell morphology and motility were examined on freshly prepared wet mounts by phase-contrast microscopy. Colony morphologies and pigmentation were determined on SG agar plates after 7 days of growth. Production of acids from sugars was examined on SG medium supplemented with 0.5% (w/v) of the sugars tested. Gram staining, catalase and oxidase activities, casein, starch, Tween 80 and gelatine hydrolysis, indole and H_2S production, nitrate reduction, and gas formation were determined as described before [23]. Anaerobic growth in the presence of L-arginine was tested according to Oren and Litchfield [19]. Appropriate positive and negative controls were run for all of the above tests, which were repeated at least three times.

Antibiotic susceptibility was tested by the disc diffussion method [4] using discs (Oxoid) containing ampicillin (10 μg), norfloxacin (10 μg), tetracycline (30 μg), bacitracin (10 IU), rifampicin (5 μg), azithromycine (15 μg), neomycin (30 μg), chloramphenicol (30 μg), penicillin G (10 IU), vancomycin (30 μg), and novobiocin (30 μg).

Total Lipid Analysis

The presence of the ether-linked lipids of the isolates was determined as described by Ross *et al.* [21]. Whole cell acid methanolysates of the strains were performed for glycerol diether moieties by thin layer chromatography on silica gel plates (Sigma, silica gel 10×10 cm, thickness 0.2 mm).

Plasmid Isolation

Plasmid DNA of the strains was isolated by using a modified method of Anderson and McKay [1].

DNA Extraction, 16S rDNA Amplification, and Sequencing

Genomic DNA was extracted from log-phase cells lysed in distilled water by phenol extraction followed by ethanol precipitation according to Dyall-Smith [6]. The gene encoding 16S rRNA was amplified by PCR with the forward primer 5'-ATTCCGGTTGATCCTGCCGG-3' (positions 1-20 according to *Halobacterium cutirubrum* NCIMB 763, GenBank Accession No. AB073366) and the reverse primer 5'-GATCCAGCCGCAGATTCCCC-3' (positions 1465-1446 according to *Halobacterium cutirubrum* NCIMB 763, GenBank Accession No. AB073366). PCR was performed for 30 cycles (3 min denaturing step at 95°C in the first cycle; 1 min denaturing at 94°C, 1 min annealing at 62°C, and 15 min polymerization at 72°C, with a final extension step at 72°C for 10 min). The amplification products were purified from agarose gel using a QIAquick PCR purification kit. Purified PCR products were sequenced by the chain termination method with dye-labeled dideoxy terminators of the Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham), using a Perkin Elmer-ABI Prism 377 automated sequencer. DNA sequencing was carried out at Iontek Company (Istanbul, Turkey).

Phylogenetic Analysis

Homology search was carried out by using the basic BLASTN search program at the NCBI Web-site. 16S rDNA sequences were aligned by using Clustal W [22]. Tree distance matrix was calculated on the basis of the algorithm of Jukes

Table 1. Aquatic parameters of 4 hypersaline lakes in Turkey [29].

Parameters	Salt Lake (Ankara)	Bolluk Lake (Konya)	Aci Lake (Denizli)	Salda Lake (Burdur)
Depth	ND	ND	0.4	45.5
Temperature (°C)	21.5	14.9	10.6	22.7
pH	7.48	8.59	7.75	9.45
Dissolved oxygen (mg/l)	6.9	3.0	13.25	7.0
Saturation (%)	80.0	35.0	100.4	96.0
Salinity (‰)	45.667	48.452	0.265	1.114
Alkalinity (meq/l)	2.0	13.0	2.6	30.6

ND: no data.

and Cantor [13]. A phylogenetic tree was constructed by the neighbor-joining method and was evaluated by bootstrap sampling (1,000 replicates) using the MEGA 3 program [15].

Nucleotide Accession Number

16S rDNA sequence data of the isolates A29, A43, A82, A85, A87B, A137, A191, A283, A317, A337, A440, B19, B36, B44A, B49, B77A, C15, C35, D1A, D50, D58A, D74, D107, D113, E7, E57B, E92B, F4A, F5, F23A, F30AI, F42A, and F100 reported in this article were submitted to GenBank and assigned the accession numbers DQ309088, DQ309094, DQ309080, DQ309079, DQ309089, DQ309083, DQ309091, DQ309093, DQ309085, DQ309092, DQ309084, DQ373052, DQ373060, DQ373062, DQ373056, DQ373047, DQ373058, DQ373061, DQ373059, DQ373053, DQ373055, DQ373050, DQ373057, DQ373048, DQ373054, DQ373049, DQ373051, DQ309082, DQ286063, DQ309087, DQ309081, DQ309086, and DQ309090, respectively.

RESULTS

Morphological and Biochemical Characteristics

A total of 33 strains were selected from six saline lakes. All isolates produced colonies on solid media, and were pink to red pigmented, circular, and with entire edges. Whereas most of the colonies were found to be convex and nonmucoid, nine isolates (A137, A317, A440, B49, D58A, D50, E7, F4A, and F5) were mucoid. Cells of the isolates exponentially growing on SG broth were observed to be extremely pleomorphic, rod, pleomorphic-rod, and coccishaped as revealed by phase-contrast microscopy. Two isolates (B49 and F5) were rod-shaped and formed weakly pink colonies, but the cells became cocci shaped forming pinkish red colonies when further incubated. The isolates C35, F23A, and F42A were rod-shaped, but became pleomorphic in old cultures. The isolates A82, A337, B19, C15, and D74 were found to be nonmotile, whereas the remaining isolates were motile. All of the strains were Gram-negative, catalase-, and oxidase-positive and were not able to grow on L-arginine under anaerobic conditions. Other biochemical tests results are tabulated in Table 2.

Antibiotic Susceptibility, Total Lipid Analyses

All isolates were found to be susceptible to bacitracin, rifampicin, and novobiocin, and resistant to ampicillin, norfloxacin, tetracycline, azithromycine, neomycin, chloramphenicol, penicillin G, and vancomycin.

Lipid analyses performed by thin layer chromatography revealed the presence of phytanyl diether moieties in all strains.

Plasmid Contents

Twenty of the isolates did not contain plasmids. Strains A440, F100, F5, and C35 had one plasmid positioned at

Table 2. Phenotypic features of halophilic archaeal isolates from Turkey.

Characteristics	Strains								
	A29	A43	A82	A85	A87B	A137	A191	A283	A317
Cell shape	Rod	Flat pleomorphic	Coccus	Rod	Rod	Rod	Rod	Rod	Pleomorphic
Motility	+	+	-	+	+	+	+	+	+
Pigmentation	Red	Orange-red	Pink	Red	Red	Pink	Red	Orange-red	Pink
Oxidase activity	+	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	-	-	-	-	-	+	+
Tween 80 hydrolysis	-	-	-	+	-	-	-	+	+
Starch hydrolysis	-	+	-	-	+[a]	-	-	+	+
Casein hydrolysis	-	-	-	-	-	-	-	-	+
Indole production	-	-	-	+	-	+	-	+	+
H_2S production	+	+	-	-	+	-	-	+	+
Nitrite from nitrate	+	+	+	+	+	+	+	+	+
Gas from nitrate reduction	-	-	-	-	-	-	-	-	+
Acid production from									
Glucose	+	+	+	+	+	+	+	+	+
Fructose	-	-	-	-	-	-	+	+	+
Arabinose	-	+	-	+	-	-	+	+	-
Galactose	+	+	-	-	+	-	+	-	-
Sucrose	-	-	+	-	-	+	+	+	+
Xylose	-	+	-	+	+	+	+	+	-
Maltose	-	-	-	-	+	+	-	-	+

Table 2. Continued.

Characteristics	Strains							
	A337	A440	B19	B36	B44A	B49	B77A	C15
Cell shape	Pleomorphic	Pleomorphic	Coccus	Rod	Pleomorphic	Rod	Rod	Coccus
Motility	-	+	-	+	+	+	+	-
Pigmentation	Red	pink	Pink	Red	Orange-red	Pink	Pink-red	Red
Oxidase activity	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+
Gelatin hydrolysis	-	+	-	-	+	-	+	-
Tween 80 hydrolysis	-	+	-	+	+	+	+	-
Starch hydrolysis	-	+	-	+[a]	+	+	-	+[a]
Casein hydrolysis	-	+	-	-	-	-	+	-
Indole production	-	+	+	-	+	-	+	-
H_2S production	+	+	+	+	+	+	+	-
Nitrite from nitrate	+	+	+	+	+	+	+	-
Gas from nitrate reduction	-	-	-	-	-	-	-	-
Acid production from								
Glucose	+	+	-	+	+	+	-	+
Fructose	+	+	-	-	+	+	-	-
Arabinose	+	-	-	-	+	+	-	+
Galactose	-	-	-	+	-	-	-	-
Sucrose	+	+	-	-	+	+	-	+
Xylose	+	-	-	+	+	+	-	+
Maltose	-	+	-	+	-	-	-	+

Characteristics	Strains							
	C35	D1A	D50	D58A	D74	D107	D113	E7
Cell shape	Rod	Rod	Rod	Rod	Pleomorphic	Pleomorphic	Rod	Rod
Motility	+	+	+	+	-	+	+	+
Pigmentation	Red	Red	Pink	Pink	Pink	Red	Pink-red	Pale-pink
Oxidase activity	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+
Gelatin hydrolysis	-	+	+	+	-	-	+	+
Tween 80 hydrolysis	-	-	+	+	-	-	-	+
Starch hydrolysis	-	+[a]	-	-	-	-	-	-
Casein hydrolysis	-	-	+	+	-	-	+	+
Indole production	-	-	-	-	-	+	+	-
H_2S production	+	+	+	+	+	+	+	+
Nitrit from nitrat	+	+	-	-	+	+	+	+
Gas from nitrat reduction	-	-	-	-	-	-	-	-
Acid production from			-					
Glucose	+	+	-	-	-	+	-	-
Fructose	+	-	-	-	-	+	-	-
Arabinose	-	-	-	-	-	-	-	-
Galactose	-	+	-	-	-	-	-	-
Sucrose	+	-	-	-	-	+	-	-
Xylose	+	+	-	-	-	-	-	-
Maltose	+	+	-	-	-	-	-	-

2.2, 5.6, 9.7, and 34.9 kb, respectively; A43, B77A, D113, and F23 had two plasmids positioned at 30, 32.9; 24.8, 30.4; 20.4, 25.8; and 2, 3 kb, respectively; A82 and F42 had three plasmids positioned at 8.8, 12.1, 13.1, and 3, 3.3, 6.1 kb, respectively; A87B, A191, and E7 had four plasmids positioned at 20.3, 26.4, 34.4, 36.9; 1, 1.2, 1.8, 29.4; and 4.9, 5.6, 6.2, 11.4 kb, respectively (Fig. 2).

16S rRNA Analysis

The fragments ranging between 1,371 bp (94%) and 1,400 bp (96%) of the 16S rDNA sequences of the isolates were determined. The close relatives and phylogenetic affiliation of the sequences of the isolates were checked via BLAST search. A phylogenetic tree constructed by the neighbor-joining method is presented in Fig. 3. All

Table 2. Continued.

Characteristics	Strains							
	E57B	E92B	F4A	F5	F23A	F30A	F42A	F100
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Pleomorphic-rod	Rod
Motility	+	+	+	+	+	+	+	+
Pigmentation	Red	Pink	Pale-pink	Pink	Red	Pink-red	Red	Red
Oxidase activity	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	-	-	-	-	+	+	-
Tween 80 hydro ysis	-	+	+	+	-	+	-	+
Starch hydrolysis	-	-	-	+	-	-	-	+[a]
Casein hydrolys s	+	+	-	-	-	+	-	-
Indole production	-	+	+	-	-	-	-	-
H_2S production	+	+	-	-	+	+	+	-
Nitrits from nitrate	+	+	+	+	+	+	+	+
Gas from nitrate reduction	-	-	-	-	-	-	-	-
Acid production from								
Glucose	-	+	+	+	+	-	-	+
Fructose	-	-	-	-	+	-	-	-
Arabinose	+	+	+	+	+	-	-	-
Galactose	-	-	-	-	-	-	-	-
Sucrose	-	-	+	-	+	-	+	-
Xylose	-	-	+	-	+	-	-	+
Maltose	-	-	-	-	-	-	+	-

[a]Weak reaction.

of the isolates showed very high similarities, which ranged from 94.8 to 100%. Among 33 isolates, 8, 5, 4, 4, 4, 3, 3, 1, and 1 isolates were clustered within the genus *Halorubrum*, *Natrinema*, *Haloarcula*, *Natronococcus*, *Natrialba*, *Haloferax*, *Haloterrigena*, *Halalkalicoccus*, and *Halomicrobium*.

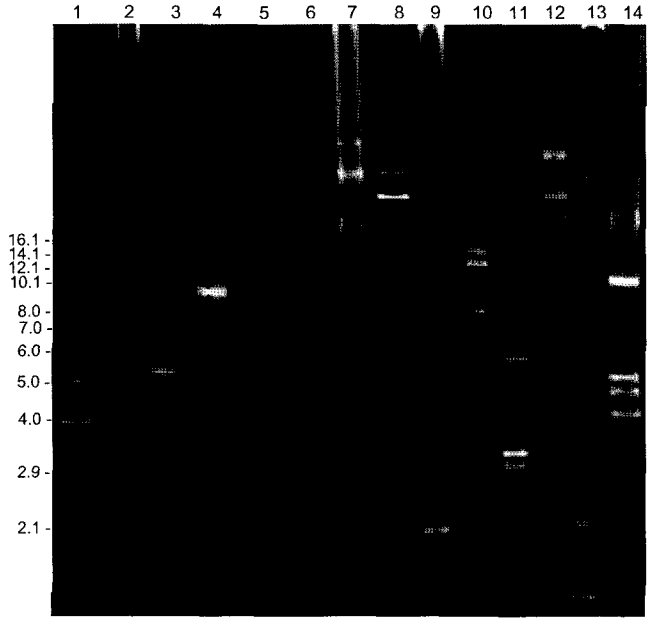

Fig. 2. The plasmid profiles (patterns) of halophilic archaeal isolates. 1, Marker (kb); 2, A440; 3, F100; 4, F5; 5, C35; 6, A43; 7, B77A; 8, D113; 9, F23A; 10, A82; 11, F42A; 12, A87B; 13, A191; 14, E7.

DISCUSSION

In this study, all of the 33 isolates examined were found to be members of the archaeal domain, the family *Halobacteriaceae*. Morphological characteristics of the isolates, such as different shades of red as colony color, a variety of morphological types from rods, cocci, to extremely pleomorphic, and growth at 25% NaCl concentration were all typical of members of the family *Halobacteriaceae* [9]. Furthermore, all isolates were found to have ether-bound membrane lipids and were resistance to antibiotics that target the bacterial peptidoglycan [4]. These results provided further evidence that our isolates are members of *Halobacteriaceae*. It was previously reported that the *Halobacteriaceae* are generally sensitive to anisomycin, bacitracin, rifampicin, and novobiocin [9]. In this study, our isolates were also found to be susceptible to these antibiotics. Characteristics such as the ether-lipid content and the antibiotic sensitivity data are accepted as the most important criteria among the proposed minimal standards for the description of new isolates in the order *Halobacteriales* [18].

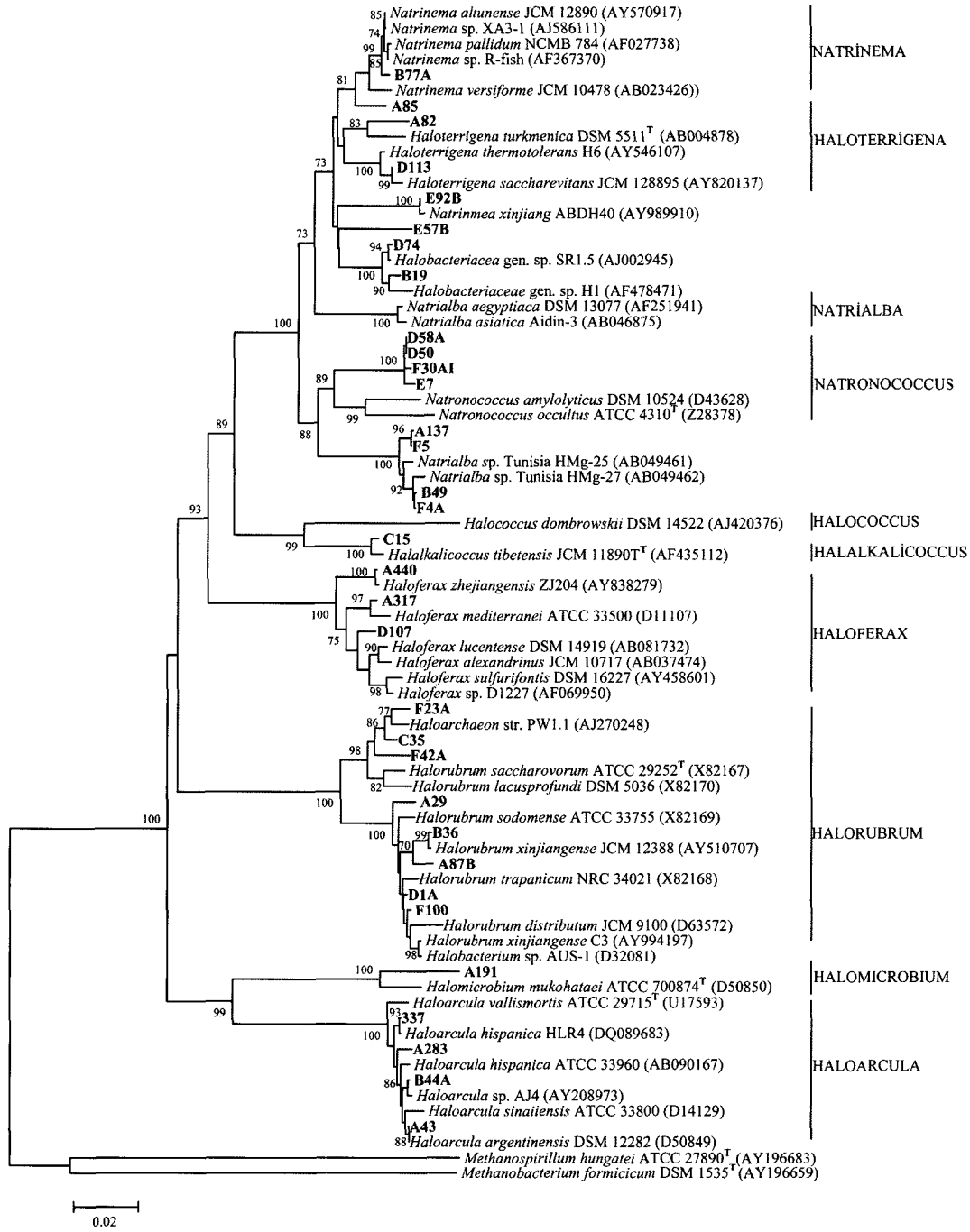

Fig. 3. Phylogenetic tree of 33 archaeal isolates from Salt Lake-Ankara (**A**), Aci Lake (**B**), Salda Lake (**C**), Seyfe Lake (**D**), Tuzla Lake (**E**), Bolluk Lake (**F**), and other related members of the *Halobacteriaceae* based on their 16S rRNA gene sequences (GenBank Accession numbers are shown in parantheses).

The tree was constructed by using the neighbor-joining method. Bootstrap values are shown at the nodes and less than 70% were omitted in this figure. The bar represents 0.02 inferred substitution per nucleotide position. 16S rDNA sequences of two methanogenic archaeal strains 16S rRNA sequences were chosen arbitrarily as the outgroup.

Halophilic archaea that contained plasmids and megaplasmids were reported previously [9, 10]. Although their presence cannot be considered as a taxonomic purpose for the description of halobacterial strains, the determination of the plasmid content has been recommended by Oren *et al.* [18]. In this work, the plasmid numbers and sizes showed differences among the isolates, and megaplasmids were not detected.

A phylogenetic tree was constructed as based on comparison of the 16S rDNA sequences of the isolates and

the reference strains in order to understand the phylogenetic position of our strains (Fig. 3). According to the comparison of the 16S rDNA sequences, our strains were clustered within nine different genera including *Natrinema*, *Haloterrigena*, *Natronococcus*, *Natrialba*, *Halalkalicoccus*, *Haloferax*, *Halorubrum*, *Halomicrobium*, and *Haloarcula*.

16S rDNA sequences of the B49, F4A, A137, and F5 strains were found to have at least 99% similarity in terms of their 16S rDNA sequences to an unidentified strain, *Natrialba* sp. Tunisia HMg-27. All of these strains displayed mucoidal colony morphology, were rod-shaped and motile, and resembled each other in their biochemical properties (Table 1). 16S rDNA sequences of the strains B77A and E92B were more than 99% similar to that of *Natrinema altunense* [27] and *Natrinema xinjiang*, respectively. Similarity of the strain A85 to *Natrinema versiforme* JCM 10478 was 98.5%, but it differed from *N. versiforme* in regard to the lack of acid production from fructose and gas from nitrate and its capability of producing acid from xylose [25]. The evolutionary distance of these 3 isolates confirmed their affiliation to the members of the genus *Natrinema*. Although a low similarity (about 96%) was observed between the strains B19 and D74 and the genus *Natrinema*, the similarity was high when they were compared with the strain *Halobacteriaceae* gen. sp. SR1.5.

The sequence and phenotypic similarity were high among the strains D58A, D50, F30AI, and E7. In the phylogenetic tree, these four strains were found to have a sequence similarity of 95% to *Natronococcus amylolyticus* DSM 10542 [14]; hence, they may represent new species. Strain A82 showed phenotypic characteristics similar to *Haloterrigena turkmenica* DSM 5511 [24], except for its ability to produce acid from fructose. The similarity between A82 and *H. turkmenica* was found to be 97.6%. A high similarity of at least 99.5% between D113 and both *H. thermotolerans* [17] and *H. saccharevitans* [26] was found. On the other hand, a low similarity rate was determined between the strain E57B and the genus *Haloterrigena*.

Strain C15 showed high similarity (99.4%) with the recently identified strain *Halakalicoccus tibetensis* JCM 11890T [28].

The strains A440, A317, and D107 closely matched to the members of the *Haloferax* genus. The phenotypic characteristics of the strain A317 considerably resembled those of *Haloferax mediterranei* ATCC 33500 with a 16S rDNA similarity of 99.2% [9, 23] (Table 1). The strains A440 and D107 showed the similarities of 99.8 and 98.8%, to *Haloferax zhejiangensis* and *Haloferax* sp. D1227, respectively.

Eight strains, namely, F23A, F42A, C35, A87B, F100, A29, B36, and D1A, had 97.6% to 99.8% similarity to each other and were clustered with the members of the *Halorubrum* genus. Furthermore, the strains F23A, C35 and F42A resembled *Halorubrum lacusprofundi* in their phenotypic properties [16]. The results of the phylogenetic tree indicated the closely related strains F100, A29, and D1A had over 99% similarty to *Halorubrum xinjiangense* C3. Two related strains, A87B and B36, were found to be similar by 98.9 to 99.8% to *Halorubrum xinjiangense* JCM 12388, respectively, and these values agreed with their phenotypic similarities [8] (Table 1).

The similarity between strain A191 and *Halomicrobium mukohatei* ATCC 70087 was 96.3%. The strain A191 differed from the latter by producing acid from fructose, lacking the ability to release gas from nitrate and to hydrolyze starch [20].

As for the strains A337, A283, A43, and B44A, on the basis of phenotypic features (Table 1) and their phylogenetic relationship (Fig. 3), it is clear that all of them belong to the genus *Haloarcula*. The closest relative of strains A337, A283, A43, and B44A was found to be *H. hispanica* HLR4, *H. argentinensis*, and *Haloarcula* sp. AJ4, with sequence similarities of 99.9, 99.4, 100, and 99.8%, respectively. However, A43 differs from *H. argentinensis* by its ability to produce acid from some sugars [11]. Except for lack of gas production from nitrate, the strain A283 shares the other phenotypic properties with *H. hispanica* ATCC 33960 [12].

In conclusion, the present study constitutes the most detailed taxonomic study on the halophilic archaea of Turkey origin conducted so far. The data presented herein are expected to contribute to archaeal taxonomy by pointing to the existence of new species, and more genome and chemotaxonomic work will be needed to further elucidate its position in the order *Halobacteriales*.

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