HALOPHILIC ARCHAEA OF *HALOFERAX* GENUS ISOLATED FROM ANTHROPOCENTRIC TELEGA (PALADA) SALT LAKE

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The paper deals with characterization of some extremely halophilic archaea isolated from surface water samples taken from Telega (Palada) salt lake, located in Prahova county, Romania. Taxonomic investigations have been conducted for several strains isolated in media with a high salt concentration. Salt deposit from Telega (formed in Neogen period) is located underground in the village with the same name in Prahova county. The area is a hillock of sub-Carpathian Mountain from which salt was extracted from antiquity until to the end of 18th century. The salt is a mixture of crystals with color varying from white until to grey and swarthy. Starting with 1872 some of the salt exploitations were relinquished and resulted in the appearance of various salt lakes from which Palada (Telega) lake have depth of 36 m, salinity around 160 g/L and pH value around 8.3. The water is also rich in calcium, magnesium and potassium. The characterization of the isolates from Telega salt lake by polyphasic approach revealed that they belonged to the genus *Haloferax*. The investigation revealed a low diversity of this salt lake, dominated by haloarchaea.

Key words: *Haloferax*; Telega salt lake; Halophilic archaea; Salt mine; Salt lake.

INTRODUCTION

The halophilic microorganisms, belonging mainly to *Archaea*, have as common feature the capacity to inhabit environment with high salt concentrations which are widely distributed through of the world[1-4] either in solid or liquid forms. Such kind of environments as salt mine and anthropocentric salt lakes that resulted after salt mine exploitation was relinquished, are commonly found in Romania either in intra or extra Carpathians area[5]. Some previous investigations lead to isolation of a number of halophilic microorganisms, both *Bacteria* and *Archaea*, in medium with high NaCl and $MgCl_2$ concentrations[6,7]. Some preliminary taxonomic studies revealed the presence of member of genera *Haloferax* and *Haloarcula* in water bodies of some man-made young salt lakes from Slănic Prahova. A new species, namely *Haloferax prahovense*, was proposed for a strain (TL 6^T) isolated from Telega (Palada) salt lake[8]. Telega village, separated by Campina (town) through Doftana River, is situated at 5 km from it, in the west part of Prahova County. The village covers a hillock of sub-Carpathian, area with medium altitude relief (450–700 m), characterized by irregularities of ground, many valleys and swales crossed by streams[9-11]. The rivulet Sarata, the Teleajen River affluent, is main water flow. Salt extraction in Telega started before 1685 and was conducted for more than 330 years,

until to 1900, by using the bell type exploitation technology[12,13]. The other data indicated that extraction has been made from antiquity[13]. Before to the year 1872 the salt exploitations point located to the east side of the rivulet were relinquished[10] resulting in the appearance of man-made salt lakes, having various depth and width detailed in Table 1[10], known today as lakes Mocanu, Palada (Telega), Stavrică, Sweet Lake, Central Bath Lake and Doftana[10]. Salt mine in Telega also have a historic importance[13]. Tourism traditionally based on mineral waters and climate stations, as well as cultural–historical and recreational resources, became a source from economical view for the area after relinquishing salt exploitation[14]. The Palada Lake, named by native also as Telega Lake is situated in the mouth of a saline having a maximum depth around of 36 m, a surface of 1416 square meters[5,10], salinity around 160 g/L and pH value around 8.3 (determined in our surface water sample). The water is also rich in calcium, magnesium and potassium. The salt in deposit of Telega (Doftana – Telega), formed in Neogen period[15], is a mixture of crystals with color varying from white until to grey and swarthy, having a surface of 2.1 square kilometers and 0.7 kilometers thickness[15]. The halophilic archaea, commonly known as halobacteria, are classified in the family *Halobacteriaceae* and various species constitute 26 recognized genera[16-18]. Between these, the genera *Haloferax* and *Haloarcula* appear to be commonly found in saline environments and are best represented. Currently *Haloferax* genus comprised eleven[19] and *Haloarcula* eight[20] validly published strains. The most of halobacteria have been shown to be pigmented pink or red even if non-pigmented strains are reported[4]. The taxonomy of these microorganisms at genus level is based particularly on membrane lipids composition and 16S rRNA gene sequence[21]. Since there were no any reports on the microbiota of Palada (Telega) lake, we undertook this study to evaluate their microbial diversity, particularly that of the halophilic archaea, by using a combination of molecular and biochemical methods. According to this polyphasic approach, this hypersaline environment, as expected, were shown to be inhabited by halophilic archaea, pertaining of genus *Haloferax*.

Table 1

The characteristics of salt lakes from Telega[5,10]

Lake	Height (m)	Surface (m^2)	Maximum width (m)	Maximum depth (m)
Doftana	413	9200	84	26
Central Bath	414	1344	38	45
Sweet Lake	424	1480	35	21
Stavrică	415	1740	52	107.5
Mocanu	415	630	22	14
Palada	416	1416	30	36

MATERIALS AND METHODS

The strains were isolated from water sample taken from the surface of Palada (Telega) lake. The strains were isolated in a medium (I) with the following composition (g/L): NaCl (125), $MgCl_2 \times 6H_2O$ (160), K_2SO_4 (5), $CaCl_2 \times 2H_2O$ (0.1), yeast extract (1), peptone (1), soluble starch (2), agar (20). The medium pH was 7.0–7.2. In some experiments the strains were grown in JCM medium No. 168 which contained (g/L): Bacto casamino acids (5), Bacto yeast extract (5), sodium glutamate (1), trisodium citrate (3), $MgSO_4 \times 7H_2O$ (29.5), KCl (2), NaCl (175.5), $FeCl_2 \times 4H_2O$ (0.036), $MnCl_2 \times 4H_2O$ (0.36 mg). The medium pH was 7.0–7.2 before autoclaving. Ten ml of the lake water were placed in a Petri dish and mixed with 30 ml of the autoclaved molten agar culture medium (I) (cooled to 55–60^{0}C). After solidification, the plates were incubated at 37^{0}C for 7–10 days. The colonies were purified by repeated transfers on the slant.

In order to determine if isolates are halophilic archaea, they were streaked on the solidified medium (I) containing taurocholic acid sodium salt, a bile acid, at a concentration of 0.25 g/L or chloramphenicol at 20 mg/L. It has been shown that cells of halophilic archaea lyse or do not grow in the presence of bile acids[22,23]. The cultures were incubated for ten days at 37^{0}C, and strains that grew on the plates with chloramphenicol were regarded as halophilic archaea.

Membrane lipids analysis was done by TLC following the method described by Kamekura [24].

Biochemical tests for catalase and oxidase activities, formation of indole and starch hydrolysis were performed according to standard procedures. Formation of sulfide was determined as described by Xin *et al.*[25] Hydrolysis of Tween 80 and gelatin were detected using the method of Gutierrez and Gonzalez [26]. Casein hydrolysis was tested on solidified JCM medium No. 168 as described previously[8]. The production of halocin (bacteriocin produced by halophilic archaea) was evaluated according to the procedure of Meseguer and Rodriguez-Valera [27]. Briefly, two ml of culture being tested as target were placed in 20 ml molten agar medium. After solidification, wells were cut in the layer, into which were deposited 100 µl of culture tested as producer, and incubated at 37^{0}C for 7–10 days. Inhibitory activity was only considered positive if clear inhibition zone surrounding a well was observed.

G+C content of the chromosomal DNA was determined by the HPLC method of Tamaoka [28].

For the 16S rRNA gene sequence analysis, total DNA was extracted and purified using the method of Tamaoka [28] adapted for halophilic archaea. The 16S rRNA genes were amplified using the PCR method, with the following forward and reverse primers: 5'- TCCGGTTGATCCTGCCG (position 8–24, *Escherichia coli* numbering) and 5'– GGAGGTGATCCAGCCG (position 1540 – 1525). The PCR products were sequenced using BigDye Terminator Cycle Sequencing Kit (Pharmacia Biotech) and Applied Biosystems ABI Prism DNA Sequencer. The sequence obtained was compared in BLAST database search and then was aligned with other reported halobacterial 16S rRNA gene sequences by using the CLUSTAL W 1.7 program. The phylogenetic tree was reconstructed by the neighbor-joining method[29].

RESULTS AND DISCUSSION

The chloride concentration of the surface water sample of the lake was 161 g/L and pH values 8.3. The lake was found to be inhabited by halophilic microorganisms able to grow in the presence of high concentration of NaCl and $MgCl_2 \times 6H_2O$, 125 and 160 g/L, respectively. The number of colonies (bacteria and archaea) was around 1100 c.f.u./mL, very low, compared to those reported for various salt lakes of similar salt concentrations. For example, the Dead Sea and Great Salt Lake are inhabited by halophilic microorganisms of the magnitude of 10^6 cells/g, although fluctuating periodically[30,31] and Lake Chaka in northwestern China by 4.8×10^6 cells/mL[32].

Isolation of halophilic archaeal strains

Twenty colonies that showed pigmentation varying from pink to different red color were picked up randomly from the plate and purified by repeated transfer on slant of the agar medium. They were then tested for grow in the presence of taurocholic acid sodium salt and chloramphenicol in order to be assumed to belong to archaea or eubacteria. Strains that were unable to grow with taurocholic acid but able to grow with chloramphenicol were assumed to belong to archaea. It should be noted that bacterial strains selected from the plate did not survive on slant medium for long time and were lost after second or third transfer. A similar behavior was observed also for some archaeal strains.

The archaeal six strains selected for further investigation, showed pink or red color, but the intensity of pigmentation decreases when the strains were transferred from plate to slant medium. Pigmentation was also affected by growth temperature. A high intensity of pigmentation was observed at high temperatures, generally up to 45^0C.

Analysis of polar lipid

Thin layer chromatography of the total lipids of the archaeal six strains revealed that they possessed the glycerol diether analogues of methyl ester of phosphatidyl glycerol phosphate (PGP-Me) and phosphatidyl glycerol (PG) as phospholipids and a major glycolipid. Phosphatidic acid was identified for two strains. All six strains possessed the same glycolipid, sulfated diglycosyl archaeol-1 (S-DGA-1). The presence of S-DGA-1 is ubiquitous in all validly published species of the genus *Haloferax*[33]: *Haloferax gibbonsii* JCM 8863, *H. denitrificans* JCM 8864, *H. volcanii* JCM 8879, *H. lucentense* JCM 9276, *H. alexandrinus* JCM 10717, *H. sulfurifontis* JCM 12327, *H. elongans* JCM 14791, *H. prahovense* JCM 13924, *H. mucosum* JCM 14792, *H. larsenii* JCM 13917, *H. mediterranei* ATCC 33500. Another glycolipid, diglycosyl archaeol-1 (DGA-1) was present in some strains as shown in Table 2.

Table 2

Membrane lipid profile of investigated strains

Strain / Lipid	TL1	TL3	TL5	TL6	21	22
S-DGA-1	+	+	+	+	+	+
DGA-1	–	+	–	–	+	–
PGP-Me	+	+	+	+	+	+
PG	+	+	+	+	+	+
PGS	–	–	–	–	–	–
PA	–	–	–	–	+	+

S-DGA-1 – ulfated diglycosyl archaeol; DGA-1 –diglycosyl archaeol; PGP-Me–phosphatidyl glycerol phosphate methyl ester; PG–phosphatidyl glycerol; PGS–phosphatidyl glycerol sulphate; PA–phosphatidic acid.

Biochemical tests

Other characteristics of investigated strains are shown in Table 3 and revealed a high degree of similarity among them. All strains were oxidase and catalase positive. Indole was produced from tryptone, and sulfide from sodium thiosulphate. The strains hydrolyzed starch and Tween 80 but not casein. There was an exception for strain 22 which hydrolyzed casein and has pink pigmentation as can be observed in Figure 1. All strains except TL6 hydrolyzed gelatin.

Fig. 1. Casein hydrolysis by some investigated strains. The clear zone surrounding colony of strain 22 showed a positive result.

Table 3

Phenotypic properties of six strains

	TL1	TL3	TL5	TL6	21	22
Mol% G+C	64.7	64.1	64.1	63.7	64.9	58.9
Lyses in distil water	+	+	+	+	+	+
H_2S from $Na_2S_2O_3$	+	+	+	+	+	+
Oxidase and Catalase	+	+	+	+	+	+
Indole from tryptone	+	+	+	+	+	+
Hydrolysis						
Casein	–	–	–	–	–	+
Gelatin	+	+	+	–	+	+
Starch	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+

Phylogenetic tree reconstruction and DNA G + C content

The tree reconstructed from the 16S rRNA gene sequences (Figure 2) strongly supported that strains suggested to belong to the genus *Haloferax* by TLC of polar lipids do belong to the genus phylogenetically as well. Strains TL1, TL5, and *Haloferax prahovense* ($TL6^T$) formed a tight cluster in the genus *Haloferax*. Probably those strains TL1, TL3 and TL5 can be assigned as *Haloferax prahovense* too. The strains 21 and 22 (sequences are not included in Figure 2 since they presented many ambiguous base) are separated by other *Haloferax* members and most probably they constitute a new species. The G + C contents of total DNA of the tested strains belonging to the genus *Haloferax* were between 58.9 and 64.9 mol% (Table 3). The contents of the *Haloferax* species reported so far were in the range of 59.5 and 64.5 mol%.

Halocin production

Halophilic archaeal strains inhabit environments in which only a few other organisms are able to grow and they probably compete each other in these environments. The production of halocins (bacteriocin-like substances)[4,34,35] could provide a mechanisms by which species avoid competition with other species having same environmental requirements. The present study revealed that three out of six tested strains were able to produce halocins active against other strains isolated from the same lake (Table 4) or different salt lakes[36]. On

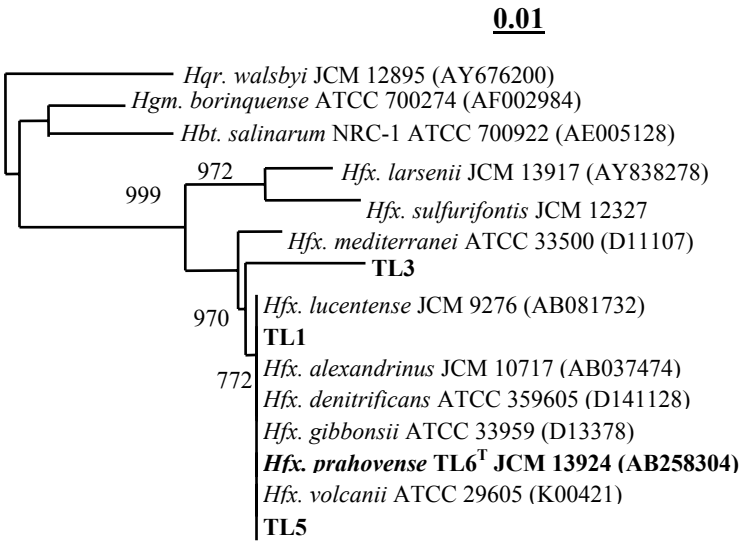

Fig. 2. Phylogenetic tree derived from the partial 16S rRNA gene sequences showing the position of the investigated strains among species of the genus *Haloferax*. The tree was reconstructed by the neighbor-joining method. Bootstrap values ≥ 70% (1000 replicates) are shown. Bar 0.01 substitutions per nucleotide position. The accession number for *Hfx. sulfurifontis* is AY458601.

the other hand, two strains (TL1 and TL3) were not sensitive to any halocins produced by other strains isolated from the same lake. The strain TL3 showed sensitivity to halocin produced by halophilic strains isolated from other artificial salt lake.

Table 4

Antagonistic interactions between investigated strains due to halocin production

Producer / Target	TL 1	TL 3	TL 5	TL 6
TL 1		–	–	–
TL 3	–		–	–
TL 5	–	+		–
TL 6	–	+	–	

Low diversity

As mentioned above, the concentration of Cl^- ion was the 161 g/l. The data from this study suggested that investigated lake showed optimum conditions for species pertaining to genus *Haloferax*. On the other hand, the low diversity could be understood taking into account the chemical composition of isolation medium. This medium was characterized by high concentrations of magnesium which most probably inhibit growth of some species. Taking into account the origin of the investigated salt lake, the predominant presence of *Haloferax* species may suggest that the underground salt deposit possesses a relatively low diversity with *Haloferax* species as the major biota of ancient origin, although species of genera *Halorubrum* and *Halobacterium*[37] or *Haloccocus* have been shown to be associated with ancient rock salt samples[38,39].

CONCLUSIONS

We have isolated and identified six halophilic archaeal strains belonging to the genus *Haloferax*. All strains possessed a glycolipid S-DGA-1, which is present in all *Haloferax* species reported so far. The strains TL 3 and 21 contained also the glycolipid DGA-1. The low diversity of haloarchaeal strains in the investigated salt lake could be due to the halocin production as a mechanism to compete in hypersaline environments.

In our preliminary study[7], we reported that TL1 as *Halobacterium*, based on simple morphological observation and lipid composition. We now consider that the affiliation of the strains based on the polyphasic approach is definitely correct.

To the best of the authors' knowledge, this is the first paper on the survey of microbiota of salt lakes from Telega - Doftana area in which salt was extracted from antiquity until to beginning of 20th century revealing the importance of the area in Romanian history and economy.

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