TAXONOMY OF A NOVEL EXTREMELY HALOPHILIC ARCHAEON BELONGING TO GENUS *HALOARCULA* ISOLATED FROM A LOW SALINE ENVIRONMENT, TECHIRGHIOL LAKE, ROMANIA

ENACHE Mădălin, COJOC Roxana, ECHIGO Akinobu, MINEGISHI Hiroaki, ITOH Takashi, KAMEKURA Masahiro

Abstract. This paper deals with the polyphasic taxonomy of an extremely halophilic strain, T1/2S95 isolated from Techirghiol Lake, Romania, a low saline environment. The strain T1/2S95 was yellow to orange pigmented, stained Gram-negative, and was able to hydrolyse Tween80, growing between 1 and 5.2 M sodium chloride. The membrane polar lipids were PGS, PGP-Me, PG, and two glycolipids, DGD-1 and S-DGD-1. Genomic DNA G+C content was 65.1 mol%. According to the analysis of 16S rRNA gene sequence (AB469844) in BLAST, the strain showed the highest degree of similarity with *Haloarcula japonica*, a haloarchaeon in the family *Halobacteriaceae*. The strain has been deposited to Japan Collection of Microoganisms (RIKEN, BioResource Center) as JCM 19933. The strain could be considered to represent a novel species of the genus *Haloarcula*.

Keywords: halophilic archaea, salt lakes, hypersaline environments, Techirghiol lake.

Rezumat. Taxonomia unui arheon halofil aparţinând genului *Haloarcula*, izolat din Lacul Techirghiol, România. Lucrarea abordează caracterizarea taxonomică polifazică a unui haloarheon, respectiv tulpina T1/2S95 izolată din Lacul Techirghiol, România, un mediu caracterizat printr-o salinitate scăzută. In baza comparaţiilor în baza de date BLAST, tulpina arată un grad mare de înrudire cu *Haloarcula japonica*. Secvenţa genică aferentă 16S ARNr provenind de la tulpina haloarheană investigată a fost depozitată la banca de gene DDBJ având numărul de acces AB469844 iar exemplarul de tulpină tip s-a depozitat la Colecţia de Microorganisme a Japoniei (RIKEN, Centrul de Bioresurse) unde are numărul de acces JCM 19933. Rezultatele obţinute în cadrul acestui studiu conduc către concluzia că tulpina T1/2S95, Gram-negativă, pigmentată, capabilă să hidrolizeze Tween80 şi să crească pe medii de cultură conţinând clorură de sodiu de la 1M până la 5.2M, având profilul lipidic membranar alcătuit din PGS, PGP-Me, PG, DGD-1 şi S-DGD-1, precum şi un conţinut în baze azotate G+C al ADN de 65.1 mol%, izolată din Lacul Techirghiol, România, poate fi considerată ca specie nouă pentru ştiinţă aparţinând genului *Haloarcula*.

Cuvinte cheie: arhee halofile, lacuri sărate, medii hipersaline, Lacul Techirghiol.

INTRODUCTION

Halophilic microorganisms belonging to the *Archaea* domain are spread in all investigated saline and hypersaline environments distributed in the entire world such as salt waters, saline soils, salt lakes, salterns and salt mines (OREN, 2013). Physiological and biochemical investigations revealed that such kind of microorganisms cope with the high ionic strength of the salty environments by either compatible solutes or salt-in strategies (OREN, 1999). The particular features of the halophilic microorganisms made them model for investigations in astrobiology (DasSARMA, 2006), taking into account that some halophiles were identified in a primary salt crystal brine dated around 250 million years old (VREELAND et al., 2000) and sodium chloride (salt) has been detected in Martian origin meteorites (GOODING, 1992). The abundance of microorganisms either bacteria or archaea in saline environments with low pH or high pH values (MINEGISHI, 2013), revealed by increasing numbers of publications in recent years, supported the high interest in the characterization and description of novel microbial strains inhabiting niches considered hostiles to normal live conditions.

In Romania, a lot of salty areas could be found as salt lakes, salt mines or saline soils harbouring halophilic archaea able to hydrolyse several polysaccharides or proteins (NEAGU et al., 2014). Among the saline and hypersaline environments in Romania, Techirghiol Lake located near the Black Sea coast is characterized by variable concentrations of salt depending on site, ranging from negligible salinity up to 60 g/L (AONOFRIESEI, 2007).

Techirghiol lake is relatively well known in terms of biological communities, biodiversity and of salinity regime, being quite well investigated for many years either from relatively high importance of therapeutic properties of the medicinal (sapropelic) mud present in the lake (ȚUCULESCU, 1965) or towards understanding of the dynamics of putative moderately halophilic microbial community (DUMITRU et al., 1996). The unique biodiversity of this area led to conclusions to include Techirghiol Lake in the European network Natura2000 in order to guarantee the protection and support for conservation status of the area (IOJĂ et al., 2010). The intensive use of medicinal mud extracted from this lake for the treatment of several diseases, mainly in rheumatology (DEMIRGIAN et al., 2012; IONESCU et al., 2012; PROFIR et al., 2012; SURDU et al., 2012) supported the significance and importance of the investigations in the area to characterize archaeal and bacterial populations. Previous investigations revealed that Techirghiol Lake harbour either moderately halophilic bacteria able to decontaminate some pesticides (ONCESCU et al., 2007) or some extremely halophilic archaea (ENACHE et al., 2009).

This paper deals with the polyphasic taxonomy of a haloarchaeal strain, T1/2S95 isolated from Techirghiol Lake. According to the analysis of 16S rRNA gene sequence in BLAST, the strain showed a high degree of similarity

with *Haloarcula japonica*. Torreblanca et al. described the genus *Haloarcula* in 1986, with *Haloarcula vallismortis* as the type species. From that time many new species were described, namely *Har. amylolytica* (YANG et al., 2007), *Har. argentinensis* (IHARA et al., 1997), *Har. hispanica* (JUEZ et al., 1986), *Har. japonica* (TAKASHINA et al., 1990; 1991), *Har. marismortui* (OREN et al., 1990), *Har. salaria* (NAMWONG et al., 2011), *Har. tradensis* (NAMWONG et al., 2011), *Har. vallismortis* (GONZALEZ et al., 1979; TORREBLANCA et al., 1986), *Har. quadrata* (OREN et al., 1999). The 16S rDNA sequence of the investigated strain has been deposited in DDBJ with accession number AB469844 and the type strain has been deposited in the Japan Collection of Microorganisms (RIKEN, BioResource Center) having access number JCM 19933.

MATERIALS AND METHODS

The strain T1/2S95 has been isolated from a water sample from Techirghiol Lake in a medium containing per litre 125 g NaCl, 160 g $MgCl_2 \cdot 6H_2O$, 5 g K_2SO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, 1 g peptone, 2 g soluble starch, and 1 g yeast extract. The pH of the medium was 7.0 – 7.2 before autoclaving. Further experiments for the characterization of the isolate were performed using the strain cultivated on JCM medium no. 168 which contained per litre: Bacto casamino acids 5 g, Bacto yeast extract 5 g, sodium glutamate 1 g, trisodium citrate 3 g, $MgSO_4 \cdot 7H_2O$ 29.5 g, KCl 2 g, NaCl 175.5 g, $FeCl_2 \cdot 4H_2O$ 0.036 g and $MnCl_2 \cdot 4H_2O$ 0.36 mg. The medium pH was also 7.0-7.2 before autoclaving. When necessary, the media were solidified by adding 20 g/l agar. Generally, the strain was cultivated at 32^0C – 37^0C, in the absence of light, with moderate agitation at 250 rpm, in 100 ml culture volume containing 10 ml inoculum and 90 ml culture medium.

The characterization of the strain T1/2S95 followed the proposed minimal standards in order to describe a new haloarchaeal strain in the order Halobacteriales (OREN et al., 1997). The classification of the strain T1/2S95 to the archaea has been evaluated in the above-described media containing 0.25 g/l taurocholic acid sodium salt or 20 mg/l chloramphenicol, taking into account that haloarchaeal cells lyse or do not grow in the presence of bile acids (KAMEKURA et al., 1998; KAMEKURA & SENO, 1991). The membrane lipids extraction and identification were performed using thin layer chromatography method (KAMEKURA, 1993). Haloarchaeal cells obtained from 100 ml cultures were mixed with distilled water and chloroform/methanol (1/2 v/v). The resulted mixture were centrifuged at 10 000 rpm and the supernatant was separated. The supernatant was treated with a mixture of chloroform/methanol. The lower chloroform phase resulted in separator funnel was isolated, dried and dissolved in the chloroform-methanol solvent mixture. The evaluation for halocin production (bacteriocin produced by halophilic archaea) was performed according to the procedure described by Meseguer and Rodriguez-Valera (1985). Two ml of culture tested as halocin target were mixed with 20 ml molten agar medium. After the solidification of this medium, 100 µl of culture tested as halocin producer were deposited into wells and incubated at 37^0C for 7-10 days. In the presence of a clear inhibition zone surrounding the well, the activity was recorded positive. The other biochemical tests were performed following the previously described protocols (ENACHE et al., 2007).

Genomic DNA of the strain T1/2S95 was isolated and purified following the method of Tamaoka (1994) adapted for halophilic archaea and the G+C content was determined by the HPLC method. The 16S rRNA genes were amplified by PCR, using the archaeal specific forward and reverse primers, 5'-TCCGGTTGATCCTGCCG (position 8 – 24 in *E. coli*) and 5'-GGAGGTGATCCAGCCG (position 1540 – 1525), respectively. The resulted DNA fragment was sequenced using BigDye Terminator Cycle Sequencing Kit (Pharmacia Biotech) and ABI Prism DNA genetic analyzer (Applied Biosystems). The orthologous 16S rRNA gene (MINEGISHI et al., 2012) was amplified with the *pyrD* primer set2: forward primer pyrD2, 5'-TCGTTGTTNARNCCCATNCGGTT-3' (corresponding to nt 346–368 of *pyrD* of *Haloarcula marismortui*); reverse primer 23SRev, 5'-GCTTWTCGCAGCTTGG-3' (corresponding to nt 56–71 of the 23S rRNA gene of *Halobacterium salinarum*). The 16S rRNA gene sequences obtained was analysed using BLAST and aligned with other reported haloarchaeal 16S rRNA gene sequences using CLUSTAL W 1.7 software. A phylogenetic tree was reconstructed by the neighbour-joining method (SAITOU & NEI, 1987).

RESULTS AND DISCUSSION

The strain T1/2S95 investigated in this work has been isolated from Techirghiol Lake, a saline lake having sodium chloride content around 60 g/l, located nearby the Black Sea coast, in Constanta county, Romania, in the proximity of Eforie city. The Gram-negative strain lysed in distilled water and was not able to grow on media supplemented with bile salts thus being assumed as haloarchaeal strain. On the solid medium colonies were elevated, circular and having whole margins and transparent. When cultivated on liquid JCM 168 medium containing various concentrations of sodium chloride (absence of sodium chloride to saturation) for 48 hours, the strain showed no pigmentation, but after 72 hours the pigmentation became weak orange in culture media with 4 – 5.2 M NaCl and yellow in media having 1 – 4 M NaCl (Fig. 1b). The strain was unable to grow bellow 1 M NaCl and grew optimally at 2.0 – 2.5 M. The strain was not able to synthetize halocins but its growth was inhibited by a halocin produced by other haloarchaeal strain, GR1 isolated from Bride Cave, a man-made salt lake located in Slănic Prahova and assigned to *Haloferax* genus as previously described (ENACHE et al., 2008). The biochemical investigations revealed that the strain was catalase and oxidase positive, sulphide was produced from sodium thiosulphate but indole was not produced

from tryptone. The strain hydrolyzed Tween80 but was negative for starch and casein hydrolysis. Gelatin liquefactions were also negative (Table 1).

The sequence of 16S rRNA gene of the strain T1/2S95, amplified with the archaeal specific forward and reverse primers, was most closely related to those of the strains of the genus *Haloarcula*.

The chemotaxonomic features, namely lipid profile and DNA G+C content complied with the already characterized members of the genus *Haloarcula*. The genomic DNA G+C content for the strain T1/2S95 was 65.1 mol% whereas members of the genus *Haloarcula* showed values from 60.1 mol% in *Har. quadrata* to 64.7 mol% in *Har. vallismortis*. Membrane lipid investigation by TLC revealed the presence of $C_{20}C_{20}$ glycerol diether derivatives of phosphatidyl glycerol sulphate (PGS), methyl ester of phosphatidyl glycerol phosphate (PGP-Me) and phosphatidyl glycerol (PG) as phospholipids (Fig. 1a). The data shown in Fig. 1a related to glycolipid analysis revealed the presence of diglycosyl archaeol-1 and their sulfated derivative (DGD-1 and S-DGD-1).

Table 1. Characteristics features of the members of *Haloarcula* genus.
1 = Formation of sulphide from sodium thiosulphate; 2 = catalase; 3 = oxidase; 4 = indole from tryptone; 5 = starch hydrolysis; 6 = Tween80 hydrolysis; 7 = Gelatin liquefactions; 8 = casein hydrolysis; 9 = range of NaCl for growth (M); 10 = optimum NaCl for growth (M); 11 = isolation site; 12 = origin of isolation site; 13 = DNA G+C content (mol%)

	1	2	3	4	5	6	7	8	9	10	11	12	13
amylolytica	+	+	+	+	+	+	+	-	2.0 -5.1	3.1	Salt lake	N	62.4
argentinensis	nd	+	+	nd	+	+	+	nd	2.0 -4.5	2.5 - 3.0	Soil of saltern	A	62
hispanica	nd	nd	nd	+/-	+	+	+	+/-	2.5 -5.2	3.5 - 4.2	Saltern	A	62.7
japonica	+	nd	nd	+	-	-	-	-	2.5 -5.0	3.5	Salted soil	N	63.3[&]
marismortui	nd	nd	nd	-	-*	-	-	nd	1.7 -5.1	3.4 - 3.9	Dead Sea	N	62
quadrata	nd	+	+	-	+**	-***	-	-	2.7- 4.3	nd	Salted pool	N	60.1
salaria	nd	+	+	-	+	+	-	-	2.5 -5.1	3.4- 4.2	Salt from fish sauce	N	61.6
tradensis	nd	+	+	-	+	+	-	-	2.5 -5.1	3.4- 4.2	Salt from fish sauce	N	62.2
vallismortis	nd	nd	nd	+	+****	-	-	-	<=2.5	4.3	Salted pool	N	64.7
T1/2S95	+	+	+	-	-	+	-	-	1.0 -5.2	2.0 - 2.5	Saline lake	N	65.1

Legend:
*= weak positive in Bergey's Manual
**= negative in paper of Namwong et al., 2011; positive in Bergey's Manual and paper of Yang et al., 2007
***= positive in paper of Namwong et al., 2011; negative in Bergey's Manual and paper of Yang et al., 2007
****= negative in paper of Yang et al., 2007
& = value corresponding to strain TR-1^T (D28872)
+/- = variable results
nd = no data available
N = natural environment; A = man-made environment

The data summarized in Table 1 suggested that the members of *Haloarcula* genus have been isolated from natural saline and hypersaline environments except two members isolated from man-made hypersaline environments, namely saltern (*Har. hispanica*) and soil from saltern (*Har. argentinensis*).

Since *Haloarcula* spp. possess multiple, heterogeneous 16S rRNA genes, the *pyrD* primer set was used to amplify the DNA fragment encompassing the orthologous 16S rRNA & tRNA-Ala with *pyrD* and 23S rRNA genes. According to the data of MINEGISHI et al. (2012) related to gene orders in the upstream of 16S rRNA genes (one or two in species of some genera, and three in *Haloarcula* spp.), orthologous 16S rRNA gene is preceded by a gene coding for dihydroorotate dehydrogenase, an enzyme involved in enzymatic synthesis process of nucleic acid base pyrimidine. Sequence of the orthologous 16S rRNA gene of the strain T1/2S95 showed high similarities with those of *Haloarcula* spp. Thus, the phylogenetic tree (Fig. 2) was reconstructed based on sequences of orthologous 16S rRNA gene. Sequence used as out-group was adopted from KEGG (Kyoto Encyclopedia of Genes and Genomes).

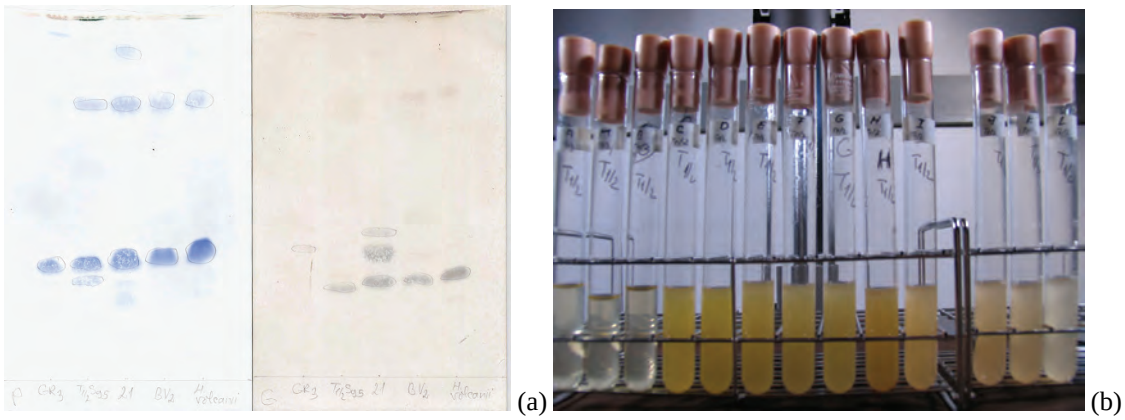

Figure 1. (a) Phospholipid (left) and glycolipid (right) pattern of the strains T1/2S95 revealing the presence of PGS, PGP-Me, PG and DGD-1 and their sulphate derivate; starting from left of either pattern the lane 1 = strain GR3, lane 2 = strain T1/2S95, lane 3 = strain 21, lane 4 = strain BV2 and the lane 5 = *Haloferax volcanii*; (b) growth and pigmentation of the strain T1/2S95 in the presence of various NaCl concentrations from nil up to saturation (from left to right) (original).

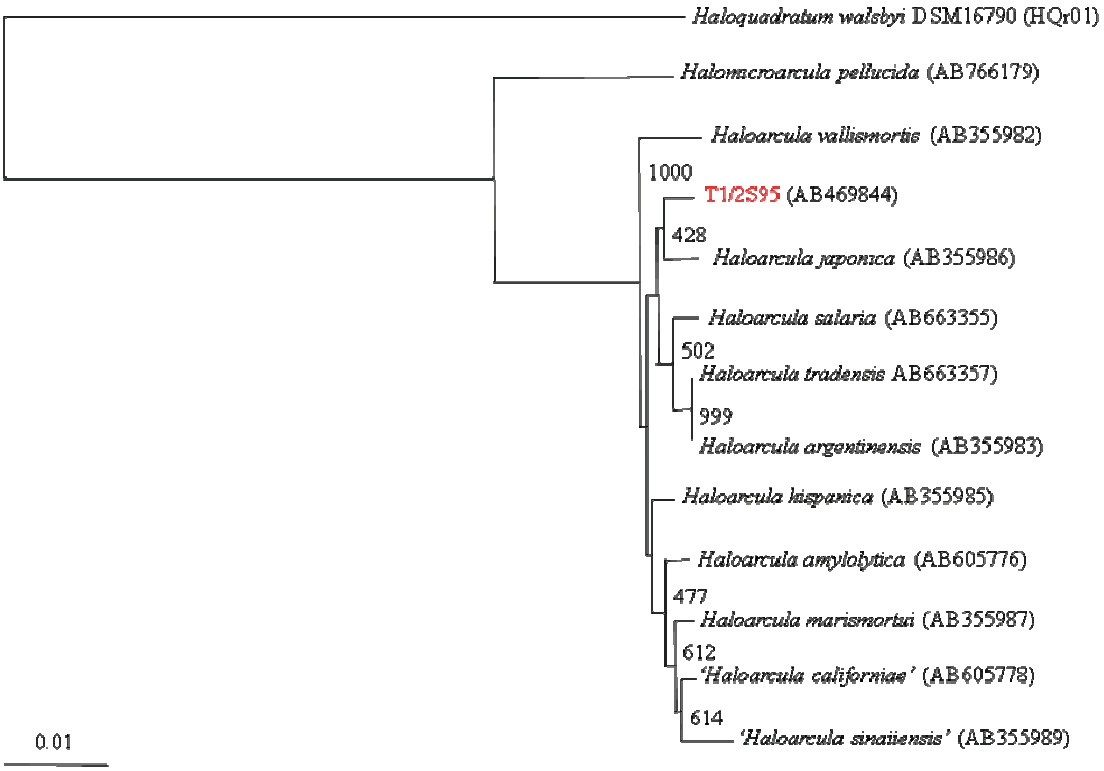

Figure 2. Phylogenetic tree revealing the position of strain T1/2S95 amongst the members of the genus *Haloarcula*. The tree was reconstructed by the neighbour-joining method. Bootstrap values over 400 are represented. Bar representing one substitution per 100 nucleotide position.

The strain T1/2S95 formed a clade together with *Har. japonica*, *Har. salaria*, *Har. tradensis* and *Har. argentinensis*. The maximum parsimony supported the group (strain T1/2S95 and *Har. japonica*) well, with a bootstrap value of 42%. The clade is also well supported by a value of probability over 30%. Another clade in Fig. 2 constituted of remaining five species, *Har. hispanica*, *Har. californiae*, *Har. sinaiiensis*, *Har. marismortui* and *Har. amylolytica*, and grouping of this clade with *Har. vallismortis* is supported very well by a probability of 100%. When compared in BLAST, the similarities of strain T1/2S95 with strains of genus *Haloarcula* forming the clade together were 99%, and 98% with remaining five species and *Har. vallismortis*.

As a general conclusion, it should be noted that pigmented Gram-negative strain T1/2S95 isolated from Techirghiol Lake in Romania, growing between 1 and 5.2 M sodium chloride concentrations, able to hydrolyze Tween80, with lipid profile constituted of PGS, PGP-Me, PG, DGD-1 and S-DGD-1, and with genomic DNA G+C content of 65.1mol%, could be considered as a novel species of the genus *Haloarcula*.

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Enache Mădălin, Cojoc Roxana
Institute of Biology Bucharest of the Romanian Academy
Splaiul Independentei 296, P.O. Box 56-53, Bucharest, Romania.
E-mail: madalin.enache@ibiol.ro; roxana.cojoc@ibiol.ro

Echigo Akinobu, Minegishi Hiroaki
BioNano Electronic Research Center, Toyo University
2100 Kujirai, Kawagoe,Saitama 350-8585, Japan.
E-mail: minehiro@toyo.jp; echigo@toyo.jp

Itoh Takashi
Japan Collection of Microorganisms, RIKEN BioResource Center
3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan.
E-mail: ito@jcm.riken.go.jp

Kamekura Masahiro
Halophiles Research Institute
Noda 278-0043, Japan
E-mail: masahirokamekura@krc.biglobe.ne.jp

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