Polish Journal of Microbiology
2012, Vol. 61, No 2, 111–117

ORGINAL PAPER

Isolation, Characterization and Phylogenetic Analysis of Halophilic Archaea from a Salt Mine in Central Anatolia (Turkey)

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Received 22 October 2011, revised 2 May 2012, accepted 5 May 2012

Abstract

The haloarchaeal diversity of a salt mine, a natural cave in central Anatolia, was investigated using convential microbiological and molecular biology methods. Eight halophilic archaeal isolates selected based on their colony morphology and whole cell protein profiles were taxonomically classified on the basis of their morphological, physiological, biochemical properties, polar lipid and protein profiles and 16S rDNA sequences. From the 16S rDNA sequences comparisons it was established that the isolates CH2, CH3 and CHC resembled *Halorubrum saccharovorum* by 98.8%, 98.9% and 99.5%, respectively. There was a 99.7% similarity between the isolate CH11 and *Halobacterium noricense* and 99.2% between the isolate CHA1 and *Haloarcula argentinensis*. The isolate CH8K and CH8B revealed a similarity rate of 99.8% and 99.3% to *Halococcus dombrowskii*, respectively. It was concluded that the isolates named CH2, CH3 and CHC were clustered in the genus *Halorubrum* and that CHA1 and CH7 in the genus *Haloarcula*, CH8K and CH8B in the genus *Halococcus* and CH11 in the genus *Halobacterium*.

Key words: Halophilic archaea, Phylogeny, Taxonomy, Salt mine, Turkey

Introduction

The increasing interest, in recent years, in microorganisms from hypersaline environments has resulted in the discovery of several new species and genera belonging to the Bacteria and Archaea domains. Halobacteria are a group of microorganisms forming a part of the domain Archaea that require high salt concentration for growth (Kamekura, 1998). The extremely halophilic archaea belong to the order Halobacteriales, which contains one family, the *Halobacteriaceae* (Ozcan *et al*., 2007). The family *Halobacteriaceae* in the domain Archaea presently is comprised of 35 genera (Euzeby, 2011).

Members of the *Halobacteriaceae* are dominant microorganisms in hypersaline environments worldwide including salt lakes, crystallizer ponds of solar salterns, salt mines, as well as hypersaline soda lakes (Oren, 2000). The haloarchaea are well adapted to hypersaline environments and require at least 1.5 M NaCl for growth (Castillo *et al.*, 2007).

Hypersaline environments are commonly present in Turkey. Several studies have been carried out to isolate and characterize halophilic archaeal strains from various saline parts of Turkey (Elevi *et al*., 2004; Ozcan *et al*. 2006; 2009; Birbir *et al*., 2007). Phylogenetic studies revealed that the halophilic archaeal isolates from Turkey clustered closely to genera *Halorubrum*, *Haloarcula*, *Natrinema*, *Halobacterium* and *Natronococcus* (Ozcan *et al*., 2007; Birbir *et al*., 2007). However, there has been little effort to identify these isolates. Current taxonomic classification of the *Halobacteriaceae* is mainly based on DNA-DNA hybridization, 16S rRNA gene sequence comparison, polar lipid composition and phenotypic characteristics (Oren *et al*., 1997; Castillo *et al*., 2006).

The main purpose of the current research was to determine the halophilic archaeal diversity of a salt mine, formerly a natural cave in central Anatolia, by determining their phenotypic characteristics, polar lipid composition and 16S rRNA gene sequence comparison.

Experimental

Material and Methods

Physico-chemical analysis of the brine samples. Physico-chemical properties of the brine samples taken from the salt mine (Çankaya Salt Mine, Çankırı-Turkey) were determined with Merc Spectroquant test

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kits and ICP-AES instrument. Test kits and Nova 60 spectrophotometer were used to detect the concentration of SO_4^{2-}, Cl^-, Ca^{2+}, Mg^{2+}, HCO^-, CO_3^{-2}, Na^+, K^+ and total hardness while a pH meter with glass electrode (WTW inoLab pH 720brand) was employed for pH measurement.

Isolation of extremely halophilic archaea. In order to isolate halophilic archaea, brine samples were collected in sterile bottles from different points in the salt mine. Each of the samples was inoculated in SG broth containing penicillin G. Aerobic enrichment cultures showing turbidity were streaked out onto solid medium containing penicillin G. Plates were incubated at 37.5°C. After two weeks incubation period, representative colonies were transferred to fresh solid SG medium in order to obtain pure culture. SG medium contained (gl^{-1}): NaCl, 250; $MgSO_4 \cdot 7H_2O$, 20; KCl, 2; sodium citrate, 3; casamino acids, 7.5; yeast extract, 1; and $FeSO_4 \cdot 7H_2O$, 0.0023. The pH was adjusted to 7.35 with 1 M KOH (Ozcan *et al*., 2006).

Phenotypic and cultural properties. Phenotypic tests of isolates were performed according to the proposed minimal standards for the description of new taxa in the order Halobacteriales (Oren *et al*., 1997). Cell motility and morphology were examined by phase-contrast microscopy of exponentially growing liquid cultures. Gram staining was carried out as described by Dussault (1955). Colony morphology was observed on optimal growth agar medium after incubation at 37.5°C for 7 days. Tests for catalase and oxidase activity and hydrolysis of starch, casein, gelatin and of esterase (Tween 20, 40, 60 and 80) were performed as explained before (Ozcan *et al*., 2006). Lipolytic activity was tested on Rhodamine agar plates (Ozcan *et al*., 2009). Nitrate reduction, H_2S formation, indole formation, utilization of sugars (Glucose, sucrose, lactose, fructose, maltose, xylose, mannose and ribose) and anaerobic growth in the presence of L-arginine and TMAO were tested according to Oren *et al*. (1997). Anaerobic growth in the presence of nitrate was tested according to Mancinelli and Hochstein (1986). The salt range for growth was determined in SG broth modified with NaCl at final concentrations of 1, 2, 3, 4, 4.5 and 5 M. The pH range for growth was determined with 50 mM MES (5.0–6.0), HEPES (6.5–7.0), tricine (7.5–8.5), CHES (9.0–9.5) and CAPS (10.0) between pH 5.0 and 10.0. Archaeal growth rates in different pH and salt concentrations were determined at 600 nm with spectrophotometric measurements.

Biochemical tests. Antibiotic susceptibility was tested by spreading cell suspensions on plates of SG medium and applying antibiotic discs (ampicillin, 10 μg; norfloxacin, 10 μg; tetracycline, 30 μg; bacitracin, 10IU; rifampicin, 5 μg; azithromycine, 15 μg; neomycin, 30 μg; chloramphenicol, 30 μg; penicillin G; 10IU; vancomycin, 30 μg; novobiocin, 30 μg; trimethoprim, 5 μg; ttreptomycin, 25 μg; erythromycin 15 μg; sulphamethazol/trimethoprim, 25 μg) (Montalvo-Rodriguez *et al*., 2000). The results were recorded as sensitive or resistant after 14 days of incubation at 37.5°C. Polar lipid analysis was performed as explained before (Oren *et al*., 1996; Oren and Litchfield, 1999). SDS-PAGE of whole-cell proteins was set up as described by Laemmli (1970) and Hesselberg and Vreeland (1995).

Phylogenetic analysis. Genomic DNA was extracted from log-phase cells lysed in purified water by phenol extraction followed by ethanol precipitation according to Dyall-Smith (2001). The gene encoding 16S RNA was amplified by PCR with the forward primer 5'-ATTCCGGTTGATCCTGCCGGAGGTC-3' (positions 1–25 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) (Ozcan *et al*., 2007). PCR was performed for 30 cycles, each of which consisted of denaturation for 1 min at 94°C, annealing for 1 min at 59°C, and polymerization for 1.5 min at 72°C. The 16S rRNA gene sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) and the phylogenetic tree were constructed with MEGA 4.1 using a neighbor joining algorithm and Kimura two-parameter corrections (Tamura *et al*., 2007).

Results and Discussion

Physico-chemical properties of brine samples. The brine samples named CSU1, CSU2A, CSU2B and CSU3 were collected from four different points in the salt mine. Table I gives the chemical parameters of these samples in which halophilic archaea strains were isolated. The results indicated that the mineral content, pH and hardness of brine are suitable for the growth of extremely halophilic archaea. The most remarkable nature of halophilic archaea is that they flourish in conditions with very high content of NaCl and KCl. Several researchers have determined that extreme halophilic archaea need at least 1.5 M NaCl for growth. They also point out that most species show optimum growth at 3.5 to 4.5 M NaCl and pH 7.0 to 7.5 (Kushner, 1985; Arahal *et al*., 1996; Oren and R.-Valera, 2001).

Extremely halophilic archaeal strains and their phenotypic properties. It was pointed out that members of *Halobacteriaceae* family displayed extreme polymorphism, having several morphologic shapes ranging from rods to pleomorphic rods, coccus, pleomorphic, square and triangles (Castillo *et al*., 2007). In the current study 8 halophilic archaeal isolates named CH2, CH3, CHA1, CHC, CH7, CH8K, CH8B and CH11,

Table I
Chemical characteristics of the brine samples studied

	Csu1	Csu2A	Csu2B	Csu3	Pw*
pH	6.61	7.03	7.06	6.72	6.0
Hardness (F)	3.8	3.1	3.8	3.7	<1.2
SO_4^{-2} (mg/L)	7400	7400	7500	7500	8
Cl^- (mg/L)	209000	230000	192000	200000	<10
CO_3^{-2} (meq/L)	–	–	–	–	–
HCO_3^- (meq/L)	1.2	1.6	2.2	1.8	–
Ca^{2+} (ppm)	790	920	970	940	–
Mg^{2+} (ppm)	530	490	510	560	–
K^+ (ppm)	210	190	190	240	–
Na^+ (ppm)	67200	18600	102300	88700	–

* Purified water

were selected on the basis of their different colony morphology and whole cell profiles for further characterization. Table II shows the morphological features of the isolates, results of several biochemical tests, antimicrobial susceptibility patterns, and NaCl and pH concentrations required for optimum growth. When whole cell protein profiles were examined (Fig. 1), seven distinct protein profiles were observed for eight different strains. CH8K and CH8B isolates were ascertained to have the same protein profile but due to their different colony pigmentations they were named differently.

CH2, CH3 and CHC isolates exhibited different protein profile (Fig. 1) and among the phenotypical features obtained, only colony pigmentation and sugar fermentation were different. CH2 and CHC isolate can produce acid from sucrose and mannose while CH3 cannot. CH2 can ferment maltose while CH3 and CHC isolate cannot. One of the notable features is that

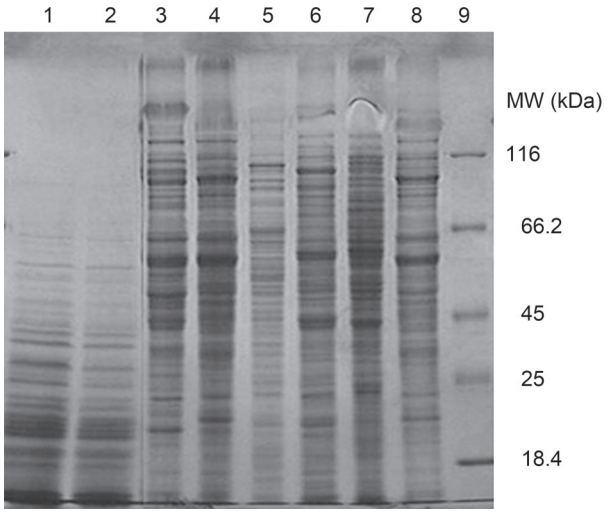

Fig. 1. Protein profiles of the archaeal strains isolated from Cankırı Cankaya Salt mine.
1: CH8K, 2: CH8B, 3: CH3, 4: CHC, 5: CH11, 6: CH7, 7: CHA1, 8: CH2, 9: Markers

CH2, CH3 and CHC isolates are resistant to the antibiotic rifampicin. Several studies point out that rifampicin has an inhibiting effect on most halophilic archaea (Pecher and Böck, 1981; Bonelo *et al*., 1984) whereas another research points out that certain halophilic archaea are resistant to rifampicin (Allen *et al*., 2008). All isolates in this study appeared to be resistant to ampicillin, norfloxacin, tetracycline, azithromycine, neomycin, chloramphenicol, penicillin G, vancomycin, trimethoprim, streptomycin, erythromycin and sulphamethazol/ trimethoprim.

CH8B and CH8K which are coccus-shaped, non motile isolates were determined to be Gram-variable while other isolates were Gram-negative. Three of six motile Gram-negative isolates were of pleomorphic shapes while the other three rod shaped. It was reported that majority of *Halobacteriales* order were of Gram-negative nature while some other members with coccus morphology such as *Natronococcus* and *Halococcus* exhibited Gram-variable feature (Oren *et al*., 1997).

It was observed that colonies of all eight strains are nonmucoid, circular shaped and convex. They have smooth surface appearance and that all strains but CH8B exhibited pink-red colony pigmentation. It was pointed out in several former studies that colonies of many Halobacterial members exhibited various tones pigmentation ranging from red to pink-orange due to high rate of carotenoid pigments in their cell membrane. In addition, they were reported to appear opaque, transparent or translucent, mucoid or non mucoid, with entire edges and convex (Oren and R.-Valera, 2001; Castillo *et al*., 2006).

It was ascertained that all strains but CH11 exhibited catalase activity and that all the isolates had oxidase activity. It was also determined that none of the isolates produced gas from the tested sugars or acid from lactose. It was established that none of the isolates exhibited lipolytic activity (Table II).

Table II
Phenotypic features of the 8 strains studied

Characteristic	CH2	CH3	CHA1	CHC	CH7	CH8K	CH8B	CH11
Colonial morphology								
Colony shape	circular	circular	circular	circular	circular	circular	circular	circular
Mukoid	–	–	–	–	–	–	–	–
Pigmentation	light pink	light red	dark red	light red	red	pink-red	white	red
Colony elevation	convex	convex	convex	convex	convex	convex	convex	convex
Colony density	transparent	translucent	opaque	transparent	opaque	opaque	opaque	transparent
Colony edge	entire	entire	entire	entire	entire	irregular	irregular	entire
Colony size (mm)	0.3–0.75	0.25–0.6	0.2–0.5	0.4–0.7	0.2–0.5	0.1–0.2	0.1–0.2	0.15–0.25
Cell morphology								
Cell shape	rod	rod	pleo.	rod	pleo.[1]	coccus	coccus	pleo.[1]
Gram reaction	Gr(–)	Gr(–)	Gr(–)	Gr(–)	Gr(–)	Gr(+/–)	Gr(+/–)	Gr(–)
Motility	+	+	+	+	+	–	–	+
Cell size (μm)	2–15	2–10	ND	2–10	ND	1–3	1–3	1–3
Acid production from								
Glucose	+	+	–	+	–	–	–	+
Sucrose	+	–	–	+	–	–	–	–
Lactose	–	–	–	–	–	–	–	–
Fructose	–	–	+	–	+	–	–	–
Maltose	+	–	+	–	+	–	–	–
Xylose	+	+	+	+	+	–	–	+
Mannose	+	–	–	+	–	–	–	–
Ribose	–	–	+	–	+	+	+	+
Anaerobic growth with								
L-Arginine	–	–	–	–	–	–	–	–
TMAO	–	–	–	–	–	–	–	–
KNO_3	–	–	–	–	+	–	–	–
Catalase activity	+	+	+	+	+	+	+	–
Oxidase activity	+	+	+	+	+	+	+	+
Gelatin hydrolysis	–	–	+	–	–	+	+	–
Casein hydrolysis	–	–	–	–	–	+	+	–
Starch hydrolysis	–	–	+	–	–	–	–	–
Lipolytic activity	–	–	–	–	–	–	–	–
Tween 20 hydrolysis	–	–	+	–	+	+	+	+
Tween 40 hydrolysis	–	–	+	–	+	–	–	+
Tween 60 hydrolysis	–	–	+	–	–	–	–	–
Tween 80 hydrolysis	–	–	+	–	–	–	–	–
H_2S production	+	+	–	+	+	+	+	–
Indole production	–	–	+	–	–	+	+	–
Nitrite from nitrate	+	+	+	+	+	+	+	–
Gas from nitrate reduction	–	–	+	–	+	–	–	–
Optimum NaCl (M)	4.5	3.5	4.5	3.5	4.5	4.5	4.5	3.5
Optimum pH	7	7.5	7	7.5	7	7	7	7
Sensitivity to								
Novobiocin (30 μg)	S[2]	S	S	S	S	S	S	S
Bacitracin (10 IU)	S	S	S	S	S	S	S	S
Rifampicin (5 μg)	R[3]	R	S	R	S	S	S	S

[1] pleomorphic, [2] sensitive, [3] resistant

No anaerobic growth was observed in any strain in the presence of L-arginine and TMAO whereas it yielded a positive result only with CH7 strain in the presence of KNO_3. Most halophilic Archaea have the ability to grow anaerobically in the presence of different chemicals due to their relatively low oxygen solubility under high salinity conditions. They were reported (Hartmann *et al.*, 1980; Mancinelli and Hochstein, 1986; Oren and Trüper; 1990; Oren and Litchfield, 1999) to have the ability to use alternative electron acceptors such as fumarate, TMAO and/or nitrate in order to perform anaerobic growth.

Polar lipid chromatograms of standard strains and the isolates from the Cankaya Salt Mine are given in Fig. 2. According to the chromatographic results, all isolates were found to contain PG and PGP-Me phospholipids. It was observed that all strains but CH8K and CH8B isolates contain PGS.

Phylogenetic analysis. Phylogenetic analysis of the extremely halophilic archaeal isolates were performed by building a phylogenetic tree which was constructed based on the 16S rRNA gene sequences (Fig. 3). When 16S rRNA sequential analyses are taken into consideration, CH11 strain was determined to resemble *Halobacterium noricense* by 99.7% ratio. CH11 isolate is a strain with a pleomorphic cellular shape, which can utilize glucose and xylose. CH11 is susceptible to bacitracin antibiotics and cannot grow anaerobically.

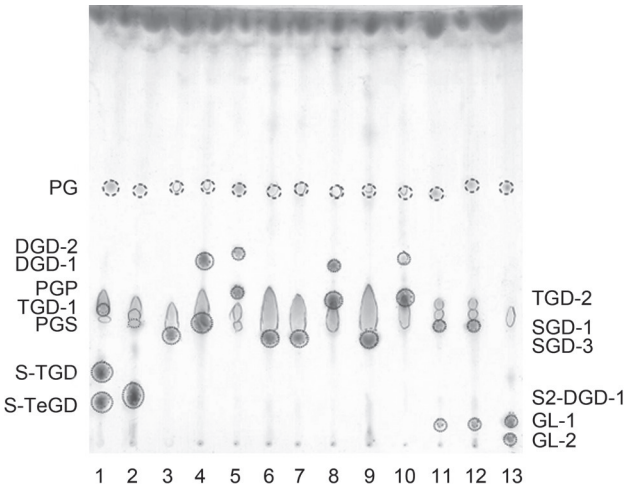

Fig. 2. Results of thin layer chromatography analysis of polar lipids extracted from halophilic archaeal isolates from the Cankaya Salt Mine

1: *Halobacterium salinarum* (DSM 3754); 2: *Natrialba asiatica* (DSM 12278); 3: *Halorubrum saccharovorum* (DSM 1137); 4: *Haloferax denitrificans* (DSM 4425); 5: *Haloarcula vallismortis* (DSM 3756); 6: CH2; 7: CH3; 8: CHA1; 9: CHC; 10: CH7; 11: CH8K; 12: CH8B; 13: CH11. (PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PGS, phosphatidylglycerolsulfate; S-DGD-3, sulfated glycosyl diether (mannose (1→4)-glucoseglyceroldiether); S-DGD-1, sulfated glycosyl diether (mannose (1→2)-glucose glycerol diether); S2-DGD-1, bis-sulfated diglycosyl diether; S-TGD, sulfated triglycosyl diether; S-TeGD, sulfated tetraglycosyl diether; DGD-2, diglycosyl diether; DGD-1, diglycosyl diether (mannose (1→2)glucose glycerol diether); TGD-1, triglycosyl diether; (galactosylmannosyl-glucosyl diether); TGD-2, triglycosyl diether GL, undefined glycolipid)

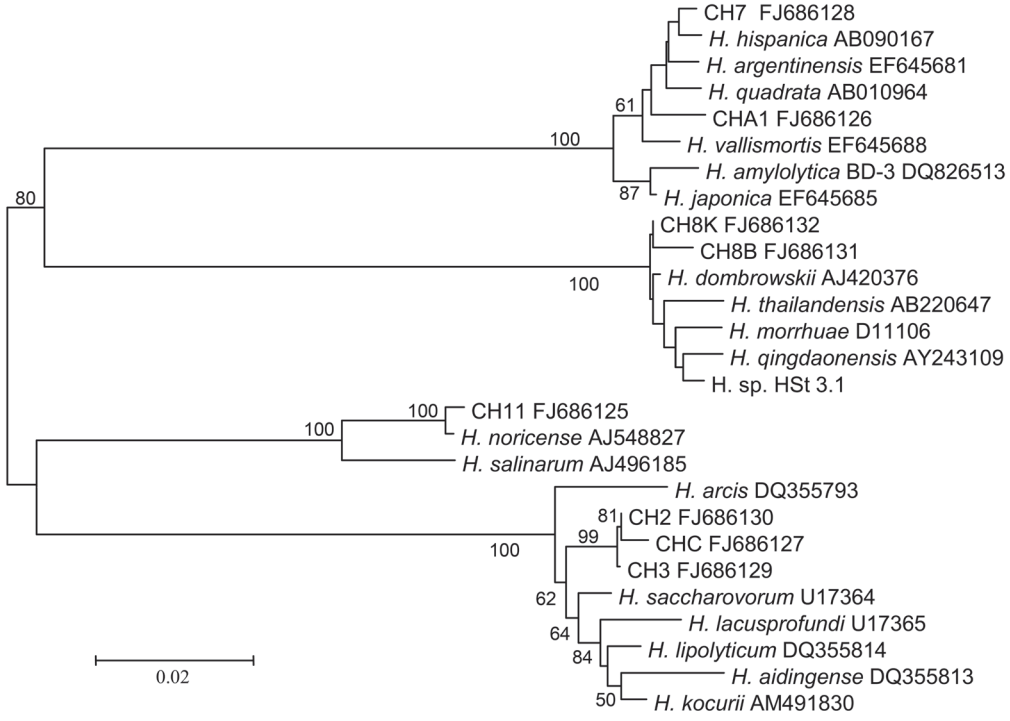

Fig. 3. Phylogenetic tree showing the relationships among the16S rRNA gene sequences of Cankaya salt mine isolates and the closest relatives within the family *Halobacteriaceae*

Bootstrap values, expressed as percentages of 1000 replicates, are shown for branches with more than 50% bootstrap support. Bar, 0.02 substitutions per site

As for *H. noricense*, it has the form of rod cells, cannot make use of glucose or xylose sugars and are resistant to bacitracin and exhibits anaerobic growth in the presence of L-Arginine (Gruber *et al*., 2004). While isolate CH11 was found to contain two undefined glycolipids in addition to PGS, PG and me-PGP lipids, it is reported that *H. noricense* contained PG, me-PGP, PGS, TGD and S-TeGD lipids (Gruber *et al*., 2004). Taking into consideration different phenotypical and chemical features between these two strains, it is suggested that isolate CH11 might be a separate species within genus *Halobacterium*.

The resemblance of CH8K and CH8B isolates to the species *Halococcus dombrowskii* is by 99.8% and 99.3%, respectively. It was revealed that CH8K and CH8B strains (the current study) and *Halococcus dombrowskii* species (Stan-Lotter *et al*., 2002) shared the properties of being coccus-shaped nonmotile cells, positive catalase, oxydase, gelatinase activities and nitrate reduction as well as containing lipids PG, PGP-me and S-DGD1 but PGS. On the other hand, it was established that CH8K and CH8B isolates, which were Gram-variable and have colony pigmentations pink-red and white respectively, could not ferment xylose or fructose sugars and contained one undefined glycolipid in addition to PG, me-PGP and S-DGD1. Meanwhile, the species *Halococcus dombrowskii* was reported to be Gram-negative, and to exhibit light red pigmentation and to be able to use fructose and xylose (Stan-Lotter *et al*., 2002). Except for colony pigmentation all other features of CH8K and CH8B isolates were found to be the same. It was reported that the number of insertion sequences in the chromosomes of halophilic archaea was rather high, and this caused a high frequency of spontaneous mutations (DasSarma, 1993). Such a mutation might be the cause of the difference in pigmentation of isolates CH8K and CH8B.

It was established that strain CHA1 resembled *Haloarcula argentinensis* by the ratio of 99.2%. CHA1 and *H. argentinensis* (Ihara *et al*., 1997) have Gram-negative and motile cells and they exhibit oxydase and catalase activities and can produce acid from fructose, maltose and ribose. Moreover, they have TGD-2 as essential glycolipid. However, unlike *H. argentinensis,* strain CHA1 cannot produce acid from glucose, sucrose and mannose. CHA1 strain differs from *H. argentinensis* by having pleomorphic cell shape and DGD-1 in addition to glycolipid TGD-2.

CH2, CH3 and CHC strains were clustered with *Halorubrum* genus and their similarity to *Halorubrum saccharovorum* species was found to be 98.8%, 98.9% and 98.5%, respectively. Isolates CH2, CH3 and CHC and *H. saccharovorum* share the properties of being rod shaped motile cells, stained Gram-negative, strict aerobic growth, positive oxidase and catalase activity and nitrate reduction (McGenity and Grant, 1996). They are also alike by having S-DGD-3 glycolipid in addition to essential phospholipids.

Isolate CH7 was determined to resemble *Haloarcula hispanica* species to the rate of 99.5%. When phenotypical characteristics of CH7 isolate and *H. hispanica* (Juez *et al*., 1986) species were compared, both strains were found to have pleomorphic motile Gram-negative cells, and positive nitrate reduction and nitrate gas formation and that they could grow anaerobically in presence of nitrate. Moreover, both strains were found to contain TGD-2 glycolipid. On the other hand, isolate CH7 did not exhibit gelatinase, caseinase and amylase activity, whereas species *H. hispanica* is positive in gelatinase, caseinase and amylase activity.

As a result, in the current study we have isolated and characterized 8 different isolates from a salt mine located in central Anatolia. The characterization of the isolates was carried out using a polyphasic approach. According to 16S rRNA sequential analysis, which is one of the most determining molecular taxonomic methods, these eight isolates were found to cluster in four different genera which are *Halobacterium*, *Halococcus*, *Haloarcula* and *Halorubrum*. The results presented herein may contribute to archaeal taxonomy, particularly to halophilic archaeal species habituating saltern cave environments.

Acknowledgements

Financial support from TUBITAK (Turkish Science and Technological Research Counsel)-Turkey and Research Council of Mustafa Kemal University-Turkey is gratefully acknowledged.

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