HALOPHILIC ARCHAEA IN THE NEOGENE SALT MASSIF FROM SLĂNIC PRAHOVA, ROMANIA

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Abstract. The archaeal component of salt embedded microbiota from a Neogene salt massif located in Unirea salt mine, Slanic, Prahova (Romania), was isolated and characterized. Salt crystals were collected from the subterranean salt block. After washing by immersion in 50 ml of sterile 20% NaCl for 5 minutes, the crystals were transferred in a fresh NaCl solution and incubated at 37^0C until completely dissolved. The number of c.f.u. present at the surface of salt crystal was at least double than that present inside the crystal, indicating most probably a contamination with salt tolerant microorganisms, rather than naturally occurring species. Biochemical and molecular analyses revealed a low diversity of haloarchaea, the predominant strains belonging to *Halorubrum* or *Haloarcula* genera. Eight out of 18 investigated strains were cultivated in the presence of 2-5.2 M NaCl, with an optimum varying from 2.5-3 M to 4.0-4.5 M. The remaining ten strains were able to grow in the presence of 2.5 M NaCl until saturation concentrations, with an optimum around 4 M NaCl. Three of the strains did not catalyse the formation of H_2S from thiosulphate. All strains were catalase positive, and able to grow in the presence of chloramphenicol and ampicillin (50 μg/ml) but not in the presence of sodium deoxycholate and novobiocin.

Keywords: rock salt, halophilic archaea, salt mine, hypersaline environments.

Rezumat. Aspecte privind prezenţa unor microorganisme halofile arheane în zăcământul de sare format în zona Slănic Prahova, România în perioada Neogene. Cristalele de sare prelevate din Mina Unirea din zona Slănic Prahova au fost imersate pentru cinci minute în 50 ml soluţie sterilă de clorură de sodiu 20% în vederea spălării acestora după care au fost transferate în soluţie proaspătă de NaCl şi ţinute la 37^0C până la dizolvarea completă. Numărul de u.f.c. prezente la suprafaţa cristalului de sare a fost mai mare comparativ cu numărul celor provenite din interiorul cristalului arătând cel mai probabil un grad ridicat de contaminare cu microorganisme capabile să tolereze concentraţii mari de sare. Analizele moleculare şi de biochimie au arătat o diversitate scăzută a haloarheelor, majoritatea aparţinând genurilor *Halorubrum* şi *Haloarcula*. Un număr de 8 tulpini dintre cele 18 investigate au crescut pe medii de cultură conţinând concentraţii de clorură de sodiu între 2 si 5,2 M cu un optim de dezvoltare în prezenţa unor concentraţii cuprinse fie în intervalul 2,5-3 M fie 4-4,5 M. Cele zece tulpini rămase au fost capabile să crească în prezenţa a 2,5 M NaCI până la concentraţiile de saturaţie, cu un optim în jurul valorii de 4 M NaCI.Trei dintre tulpinile investigate nu au produs H_2S din tiosulfat. Toate tulpinile testate au avut activitatea cataliză pozitivă şi au fost capabile să se dezvolte în prezenţa cloramfenicolului şi a ampicilinei (50 μg/ml) însa nu şi în prezenţa deoxicolatului de sodiu şi a novobiocinei.

Cuvinte cheie: evaporite, halofile arhea, mina de sare, medii hipersaline.

INTRODUCTION

Halophilic microorganisms belonging to the domain Archaea have been identified in a variety of saline and hypersaline environments. Due to their unique structural and functional characteristics, which support their growth, they flourish in such extremely severe conditions. Haloarchaea were isolated or detected by PCR techniques (even if in some cases they are non-cultivable) in a wide range of environments with salinity varying from negligible (ELSHAHED et al., 2004; LEUKO et al., 2008) to saturation (32 g/L NaCl), such as Dead Sea (OREN, 1983; 2002), solar salterns (BENLLOGH et al., 2001; LITCHFIELD & GILLVERT, 2002; OCHSENREITER et al., 2002) or unusual salted habitat like nostrils salt glands of *Calonectris diomedea*, a sea bird (BRITO-ECHEVERRIA et al., 2009).

Such kind of microorganisms were isolated from a fluid inclusion in salt crystals (NORTON et al., 1993) and in the last decade a novel member, *Halobacterium noricense* was characterized as a new species of halophilic archaea as a novel member of the genus *Halobacterium* (GRUBER et al., 2004). Moreover, a recent study revealed that *Halobacterium* species are adapted to survive in halite for long periods of time (GRAMAIN et al., 2011), and a number of viable haloarchaea were isolated from the rock salts (GRANT et al., 1998; MCGENITY et al., 2000), suggesting that several and various populations of halophilic microorganisms are relict populations derived from the ancient sea.

Salt (halite) deposits resulting from the precipitation turnover through the years of older salty environments were identified worldwide. Taking into account that one of the oldest data which could be attributed to the presence of microbial species associated with rock salts (FISH et al., 2002; KAMEKURA, 2003; 2007), they can be also considered as salted relics of brines that presumably hosted some populations of halophilic archaea.

Some salt deposits that were formed in the Neogene period are distributed in Romania, mainly in the proximity of the Carpathian areas, such as Slănic, Prahova (HAR et al., 2006; MĂRUNŢEANU, 1999). The exploitation of the halite from this deposit was active since 1685 until nowadays using several technologies. However, little is known about their microbiota, except recent investigations that revealed for the first time the presence of both halophilic archaea and bacteria (COJOC et al., 2009; ENACHE et al., 2000; 2008ab; 2012). The underground salt deposit from Slănic is located 45.5 m to 499 m deep (DRĂGĂNESCU, 1990), and most probably dated from the Neogene period (23.03 - 2.588 MY ago). The deposit showed a grey and swarthy colour, and is variegated as a consequence of turnovers that took place during precipitation processes, due to climatic and sedimentary variations (HAR et al., 2006). Currently this deposit

represents one of the most important salt exploitation in Romania. In this study, we investigated whether haloarchaea inhabit this salt mine, and if they represent relict populations of ancient haloarchaea.

MATERIALS AND METHODS

Sampling of rock salt and isolation of halophilic microorganisms

The rock salt crystal were collected from subterranean salt mine Unirea, located in Slănic, Prahova, Romania, at about 208 meters depth. The crystal with no apparent strong clay or soil content was broken in three pieces and washed with sterile 20% NaCl solution. The resulted brine was used to remove the microorganisms located on the crystal surface. After washing, the crystal was sterilized in flame for few seconds, and dissolved in fresh sterile 20% NaCl. The resulted brine was used to isolate haloarchaeal strains located inside the crystal. Aliquots of 1 ml of resulted brines (surface and inside) were mixed with 25–30 ml of molten agar medium JCM 168 which contained (g/L): Bacto casamino acids (5), Bacto yeast extract (5), sodium glutamate (1), trisodium citrate (3), $MgSO_4x7H_2O$ (29.5), KCl (2), NaCl (175.5), $FeCl_2x4H_2O$ (0.036), $MnCl_2x4H_2O$ (0.36 mg). The medium pH was 7.0-7.2 before autoclaving. After that the samples were incubated at 37^0C for several weeks until the appearance of red and pink colonies.

Biochemical assays

A biochemical characterization of the isolated haloarchaeal strains was performed following the minimal standard procedure for description of new taxa, as previously described in the case of halobacteria (ENACHE et al., 2000; 2007; 2008ab; 2009). The NaCl concentration range and the optimum NaCl concentration for growth were determined by cultivation at 37^0C in JCM 168 medium for up to seven days. The catalase and oxidase activities and the presence of metabolic pathways for producing H_2S from thiosulphate, of indole from tryptophan and starch hydrolysis were investigated according to previous protocols (ENACHE et al., 2007; 2008ab). The antibiotic and bile salts resistance was also tested, using 50 µg/ml of chloramphenicol, penicillin, ampicillin erythromycin and novobiocin and sodium deoxycholate in order to differentiate archaeal and bacterial strains, as previously indicated (ENACHE et al., 2007). In addition, the Gram staining and the cell shape were determined as previously described (ENACHE et al., 2007; 2009)

16S rRNA gene sequence analysis

Total DNA was extracted and purified using the method of Tamaoka adapted for halophilic archaea (ENACHE et al., 2008ab). The 16S rRNA genes were amplified by PCR, using the archaeal specific forward and reverse primers 5'-TCCGGTTGATCCTGCCG (position 8-24) and 5'-GGAGGTGATCCAGCCG (position 1540-1525), respectively. The resulted DNA fragments were sequenced using BigDye Terminator Cycle Sequencing Kit (Pharmacia Biotech) and ABI Prism DNA genetic analyzer (Applied Biosystems). The sequences obtained were 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.

RESULTS AND DISCUSSIONS

Isolation of halophilic microorganisms and number of strains

The analysis of the salt crystal extracted from Slănic salt mine revealed the presence of inhabiting halophilic microorganisms able to grow in the presence of sodium chloride concentrations varying from 2M to saturation (5.2M). The number of colonies from the surface of the crystal ranged from 340 c.f.u./weight of crystal part (Table 1), in the case of the soil-contaminated crystal region, to none for another part of the crystal. In another part of the crystal the number of colonies was huge. The brine obtained from dissolving the washed crystal contained a variable c.f.u. number ranging from 6 to 120/weight of crystal part (Table 1). This number appears to be relatively high as compared with those reported in literature (DOMBROWSKI, 1963; NORTON et al., 1993). Figures 1 and 2 showed colonies obtained during the experimental procedure from the brine obtained by washing the salt crystal with sterile 20% NaCl solution.

Considering the number of colonies and based on the appearance of their pigmentation, predominantly dark red, 18 colonies were randomly picked up from the plate and transferred to agar slant for further investigation (Table 1). The strains unable to grow in the presence of taurocholic acid but able to grow in the presence of chloramphenicol were assigned as belonging to archaea.

Table 1. Samples characterization.

Crystal	Apparent contamination	Weight of crystal before washing step	Total observed colonies (colony forming units)		Strains		Observations
			Surface	Inside	Surface	Inside	
1	Apparent contaminated with trace of soil	5.86 g	340	120	7, 8, 9, 10	12, 13, 14, 15	Colony 14 is difficult to cultivate on agar slant
2	No contamination	4.05 g	absent	20	-	4, 5, 6	
3	No contamination	1.73 g	high number	6	3, 11	1,2, 16, 17, 18, 19	Colonies observed on brine from surface appear to be small and similar.

All the selected strains appear to belong to Archaea, suggesting that, at least inside of the salt crystal, the microorganisms associated to the salt crystal are not members of Eubacteria. In the case of crystal 3, colonies observed on brine from surface appear to be similar and were located in the small area of the plate. The strain 16 was lost after the second passage. The strains 1, 2 and 14 are difficult to cultivate on agar slant.

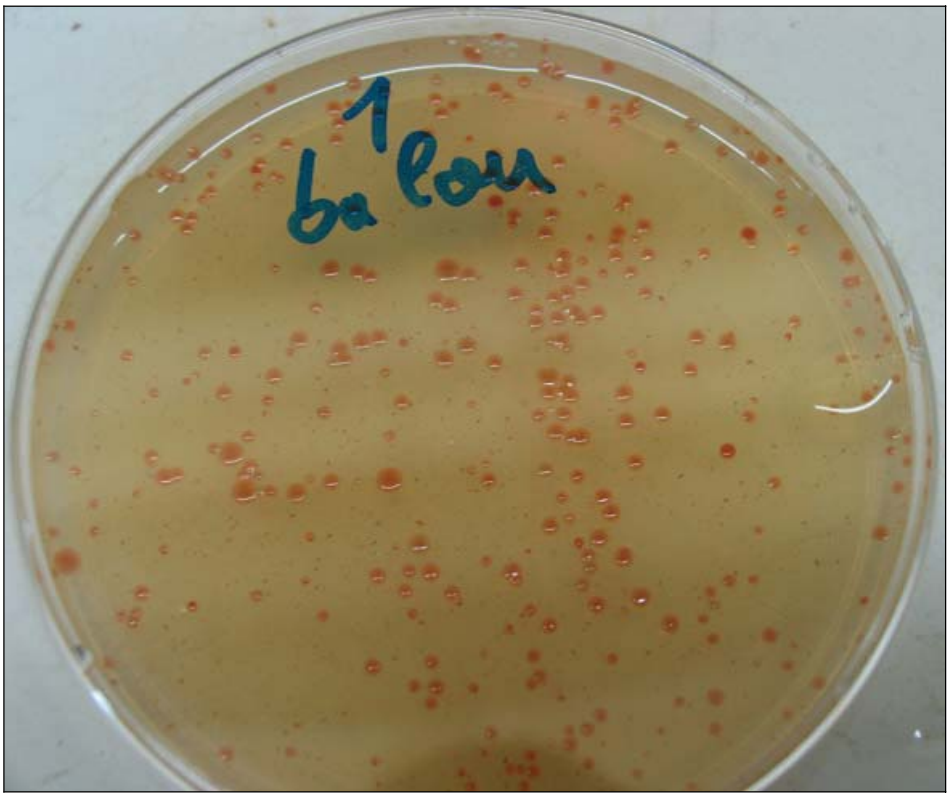

Figure 1. Colonies of red-pigmented haloarchaea obtained from the brine resulted by washing the salt crystal part 1 with sterile 20% NaCl solution (original).

Figure 2. Colonies of red-pigmented haloarchaea obtained from the brine resulted by washing the salt crystal part 3 with sterile 20% NaCl solution (original).

Biochemical characterization

The biochemical characteristics of the investigated strains are shown in table 2. All strains showed a negative Gram staining, and presented catalase activity. All strains were rod–shaped, with the exception of strain 6, isolated from inside of the crystal region that was not contaminated with soil traces, which showed an irregular shape.

The growth range of NaCl concentrations is indicated for all strains (Table 2). Eight out of the investigated strains were able to grow in a range of 2M to 5.2M, seven strains from 2.5M to 5.2M NaCl, strain 17 from 1.5M to 5.2M, and strain 19 from 3.0 to 5.2M. The optimum NaCl concentrations were also determined (Table 2). All these strains presented cell lysis when the cell suspension was diluted in distilled water, indicating halophilic specific behaviour.

Moreover, these strains were not catalysing the tryptophan deamination reaction. Strains 5, 6 and 14 were not able to catalyse the thiosulphate reduction to H_2S. Nine strains (3, 4, 5, 6, 7, 8, 9, 11 and 12) were able to hydrolyse the starch. In addition, three strains (13, 18 and 19) were not capable to catalyse oxidative reactions (Table 2).

The sensitivity to antibiotics (50µg/ml) of strains 3-17 was tested for chloramphenicol, penicillin, ampicillin erythromycin and novobiocin (Table 3). All the strains investigated presented resistance to chloramphenicol and ampicillin, and were not able to grow in the presence of sodium deoxycholate and novobiocin. Among the surface and inside strains, only one strain (13) showed no resistance to penicillin, and five strains (5, 12, 13, 15, 17) were not capable to grow in the presence of erythromycin.

Table 2. Biochemical features of investigated strains.

Test / Tested strain	A	B	C	D	E	F	G	H	I
1	2.0-5.2	2.5-3.0	N	N	N	N	N	N	N
2	2.0-5.2	3.0-4.0	N	N	N	N	N	N	N
3	2.0-5.2	2.5-3.5	-	B	+	+	+	-	+
4	2.5-5.2	3.5-4.0	-	B	+	+	+	-	+
5	2.5-5.2	4.0-4.5	-	CB	+	+	-	-	+
6	2.5-5.2	4.0-4.5	-	I	+	+	-	-	+
7	2.0-5.2	2.5-3.0	-	B	+	+	+	-	+
8	2.5-5.2	3.5-4.0	-	CB	+	+	+	-	+
9	2.5-5.2	2.5-3.0	-	B	+	+	+	-	+
10	2.5-5.2	3.5-4.0	-	CB	+	+	+	-	-
11	2.0-5.2	3.5-4.0	N	N	+	+	+	-	+
12	2.0-5.2	3.5-4.5	-	B	+	+	+	-	+
13	2.0-5.2	2.5-3.5	-	B	+	-	+	-	-
14	N	N	-	B	+	+	-	-	-
15	2.0-5.2	4.0-4.5	-	B	+	+	+	-	-
17	1.5-5.2	4.0-4.5	-	B	+	+	+	-	-
18	2.5-5.2	4.0-4.5	-	CB	+	-	+	-	-
19	3.0-5.2	4.0-4.5	-	CB	+	-	+	-	-

Legend: N = no data available; B = rod form; CB = irregular rod; I = irregular form; + = activity is present; - = activity is absent; red number represent strains isolated from the surface of the salt crystal; A = range of NaCl for growth (M); B = Optimum NaCl (M); C = Gram staining; D = Shape; E = Catalase; F = Oxidase; G = H_2S from thiosulphate; H = Indole from tryptophan; I = Starch hydrolysis**.**

Table 3. Antibiotic and bile salt resistance. Strain growth was carried out in the presence of various antibiotics (50 µg/ml) and bile salt, as described in methods. Strains isolated from the surface of the salt crystal (red), and inside the salt crystal (black); (+) strains able to grow; (-) strains not able to grow.

Antibiotic / Strain	Chl	NaDeox	Pen	Amp	Eryth	Nov
3	+	-	+	+	+	-
4	+	-	+	+	+	-
5	+	-	+	+	-	-
6	+	-	+	+	+	-
7	+	-	+	+	+	-
8	+	-	+	+	+	-
9	+	-	+	+	+	-
10	+	-	+	+	+	-
11	+	-	-	+	-	-
12	+	-	+	+	-	-
13	-	-	+	+	-	-
15	+	-	+	+	-	-
17	+	-	+	+	-	-

Legend: Chl = chloramphenicol, NaDeox = Sodium deoxycholate; Pen = penicillin; Amp = ampicillin; Eryth = erythromycin; Nov = novobiocin.

Phylogenetic tree reconstruction

The phylogenetic tree reconstructed from the 16S rRNA gene sequences (Fig. 3) revealed that most of the investigated strains grouped closely with *Halorubrum saccharovorum*. Other strains (18 and 19) were grouping with *Halobacterium noricense*, an organism isolated from Permian salt deposit in Austria (GRUBER et al., 2004), and strain 14 with *Haloarcula japonica*. This distribution is in accordance, most probably, with a high intragenomic heterogeneity occurring within this haloarchaeal genus. The strains 12 and 13, isolated from the inside of crystal apparently contaminated with trace of soil, clustered together with *Halorubrum* genus, suggesting that they constitute a new species. The other 11 strains (3, 4, 5, 6, 7, 8, 9, 10, 11, 15 and 17) (Fig. 3) were grouped in a tight cluster with *Halorubrum saccharovorum*, suggesting an intragenomic heterogeneity also within genus *Halorubrum*.

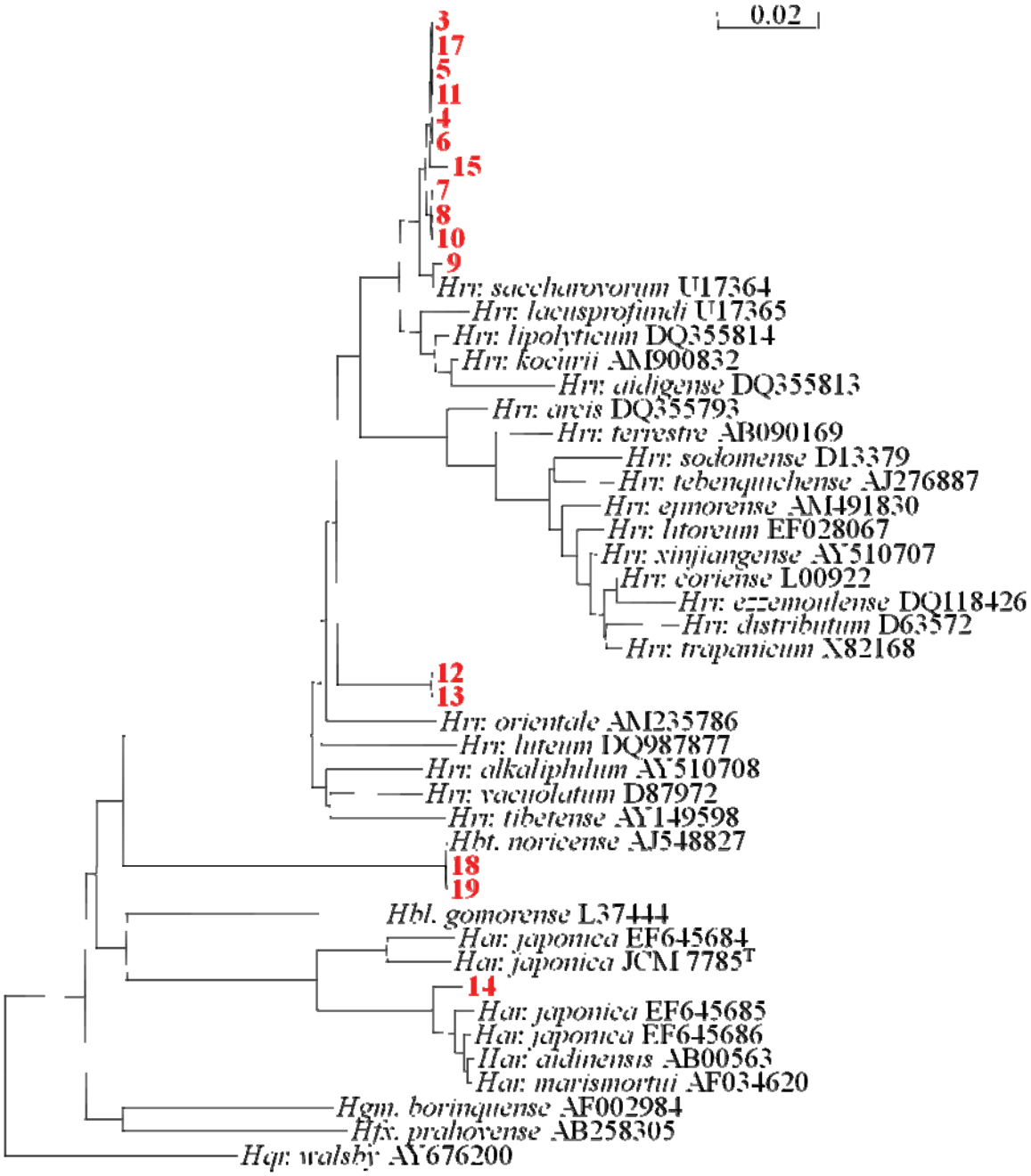

Figure 3. Phylogenetic tree based on partial sequences of 16S rDNA revealed the position of the investigated strains (in red) among species of genera *Halorubrum*, *Halobacterium* and *Haloarcula*. The tree was reconstructed by neighbour-joining method. Bootstrap values ≥ 70% (1000 replicates) are shown. Bar 0.02 substitutions per nucleotide position.

Predominant presence of Halorubrum strains

As mentioned above, the majority of the investigated strains (13 out of 18) appear to be either new species of *Halorubrum* (strains 12 and 13) or strains closely related to *H. saccharovorum* (11 strains). The strains 12 and 13 were isolated from inside of a salt crystal. From the total of 11 strains grouping with *Hrr. saccharovorum*, five were also isolated from inside of the salt crystal.

Taking into account the origin of salt deposit from Slănic, Prahova and previous data (ENACHE et al., 2008a, b) concerning halophilic microorganisms from the salted lakes surface from the same area, the predominance of *Halorubrum* species both inside and on the surface of the salt crystal suggested that the underground salt deposit host *Halorubrum* species as the dominant biota component of ancient origin, in contrast with surface salted lakes where the predominant biota are represented by members of the genus *Haloferax*.

CONCLUDING REMARKS

This work reported the first isolation and cultivation of haloarchaeal species from an underground salt massif, formed in the Neogene period, located in the area of Slănic, Prahova.

The phylogenetic tree of the 16S rRNA gene sequences from the investigated 18 strains revealed that these isolates belong to the *Halorubrum*, *Haloarcula* and *Halobacterium* genera. The strains grouping within *Halobacterium* genus were very similar with *Halobacterium noricense*, a cultivable haloarchaeal strain identified from ancient salt, salt mine environments, halite crust, and halite crystal from a saltern (GRAMAIN et al., 2011). The age of the salt hosting *Hbt. noricense* varies from 1.8 until to 250 MY (GRAMAIN et al., 2011), supporting the hypothesis of an ancient age for this haloarchaeal strains.

In accordance, our data revealing the absence of a tryptophan metabolizing pathway suggest the older age of the strain, while the capacity to transform thiosulphate argued for an age of at least 2.8 – 3.2 MY (BLANK, 2009).

Since the salt deposit from Slănic was formed during the Neogene period, there is a possibility that microorganisms isolated from this salt block to be relics of the life forms that existed in that area, since the occurrence of the first elements which subsequently created the deposit of salt.

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