World J Microbiol Biotechnol (2007) 23:309–316
DOI 10.1007/s11274-006-9223-4

ORIGINAL PAPER

Extremely halophilic Archaea from Tuz Lake, Turkey, and the adjacent Kaldirim and Kayacik salterns

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Received: 22 February 2006 / Accepted: 14 June 2006 / Published online: 28 January 2007

Abstract Tuz Lake is a hypersaline lake located in Central Anatolia, Turkey. The lake and its salterns, Kaldirim and Kayacik, are the major sources of solar salt for industrial applications in Turkey, especially in the food and leather industries. Use of the crude solar salt often results in microbial deterioration of the products. We therefore initiated a thorough characterization of the microbial communities in Tuz Lake and its adjacent salterns, and we present here the results of investigations on diversity of extremely halophilic Archaea. Twenty-seven colonies of aerobic red or pink Archaea (family *Halobacteriaceae*) were selected according to colony shape, size, consistency and pigmentation, and characterized according to their phenotypic characteristics, polar lipid contents, and antibiotic sensitivities. Furthermore, 16S rRNA genes of the isolates were screened by DGGE analysis and partially sequenced. Phylogenetic analysis showed that most isolates belonged to the genera *Haloarcula*, *Halorubrum* and *Halobacterium*. *Haloarcula* was found to be dominant both in Tuz Lake and in the saltern samples. *Halorubrum* species were isolated from Tuz Lake and from the Kaldirim saltern, and *Halobacterium* species were recovered from Tuz Lake and from the Kayacik saltern. All strains showed various activities of hydrolytic enzymes (proteases, amylases, cellulases, and others), activities which are responsible for the detrimental effects of the crude salt in food and leather products.

Keywords Tuz Lake · Kaldirim saltern · Kayacik saltern · Halophilic Archaea

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Introduction

Tuz Lake is the largest salt lake in Central Turkey. The inland hypersaline lake occupies a depression in the dry central plateau of Turkey, 105 km northeast of the city of Konya. The lake is shallow (1–2 m) and for most of the year has an area of about 1500 km^2. The salt concentration of its waters reaches values up to 33%. The Lake and the salterns constructed at its shores provide a major source of solar salt: more than 200 million tons of salt is produced from the lake, which amounts to 73% of the salt consumption of Turkey (Birbir and Sesal 2003).

Salt lakes and the crude solar salt produced from them contain large numbers of prokaryotes, mainly

extremely halophilic Archaea of the family *Halobacteriaceae*. Numbers of 10^7–10^8 c.f.u. of halophilic Archaea per ml brine are not uncommon, and unprocessed solar salt often contains 10^5–10^6 c.f.u. per gram. Members of the *Halobacteriaceae* are the dominant microorganisms in hypersaline environments worldwide, including salt lakes, crystallizer ponds of solar salterns, salt mines, as well as hypersaline soda lakes (Grant et al. 1998; Oren 2000). They generally require at least 1.5–2 M NaCl for growth, and most species thrive optimally between 2 and 4 M NaCl. When present in large numbers, their presence is conspicuous because of their red-pink to orange pigmentation, due to 50-carbon carotenoids (α-bacterioruberin and derivatives) which have a photoprotective role, sometimes accompanied by the purple bacteriorhodopsin, which serves as a light-driven proton pump (Oren 2000).

The salts extracted from Tuz Lake and its salterns are commonly used in the Turkish leather industry in the brine curing of hides. Extensive growth of halophilic Archaea on the hides during the curing process often causes deterioration of the product as a result of the activity of hydrolytic enzymes, notably proteases, excreted by these organisms (Bailey and Birbir 1993; Birbir and Ilgaz 1996; Bailey and Birbir 1996; Birbir et al. 1996; Birbir 2004). The detrimental activity of such halophilic Archaea in the production of salted meat and fish products is also well known (Birbir et al. 2004; Grant et al. 1998). Cellulase activity of haloarchaeal strains is of special interest. The ability to digest cellulose is widely distributed among many genera in the domain *Bacteria* and in fungal groups within the domain *Eucarya.* However, only a few archaeal cellulases have been reported thus far (Bauer et al. 1999; Limauro et al. 2001). We earlier reported the occurrence of cellulase activity in representatives of the genera *Halobacterium*, *Haloarcula*, *Halorubrum* and *Natrinema* (Birbir et al. 2004). Cellulases have a huge economic potential in the conversion of plant biomass into fuel and chemicals, and also find applications in the food and detergent industries.

We earlier presented data on the halophilic archaeal community of the Ayvalik saltern, located in the northeastern part of Turkey (Elevi et al. 2004). However, the microbiology of Tuz Lake and its salterns has remained unexplored. In view of the economic interest of the salt extracted from the lake and its microbiological properties, especially for the leather and food industries, we have initiated a study of the microbiology of these brines. Here we present data on the diversity of the halophilic Archaea detected in samples from Tuz Lake and its Kaldirim and Kayacik salterns and on the hydrolytic enzymatic activities displayed by them.

Materials and methods

Brine and salt samples

Salt samples were collected in September 1999 from six different locations in Tuz Lake, three locations in the Kaldirim saltern and two locations in the Kayacik saltern. In addition we collected three brine samples from Tuz Lake and one from the Kaldirim saltern. Chemical parameters of the samples (pH, moisture content, total organic material, Na^+, K^+, Ca^{2+}, Mg^{2+}, HCO_3^- and Cl^-) were determined as described earlier (Birbir and Sesal 2003; Elevi et al. 2004).

Isolation and phenotypic characterization of halophilic microorganisms

We used a membrane filter technique to determine the numbers of extremely halophilic microorganisms in the samples (Birbir et al. 2002). Membranes were incubated on agar plates containing medium (per liter): yeast extract, 5 g; tri-Na-citrate, 3 g; $MgSO_4{\cdot}7H_2O$, 20 g; KCl, 2 g; NaCl, 250 g, and agar, 20 g. After six weeks of incubation at 40°C colonies were enumerated, and selected colonies differing in shape, size and pigmentation were restreaked several times to obtain pure cultures. The colonies were characterized as described by Oren et al. (1997). Cell morphology and motility were examined microscopically in exponentially growing liquid cultures. Salt requirement and tolerance were examined on plates of the above-described medium in which the NaCl concentration was varied (0, 3, 5, 6, 7, 8, 10, 15 and 25%), and the pH tolerance was tested in 25% NaCl medium adjusted to pH values of 4.5, 6.0, 7.0 and 7.5. Gram staining was performed using acetic acid-fixed samples. Antibiotic sensitivity was examined by spreading bacterial suspensions on plates containing the standard growth medium and applying antibiotic discs (amikacin, 30 μg; ampicillin, 10 μg; bacitracin, 10 U; cefadroxil, 30 μg; chloramphenicol, 30 μg; ciprofloxacin, 5 μg; erythromycin, 15 μg; neomycin, 30 μg; novobiocin, 5 μg; penicillin G, 10 U; spiramycine, 100 μg; streptomycin, 25 μg, and sulfamethoxazole-trimethoprim, 25 μg). The results were recorded as sensitive or resistant after 14 days of incubation at 40°C (Birbir et al. 2004).

Polar lipid analyses

Cell pellets from 50 ml culture were suspended in 1 ml 4 M NaCl and extracted with 3.75 ml methanol-chloroform (2:1, v/v) for 4 h. The extracts were centrifuged and pellets were extracted with 4.75 ml

methanol–chloroform–water (2:1:0.8, v/v). Then 2.5 ml of chloroform and 2.5 ml of water were added to the combined supernatants and the tubes were centrifuged to achieve phase separation. The chloroform phase was collected and dried in a vacuum desiccator. Lipids were dissolved in a small volume of chloroform, applied to silica gel plates (20 × 20 cm, Aldrich) and separated by single development with chloroform-methanol-acetic acid-water (85:22.5:10:4, v/v). Lipid spots were detected by spraying with orcinol-ferric chloride reagent (Sigma) and heating of the plates at 150°C, enabling differential detection of glycolipids, and with molybdenum blue reagent (Sigma) for detection of phospholipids (Oren et al. 1996; Oren and Litchfield 1999).

Biochemical tests

All test media contained 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, and 2 g KCl per liter. Oxidase, catalase, β-galactosidase, DNase, degradation of gelatin, casein, starch and Tween 80 and indol production were tested using earlier described procedures (Gónzalez et al. 1978; Quesada et al. 1982; Bailey and Birbir 1993). Substrate concentrations used were 2% gelatin, 1% casein, 1% soluble starch, and 0.1% Tween 80, respectively. Cellulase activity was detected on solid medium containing carboxymethylcellulose, 2 g; yeast extract, 1 g; casamino acids, 1 g; $MgSO_4 \cdot 7H_2O$, 20 g; KCl, 2 g; NaCl, 250 g and agar, 20 g per liter. The plates were stained with 0.1% (w/w) Congo red solution for 30 min and washed with 1 M NaCl for 10 min. Cellulase activity was observed as clear zones around the colonies against a red background (Limauro et al. 2001; Birbir et al. 2004). Reduction of nitrate to nitrite was determined in tubes containing 20 ml of liquid medium supplemented with 1% KNO_3. Durham tubes were used to detect gas formation, and cultures were examined periodically for the presence of gas and nitrite accumulation (Quesada et al. 1982; Birbir et al. 2004). Production of acid from D-glucose, maltose, sucrose and lactose was tested in medium without sodium citrate, supplemented with 0.001% phenol red. The test sugars, sterilized by filtration, were added to a final concentration of 1% (Arahal et al. 1996). Appropriate positive and negative controls were used in all tests.

Amplification and sequencing of 16S rRNA genes

Cells were disrupted in a bead beater, and the released DNA was isolated and purified with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v) as described (Calli et al. 2003). For DGGE analysis, fragments (approx. 480 bp) of the 16S rRNA genes were amplified using the primer set A-109 (F) (5′-AC(G/T)GCTCAGTAACACGT-3′) (Groskopf et al. 1998) and GC-515 (R) (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGCACGGGGGGATGTATTACCGCGGCTGCTGGCAC-3′) (Lane 1991). We used a Progene thermocycler (Techne) with programs given by Calli et al. (2003). PCR products were viewed on ethidium bromide-stained agarose gels. DGGE analyses were performed with the BioRad D-Code System according to Nübel et al. (1996), using an 8% polyacrylamide gel and a denaturing gradient from 30 to 50%. After a 5 min electrophoresis at 200 V, the gels were run for 16 h at 85 V at 60°C, and were then silver-stained (Sanguinetti et al. 1994). Strains showing different DGGE banding patterns were selected for sequencing. For the amplification of a longer portion of the 16S rRNA gene, primers A-109 (F) as above and 1510 (R) (5′-GGTTACCTTGTTACGACTT-3′) (Ficker et al. 1999) were used. Sequencing analysis was carried out by the SeqLab Sequence Laboratories, Göttingen, Germany. The derived partial (approx. 800 bp) 16S rRNA gene sequences were compared with sequences in the GenBank database using the BLAST search program (Altschul et al. 1990) and aligned by using the multiple alignment Clustal W program (Thompson et al. 1994). A neighbor-joining phylogenetic tree (Saitou and Nei 1987; Page 1996) was constructed with the Molecular Evolutionary Genetics Analysis package (MEGA version 2.1) (Kumar et al. 2001) with the Jukes–Cantor algorithm.

Nucleotide sequence accession numbers

16S rRNA gene sequence data of the isolates 3KYS1, 3TL6, 2KYS1, 5TL6, 3TL4, 2TL9 reported in this article have been deposited in the NCBI, NLM, NIH and GenBank nucleotide sequence databases under the accession numbers DQ352855, DQ352856, DQ352857, DQ352858, DQ352859 and DQ352860, respectively.

Results and discussion

Using the nutrient-rich (0.5% yeast extract), high-salt (25% NaCl) medium, we obtained between 7×10^3 and 1.8×10^5 c.f.u. from Tuz Lake brine (3 samples). Salt collected from Tuz Lake yielded 7×10^4–1.1×10^6 c.f.u. per gram (6 samples). We grew 1.5×10^5 c.f.u. per ml from a sample of Kaldirim saltern brine. Salt from the Kaldirim and the Kayacik salterns gave 1.5×10^5 –1.6×10^7 and 1.1–3.5×10^5 c.f.u. per gram (3 samples

Table 1 Characteristics differentiating the 27 strains isolated from Tuz Lake and the Kaldirim and Kayacik salterns

Characteristics	Percent positive strains		
	Tuz Lake	Kaldirim saltern	Kayacik saltern
Rod-shaped	50	33	25
Pleomorphic	50	67	75
Growth between 10–25% NaCl	36	100	75
Growth between 15–25% NaCl	57	0	0
No growth below 25% NaCl	7	0	25
Gelatin hydrolysis	64	89	100
Casein hydrolysis	14	22	0
Starch hydrolysis	64	100	50
β-Galactosidase activity	7	33	0
DNase activity	50	67	50
Cellulase activity	57	100	100
Tween 80 hydrolysis	100	56	50
Indol production	36	44	100
Reduction of nitrate to nitrite	100	100	25
Formation of gas from nitrate	100	100	25
Acid production from glucose	50	78	75
Acid production from maltose	92	78	25
Acid production from sucrose	29	11	75
Acid production from lactose	14	33	25
Sensitivity to amikacin	14	0	0
Sensitivity to cefadroxil	14	0	0
Sensitivity to sulfamethoxozole + trimethoprim	50	33	0

and 1 sample, respectively). Generally over 95% of the colonies were pink-red or orange pigmented; however, one of the Tuz Lake salt samples and the Kayacik saltern brine sample yielded 57 and 31% unpigmented colonies, respectively.

Twenty-seven colonies were selected according to colony shape, size, consistency and pigmentation: 14 from Tuz Lake, 9 from Kaldirim, and 4 from Kayacik. They were isolated in pure culture, and the isolated strains were subjected to a range of phenotypic tests. All isolates grew optimally at 25% NaCl at 40°C and a pH of 7.5. All were motile, all stained Gram-negative, showed positive oxidase and catalase reactions, were sensitive to bacitracin and to novobiocin, and resistant to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, neomycin, penicillin, spiramycin, and streptomycin. These properties, together with the colony pigmentation and the requirement for high salt concentrations, suggest that all are members of the archaeal family *Halobacteriaceae* (Grant et al. 1998; Oren 2000). Dominance of halophilic Archaea in the lake and its salterns was also suggested by a reddish-pink color of their waters. Table 1 presents a number of additional properties tested, for which different reactions were obtained among the strains.

To obtain further information about the taxonomic affiliation of the strains, we characterized their polar lipids. The genera within the family *Halobacteriaceae* differ with respect to the types of polar lipids found in their membranes. While all contain the diether derivatives of phosphatidylglycerol (PG) and the methyl ester of phosphatidylglycerolphosphate (PGP-Me), the presence or absence of phosphatidylglycerolsulfate (PGS) and of different sulfated or non-sulfated glycolipids is characteristic for certain genera only. These differences have been exploited to obtain

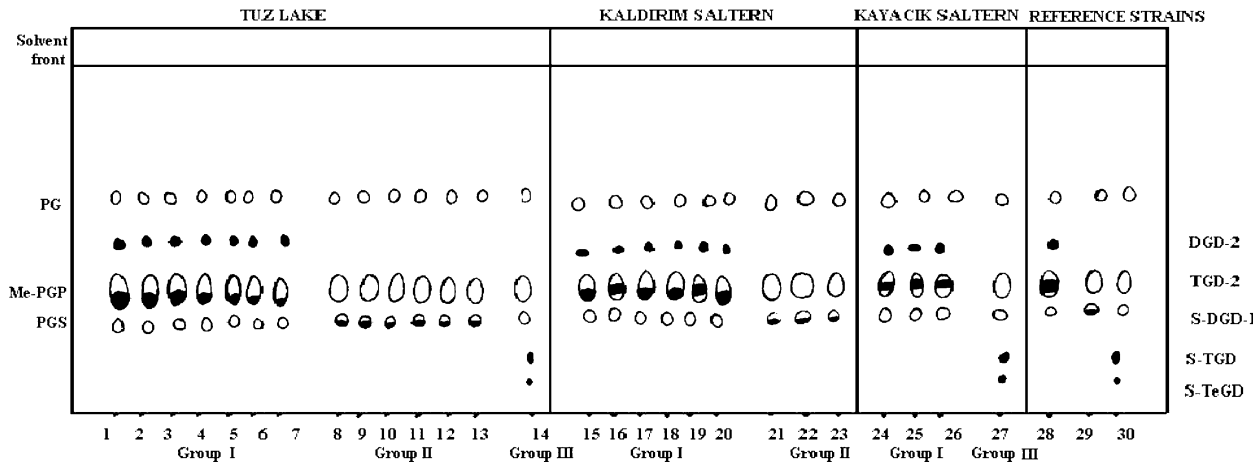

Fig. 1 Thin layer chromatogram of polar lipids extracted from halophilic archaeal isolates from Tuz Lake – lanes 1–7: Group I (strains 1TL3, 2TL4, 3TL4, 1TL5, 2TL6, 3TL6, 4TL6), lanes 8–13: Group II (strains 1TL1, 1TL4, 1TL6, 5TL6, 1TL8, 1TL9), lane 14: Group III (strain 2TL9), the Kaldirim saltern – lanes 15–20: Group I (strains 1KS1, 2KS1, 1KS4, 2KS4, 3KS4, 4KS4), lanes 21–23: Group II (strains 3KS1, 1KS2, 1KS3), and the Kayacik salterns – lanes 24–26: Group I (strains 1KYS1, 2KYS1, 1KYS2), lane 27: Group III (strain 3KYS1), as compared to reference strains (lane 28: *Haloarcula vallismortis*, lane 29: *Halorubrum sodomense*, lane 30: *Halobacterium salinarum*). The plates were stained with molybdenum blue to visualize phospholipids, followed by orcinol ferric chloride spray reagent to visualize glycolipids (marked as black spots). The tentative identification of the lipid spots, as based on literature data (Oren and Gurevich 1993; Oren and Litchfield 1999), is indicated

Table 2 Phenotypic characteristics, biochemical tests and lipid patterns of 6 representative isolates

Characteristics	Group I			Group II	Group III	
	3TL4	3TL6	2KYS1	5TL6	2TL9	3KYS1
Pigmentation	Light pink	Light pink	Brick red	Brick red	Light red	Light blood red
Cell morphology	Pleomorphic cells	Pleomorphic cells	Pleomorphic cells	Pleomorphic rods	Pleomorphic rods	Pleomorphic rods
Cell size; width and length (μm)	2.5 × 5.0	2.5 × 2.5	1.0 × 3.0–5.0	2.5 × 5.0–7.5	2.5 × 5.0–7.5	1.5–2.5 × 5.0–12.5
Growth at 40°C						
0–8% NaCl	–	–	–	–	–	–
10% NaCl	–	–	–	–	–	–
15% NaCl	+	+	+	+	+	–
25% NaCl	+	+	+	+	+	+
Growth at pH 4.5	–	–	–	–	–	–
Growth at pH 7.5	+	+	+	+	+	+
Oxidase activity	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	–	+	+
Casein hydrolysis	+	–	–	–	+	–
Starch hydrolysis	–	+	+	+	+	–
β-galactosidase activity	–	–	–	–	+	–
DNase activity	–	–	–	+	–	+
Cellulase activity	+	+	+	–	+	+
Tween 80 hydrolysis	+	+	–	+	+	+
Indol production	–	+	+	–	–	+
Reduction of nitrate to nitrite	+	+	–	+	+	–
Formation of gas from nitrate	+	+	–	+	+	–
Ammonia production from peptone	+	+	+	+	+	+
Acid production from:						
D-Glucose	–	+	+	–	–	+
Maltose	+	+	–	+	+	+
Sucrose	+	–	+	–	–	+
Lactose	–	–	–	–	–	+
Polar lipids:						
PG, PGP-Me, PGS	+	+	+	+	+	+
DGD-2, TGD-2	+	+	+	–	–	–
S-DGD-1	–	–	–	+	–	–
S-TeGD, S-TGD	–	–	–	–	+	+

information on the archaeal communities in saltern crystallizer brines (Oren et al. 1996), as well as in the characterization of isolates obtained from such brines (Oren and Litchfield 1999; Elevi et al. 2004). We divided the isolates into three groups based on their morphology and lipid analysis. Group I was defined as consisting of pleomorphic, irregular or triangular shaped. Group II consisted of short or long thin rods, in part with curved or swollen cells. Isolates classified in Group III were pleomorphic club-shaped, bent or swollen rods. Strains classified in Group I possessed PG, PGP-Me, PGS, and two glycolipids: DGD-2 and TGD-2 (Fig. 1), a pattern characteristic of the genus *Haloarcula*. The polar lipid compositions of strains in Group II were PG, PGP-Me, PGS and S-DGD-1, a pattern resembling that of the genus *Halorubrum*. Finally, Group III strains, recovered from Tuz Lake and Kayacik salterns possessed PG, PGP-Me, PGS, S-TeGD and S-TGD, a pattern known from the genus *Halobacterium* (Grant et al. 1998; Oren 2000). Isolates belonging to Group I were recovered from Tuz Lake salt (4 salt samples), Kaldirim saltern salt and brine samples (1 salt and 1 brine samples), and Kayacik saltern salt (2 samples), isolates belong to Group II were isolated from Tuz Lake salt and brine samples (3 salt and 2 brine samples), and Kaldirim saltern salt (3 samples). Strains of Group III were recovered from Tuz Lake brine (1 sample) and Kayacik saltern salt (1 sample).

All isolates yielded PCR products of amplified 16S rRNA-gene-derived sequences with Archaea-specific primers. To compare phylogenetically similar isolates, the V6–V8 region of their 16S rRNA genes were amplified and analyzed using DGGE. Isolates having similar DGGE banding patterns were classified into three sub-groups, and six representative isolates, belonging to these sub-groups (Table 2), were selected for 16S rRNA gene sequencing. These six isolates were grouped within the genera *Haloarcula* (Group I), *Halorubrum* (Group II), and *Halobacterium* (Group

III), with high similarities to known species within these genera (>96%) (Fig. 2). The phylogenetic identification results of these six isolates were found to be consistent with the tentative identification based on polar lipid contents (Fig. 1; Table 2).

The genus *Halobacterium* (corresponding to group III) is not known to dominate the archaeal communities of salt lakes (Oren 2000; Oren and Litchfield 1999; Rodriguez-Valera et al. 1985). We recovered two strains from Tuz Lake and the Kayacik saltern that can be classified within the genus *Halobacterium* on the basis of their phenotypic characteristics, lipid composition and 16S rRNA gene sequence. The very high concentrations of salt and organic nutrients used in the isolation plates may have been a selective factor favoring recovery of *Halobacterium* colonies.

The diversity of halophilic Archaea described above is undoubtedly only a small fraction of the true diversity of *Halobacteriaceae* in Tuz Lake and its salterns. Red brines are expected to contain 10^7–10^8 archaeal cells per ml, and the number of colonies recovered in our studies was much lower. Moreover, the high salt, high nutrient medium used for colony enumeration and isolation is highly selective. Far better recovery and coverage of all taxa present can be achieved using low nutrient media and prolonged incubation times, as shown for example in the elegant study by Burns et al. (2004) of the haloarchaeal community in Australian saltern ponds.

All isolates obtained were able to degrade a wide variety of macromolecules, as shown by their high protease, lipase, DNAse and amylase activities. Such hydrolytic activities are well known in many representatives of the *Halobacteriaceae* (Grant et al. 1998), and have been described from isolates obtained from other Turkish salt sources as well (Birbir et al. 2004; Elevi et al. 2004). Cellulase activity has earlier been reported in halophilic archaeal strains recovered from the Tuzköy salt mine in Turkey (Birbir et al. 2004). Similarly, we found that 57% of the Lake isolates and all of the Kaldirim and Kayacik salterns isolates showed cellulase activity. Cellulase activity may thus be a common property of haloarchaeal strains. A study of the salt relationships of the cellulase activities of haloarchaeal isolates from the Tuzköy salt mine, grown in liquid media containing 10%, 15% and 25% NaCl, showed that cellulase activity was optimal in 2.5 M NaCl, 25°C, and pH 7 (Gozuacık et al. 2004). Whether such cellulase-positive halophilic Archaea are indeed capable of growth on cellulose as carbon and energy source remains to be ascertained.

Activities of hydrolytic enzymes of halophilic Archaea, especially proteases, are well known to be detrimental in the preservation of salted food and hides (Kallenberger 1984; Bailey and Birbir 1993; Bailey and Birbir 1996; Birbir and Ilgaz 1996). Salts from Tuz Lake and the Kaldirim and Kayacik salterns are directly used in preservation of hides and food

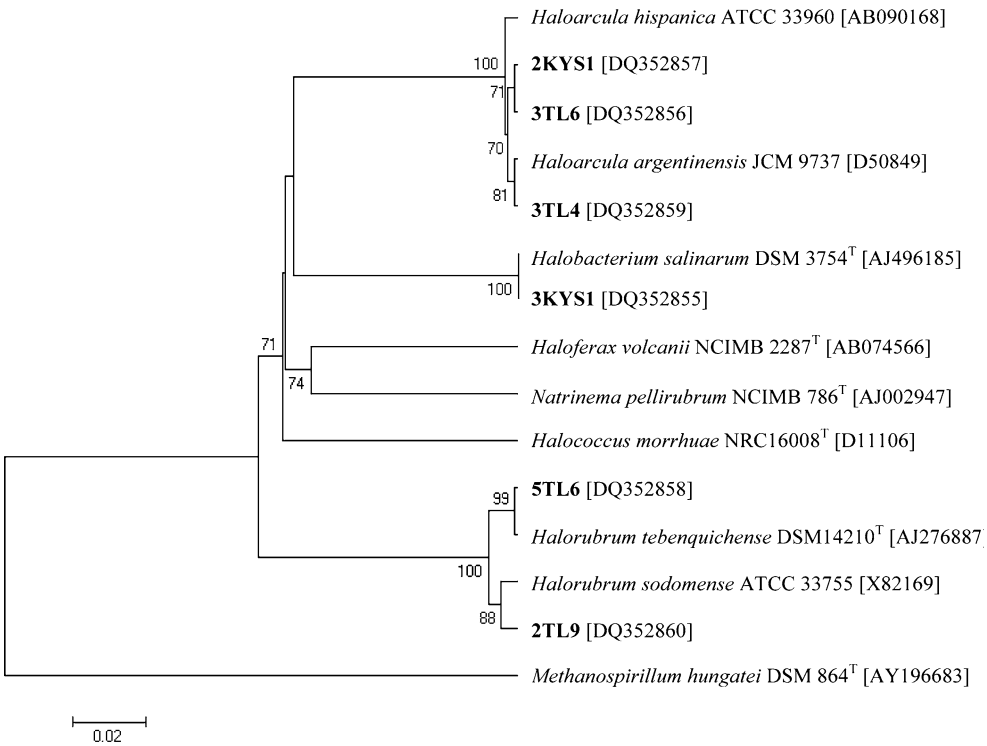

Fig. 2 Phylogenetic tree showing the relationship of six selected isolates with other species of the family *Halobacteriaceae*. Evolutionary distance was calculated using the method of Jukes and Cantor (1969), and the topology was inferred using the neighbor-joining method (Saitou and Nei 1987), based on bootstrap analysis of 1000 trees. The scale bar represents 0.02 inferred substitutions per nucleotide position

such as fish, vegetables, cheese, green and black olives, grape leaves and tomato paste. The finding that hydrolytic enzymes are widespread in the Archaea present in the brines and in the crude solar salt produced from them explains why the use of such salts often causes serious deterioration of salted products and results in significant economic losses. Therefore it is recommended that salt from Tuz Lake and its salterns should be kilned or treated with effective bactericides before use in the food and hide industries.

Acknowledgements This work was partially supported by the Scientific Research Project Commission of Marmara University, Project No: 2005 FEN BSE-072/171001. We thank the Scientific Research Project Commission and the Center for Wild Plants and Water Products of Marmara University for supporting this research. Assistant Prof. Yasar Birbir, Assistant Prof. Musa. Sahin Ugur and Mr. Yemilhan Atilgan are acknowledged for supply of the samples. We express our appreciation to Mr. Hüseyin Çimen, Mrs. Didem Berber, Miss Selçen Durmaz and Miss Hüsniye Hacioglu for their assistance. We thank also Sigma Co. (St Louis, USA) for donating the lipid detection spray reagents.

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