Halophilic microorganisms from man-made and natural hypersaline environments: physiology, ecology, and biotechnological potential

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1 Introduction

What are hypersaline environments? Geologists or geochemists define saline lakes *sensu lato* as bodies of water with salinity more than 3 g/l (0.3%), while those *sensu stricto* (hypersaline) are bodies of water that exceed the modest 35 g/l (3.5%) salt of oceans (Williams 1998). Many microbiologists use the term hypersaline to denote the well-known salt lakes, such as the Dead Sea and the Great Salt Lake or crystallizer ponds of solar salterns, environments almost saturated with salt.

A lot of hypersaline environments are found in nature throughout the world, natural or man-made (Javor 1989a). Rock salt from salt deposits has been a source of sodium chloride for human beings from prehistoric period (Multhauf 1978). The subterranean salt deposit of the Mediterranean Sea is the result of Messinian salinity crisis 5.96–5.33 My ago (Duggen et al. 2003). Strong brines occur in both marine-derived (thalassic) and nonmarine (athalassic) systems. In arid coastal zones, large scale sabkhas, salt flats, or strong brines of tiny scales are observed (Javor 1989b). These hypersaline environments are too harsh for normal life to exist, but a variety of microbes, both Bacteria and Archaea, survive.

2 Halophilic microorganisms

When microbiologists are confronted with the question of how to define halophilic microorganisms, an answer is the classical definition by Kushner (1985), who categorized them into slight, moderate and extreme halophiles, depending on the NaCl concentration that supported optimal growth. The term "halotolerant" is

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generally used for organisms that are able to grow in media without added NaCl, but are able to grow at high salt concentration, while "halophilic" is for those that require addition of NaCl or other salts to media for their growth. The definition seems clear, but the fact is that many "halotolerant" microorganisms are able to grow at higher salt concentrations than some "halophilic" microorganisms.

Another answer is an operational definition by Oren (2002): "microorganisms that are able to grow well above 100 g/l salt." Since the time Oren compiled the data, numerous papers on the "halophilic microorganisms" have been published proposing new genera and species, both Bacteria and Archaea. In this chapter, we arbitrarily define halophilic microorganisms as those that require NaCl higher than 30 g/l for growth and are able to grow well above 200 g/l, irrespective of the salt concentration of the origin of isolation. Although most origins are hypersaline, above 100 g/l salt as defined by Oren, sometimes data of salt concentration of bodies of water are not described in the original papers. When the sources are soil samples (Ventosa et al. 2008) or materials collected on seashores or leaves of plants, it is difficult to measure the exact salt concentration of the very small niches in those samples where halophilic microorganisms are thriving.

2.1 Haloarchaea

Haloarchaea, members of the family *Halobacteriaceae*, are a group of extremely halophilic microorganisms, forming a part of the domain Archaea. The early investigations on the general bacteriology of red pigmented halophilic bacteria as the cause of the reddening of salted hides (red heat) and fish (red eye) began in the 1920s. The scientists came to the conclusion that marine or solar salts and rock salts are contaminated by the "halophilic bacteria" (Anderson 1954; Juez 1988). The eighth edition of Bergey's Manual of Determinative Bacteriology placed rod-shaped extreme halophiles in the genus *Halobacterium* with two species, *Halobacterium salinarium* and *Halobacterium halobium*, and coccoid extreme halophiles in the genus *Halococcus*, with only the species *Halococcus morrhuae* (Gibbons 1974). Soon, *Hbt. halobium* was suggested to be a member of *Archaebacteria* by Magrum et al. (1978). Tindall et al. (1980) were the first to isolate alkaliphilic haloarchaea that grow only in media of pH higher than 7.5, and they introduced the novel genera *Natronobacterium* and *Natronococcus* (Tindall et al. 1984). Two more genera, *Haloarcula* and *Haloferax*, were proposed to accommodate some new isolates and several species of the genus *Halobacterium* (Torreblanca et al. 1986). Since then, numerous strains have been isolated from hypersaline environments distributed all over the world. Neutrophilic strains were differentiated at the generic

Table 1A. Halophilic archaea and their distribution around the world[a]

Country	Saline environment	Archaea
Algeria	Ezzemoul sabkha	*Halorubrum ezzemoulense*
Antarctica	Deep Lake	*Halorubrum lacusprofundi*
Argentine	Salt flats (not specified)	*Haloarcula argentinesis* *Halomicrobium mukohataei*
Australia	Hamelin Pool, Shark Bay, Western Australia	*Halococcus hamelinensis* *Haloferax elongans* *Haloferax mucosum*
	Cheetham Salt Works, Geelong, Victoria	*Halonotius pteroides* *Haloquadratum walsbyi* *Halorubrum coriense* *Natronomonas moolapensis*
Austria	Salt mine, Altaussee	*Halobacterium noricense*
	Rock salt, Bad Ischl salt mine	*Halococcus dombrowskii* *Halococcus salifodinae*
Canada	Salted cowhide	*Halobacterium salinarum*[b]
Chile	Lake Tebenquiche, Atacama Saltern	*Halomicrobium katesii* *Halorubrum tebenquichense*
China	Saline soil, Daqing, Heilongjiang Province	*Haloterrigena daqingensis*
	Fuqing solar saltern, Fujian Province	*Halorubrum litoreum*
	Rudong marine solar saltern, Jiangsu Province	*Haladaptatus litoreus* *Halogeometricum rufum* *Halogranum rubrum* *Halopelagius inordinatus* *Haloplanus vescus* *Halosarcina limi*
	Solar saltern, Zhoushan archipelago, Zhejiang Province	*Haloferax larsenii*
	Sea salt, Qingdao, Shandong Province	*Halococcus qingdaonensis*
China (Inner Mongolia Autonomous Region)	Baerhu Soda Lake	*Natronolimnobius baerhuensis* *Natronolimnobius innermongolicus*
	Lake Bagaejinnor	*Halorubrum kocurii*
	Lake Chagannor, 17(C, pH 10.5	*Halorubrum luteum* *Natronorubrum sediminis*
	Chahannao soda lake	*Halobiforma nitratireducens* *Natrialba chahannaoensis*
	Lake Ejinor	*Halorubrum ejinorense* *Halorubrum orientale*

(*continued*)

Table 1A. (*continued*)

Country	Saline environment	Archaea
		Halovivax asiaticus *Natrinema ejinorense*
	Jilantai salt lake	*Halobacterium jilantaiense*
	Lake Shangmatala	*Halopiger xanaduensis*
	Lake Xilin Hot	*Halostagnicola larsenii* *Haloterrigena salina* *Halovivax ruber*
	Unnamed soda lake, Hulunbeir prefecture	*Natrialba hulunbeirensis*
China (Tibet Autonomous Region)	Bange salt-alkaline lake, pH 10	*Natronorubrum bangense* *Natronorubrum tibetense*
	Lake Zabuye, pH 9.4	*Halalkalicoccus tibetensis* *Halorubrum tibetense*
China (Xinjiang Uygur Autonomous Region)	Aibi (or Ebinur) salt lake	*Haloarcula amylolytica* *Halorubrum lipolyticum* *Haloterrigena limicola* *Haloterrigena longa* *Haloterrigena saccharevitans* *Natrinema versiforme* *Natronorubrum aibiense*
	Aiding salt lake	*Halorubrum aidingense* *Natronorubrum sulfidifaciens*
	Xiao-Er-Kule Lake	*Halorubrum xinjiangense*
	Saline lake (sampling site not specified)	*Halorubrum alkaliphilum*
	Ayakekum salt lake, Altun Mountain, pH 7.8	*Halobiforma lacisalsi* *Halorubrum arcis* *Natrinema altunense*
Egypt	Brine pool, Sinai	*Haloarcula quadrata*
	Saline soil, Aswan	*Halobiforma haloterrestris* *Halopiger aswanensis* *Natrialba aegyptiaca*
	Solar saltern, Alexandria	*Haloferax alexandrinus*
	Wadi Natrun	*Natronomonas pharaonis*
Israel/Jordan	The Dead Sea	*Haloarcula marismortui* *Halobaculum gomorrense* *Halococcus morrhuae*[c] *Haloferax volcanii* *Haloplanus natans* *Halorubrum sodomense*

(*continued*)

Table 1A. (*continued*)

Country	Saline environment	Archaea
Italy	"Red heat" in salted hides	*Natrinema pellirubrum*[d]
	Solar salt, Trapani, Sicily	*Halorubrum trapanicum*[d]
Japan	Solar salt, Niigata	*Natronoarchaeum mannanilyticum*
	Salt field, Ishikawa	*Haloarcula japonica*
	Sea sand (sampling site not specified)	*Natrialba asiatica*
Kenya	Lake Magadi	*Halorubrum vacuolatum* *Natrialba magadii* *Natronobacterium gregoryi* *Natronococcus amylolyticus* *Natronococcus occultus*
Mexico	Solar saltern, Baja California	*Halorubrum chaoviator*
Phillipines	Solar salt (sampling site not specified)	*Halarchaeum acidiphilum*
Puerto Rico	Solar saltern, Cabo Rojo	*Halogeometricum borinquense* *Haloterrigena thermotolerans*
Red Sea	Shaban Deep, bride–sediment interface (depth of 1,447 m, pH 6.0)	*Halorhabdus tiamatea*
Romania	Telega Lake, Prahova	*Haloferax prahovense*
South Korea	Jeotgal (salty condiment)	*Haladaptatus cibarius* *Halalkalicoccus jeotgali* *Halorubrum cibi* *Haloterrigena jeotgali* *Natronococcus jeotgali*
Spain	Fuente de Piedra salt lake, Malaga	*Haloterrigena hispanica*
	San Fernando solar saltern, Cadiz	*Halococcus saccharolyticus*
	Santa Pola solar saltern, Alicante	*Haloarcula hispanica* *Haloferax gibbonsi* *Haloferax lucentense* *Haloferax mediterranei*
Taiwan	Solar salt (sampling site not specified)	*Natrialba taiwanensis*
Thailand	Fermented salty foods (Ka-pi, Nam-pla, Pla-ra)	*Halobacterium piscisalsi* *Halococcus thailandensis* *Natrinema gari*
Turkmenistan	Saline soil	*Halorubrum distributum* *Halorubrum terrestre* *Haloterrigena turkmenica*
USA	The Great Salt Lake, Utah	*Halorhabdus utahensis*
	Death Valley, California	*Haloarcula vallismortis*

(*continued*)

Table 1A. (*continued*)

Country	Saline environment	Archaea
	Saltern, San Francisco Bay, California	*Haloferax denitrificans* *Halorubrum saccharovorum*
	Cargill Solar Salt Plant, Newark, California	*Halorubrum californiense*
	Rock salt crystals, Carlsbad, New Mexico	*Halosimplex carlsbadense*
	Zodletone Spring, Oklahoma	*Haladaptatus paucihalophilus* *Haloferax sulfurifontis* *Halosarcina pallida*

[a] *Natrinema pallidum* NCIMB 777 was isolated from salted cod fish, but the site of isolation is not clear
[b] Lochhead (1934)
[c] Kocur and Hodgkiss (1973)
[d] On-line catalog of NCIMB

level based on physiological characteristics and on the presence or absence of specific membrane lipids, phosphatidylglycerosulfate (PGS) and glycolipids (Kates 1995; Grant et al. 2001). Alkaliphilic strains, on the other hand, were devoid of detectable amount of glycolipids. The introduction of the PCR technique (Saiki et al. 1988) made analysis of 16S rRNA gene sequences easy in microbial taxonomy (Weisburg et al. 1991). The first phylogenetic tree of haloarchaea was reconstructed in 1993 using 19 sequences available at that time, demonstrating that species of the genera *Halobacterium, Halococcus, Haloarcula, Haloferax,* and *Natronobacterium* formed coherent clusters (Kamekura and Seno 1993).

At present, haloarchaeal strains are classified in 119 species of 32 genera (as of February 2010; Table 1A) (Enache et al. 2007b; Burns et al. 2010; Minegishi et al. 2010; Shimane et al. 2010). All strains of halophilic Archaea require NaCl higher than at least 4.7% (0.8 M) and are able to grow up to at least 23% (4.0 M) NaCl. Although the majority are neutrophiles, alkaliphilic species have been isolated from soda lakes or Wadi Natrun in Kenya, China, India, Egypt, etc. (Horikoshi 1999; Rees et al. 2004). They are accommodated in the genera *Natronobacterium, Natronococcus, Natronomonas* (Kamekura et al. 1997), *Halalkalicoccus* (Xue et al. 2005) and *Natronolimnobius* (Itoh et al. 2005). The genera *Halobiforma, Halorubrum, Natrialba* and *Natronorubrum* consist of both neutrophilic and alkaliphilic species.

Halophilic Archaea are believed to survive for long times in fluid inclusions of halite (Grant et al. 1998; Kunte et al. 2002; Stan-Lotter et al. 2003; Park et al. 2009). Recently, Fendrihan et al. (2009b) suggested the use of Raman spectroscopy as a potential method for the detection of extremely halophilic Archaea embedded in halite.

Table 1B. Halophilic bacteria and their distribution around the world

Country	Saline environment	Bacteria	Range of NaCl (%)	Optimum NaCl (%)
Algeria	Ezzemoul sabkha	*Halomonas sabkhae* *Salicola salis*	5–25 10–25	7.5 15–20
Canada	Contaminant on agar plate	*Actinopolyspora halophile*	10–33	15–20
Chile	Solar saltern, Cahuil, Pichilemu	*Halomonas nitroreducens*	3–20	5–7.5
China	Xiaochaidamu salt lake, Qinghai province	*Gracilibacillus halophilus*	7–30	15
China (Inner Mongolia Autonomous Region)	Lake Chagannor, 17°C, pH 10.5	*Bacillus chagannorensis* *Salsuginibacillus kocurii*	3–20 (salts) 3–20 (salts)	7 (salts) 10 (salts)
	Lake Shangmatala	*Aquisalibacillus elongatus*	3–20	10
	Lake Xilin Hot	*Virgibacillus salinus*	3–20	10
	Xiarinaoer soda lake	*Salsuginibacillus halophilus*	9–30	19
China (Xinjiang Uygur Autonomous Region)	Aiding salt lake	*Alkalibacillus salilacus* *Bacillus aidingensis* *Lentibacillus halodurans* *Prauserella sedimina* *Salinibacillus aidingensis*	5–20 8–33 5–30 5–20 5–20	10–12 12 8–12 10 10
	Qijiaojing Lake	*Haloechinothrix alba*	9–23	15
	Saline lake (sampling site not specified)	*Bacillus salarius* *Lentibacillus lacisalsi* *Saccharopolyspora halophilia*	3–20 5–25 3–20	10–12 12–15 10–15
	Saline soil (sampling site not specified)	*Alkalibacillus halophilus* *Nocardiopsis salina* *Prauserella halophila* *Saccharomonospora* *paurometabolica* *Streptomonospora alba* *Streptomonospora amylolytica* *Streptomonospora flavalba* *Streptomonospora halophila*	5–30 3–20 5–25 5–20 5–25 5–20 5–25 5–20	10–20 10 10–15 10 10–15 10 10 10
Congo	Oil-well head sample	*Halanaerobium congolense*	4–24	10
Egypt	Wadi Natrun	*Natranaerobius thermophilus* *Natranaerobius trueperi* *Natronovirga wadinatrunensis* *Thiohalospira alkaliphila*	18–29 19–31 19–31 3–23	19–23 22 23 12
France	Salin-de-Giraud saltern, Camargue	*Halanaerobacter salinarius* *Halorhodospira neutriphila* *Thiohalocapsa halophila*	5–30 6–30 3–20	14–15 9–12 7

(*continued*)

Table 1B. (*continued*)

Country	Saline environment	Bacteria	Range of NaCl (%)	Optimum NaCl (%)
Greece	Saltworks, Mesolongi	*Bacillus halochares*	6–23	15
Iran	Lake Aran-Bidgol	*Lentibacillus persicus*	3–25	7.5–10
	Howz Soltan Lake	*Bacillus persepolensis*	5–20	10
Iraq	Saline soil	*Actinopolyspora iraqiensis*	5–20	10–15
Israel/Jordan	The Dead Sea	*Rhodovibrio sodomensis* *Salisaeta longa* *Selenihalanaerobacter shriftii* *Virgibacillus marismortui*	6–20 5–20 10–24 5–25	12 10 21 10
Japan[a]	Solar salt	*Nesterenkonia halobia*	3–25	–
	Salted foods	*Chromohalobacter japonicus* *Halanaerobium fermentans*	5–25 7–25	7.5–12.5 10
Kenya	Lake Magadi	*Natroniella acetigena*	10–26	12–15
Kuwait	Salt marsh soil	*Saccharomonospora halophila*	10–30	–
Mexico	Solar saltern, Baja California	*Halospirulina tapeticola*	3–20	10
	Brine water, Gulf of Mexico	*Halanaerobium acetethylicum*	5–22	10
Mongolia	Barun-Davst-Nur	*Halovibrio denitrificans*	12–30	12–15
Nauru (South Pacific)	Sea shore wood	*Salimicrobium halophilum*	3–30	–
Peru	Maras salterns, Andes	*Salicola marasensis*	10–30	15
Portugal	Terminal pond of a saltern	*Rhodovibrio salinarum*	3–24	9–15
Puerto Rico	Black mangrove, solar saltern of Cabo Rojo	*Halobacillus mangrove*	5–20	10
Russia	Kulunda Steppe, Altai	*Halospina denitrificans* *Methylohalomonas lacus* *Thiohalorhabdus denitrificans* *Thiohalospira halophila* *Thiomicrospira halophila*	12–30 3–23 9–23 12–30 3–20	15–18 12 18 15–18 9
Senegal	Retba Lake	*Halanaerobium lacusrosei*	7.5 to saturated	18–20
South Korea	Byunsan solar saltern, Yellow Sea	*Lentibacillus salinarum*	3–24	10–12
	Solar saltern, Yellow Sea	*Alkalibacillus flavidus*	4–26	10
	Seawater, Yellow Sea	*Salinisphaera dokdonensis*	4–21	10
	Kunsan solar saltern	*Nocardiopsis kunsanensis*	3–20	10
	Jeotgal (salty condiment)	*Lentibacillus jeotgali*	3–20	10–15

(*continued*)

Table 1B. (*continued*)

Country	Saline environment	Bacteria	Range of NaCl (%)	Optimum NaCl (%)
Spain	Soil from Fuente de Piedra, saline wetland, Malaga	*Halomonas fontilapidosi*	3–20	5–7.5
	Cabo de Gata solar saltern, Almeria	*Halomonas almeriensis* *Kushneria indalinina* *Salinicola halophilus*	5–25 3–25 3–25	7.5 7.5–10 7.5–10
	Mallorca solar saltern, Balearic Islands	*Salinibacter rubber*	15–33 (salts)	20–30 (salts)
	Santa Pola solar saltern, Alicante	*Halomonas cerina* *Virgibacillus salexingens*	7.5–20 7–20 (salts)	7.5–10 10 (salts)
Thailand	Fermented salty foods (Ka-pi, Nam-pla, Pla-ra)	*Lentibacillus halophilus* *Lentibacillus juripiscarius* *Lentibacillus kapialis*	12–30 3–30 5–30	20–26 10 15
Tunisia	Chott El Guettar	*Halothermothrix orenii*	4–20	10
	Chott El-Djerid	*Halanaerobaculum tunisiense*	14–30	20–22
Ukraina	Lake Sivash, Crimea	*Halanaerobium saccharolyticum* *Halocella cellulosilytica* *Orenia sivashensis*	3–30 5–20 5–25	10 15 7–10
USA	The Great Salt Lake, Utah	*Halomonas variabilis*	7–29	9
	Death Valley, California	*Actinopolyspora mortivallis*	5–30	10–15
	Saltern, San Francisco Bay, California	*Halanaerobacter chitinivorans*	3–30	12–18
	Evaporated sea water, Oregon	*Rhodothalassium salexigens*	5–20	12
	Saline oil field brine, Oklahoma	*Arhodomonas aquaeolei* *Halanaerobium salsuginis*	6–20 6–24	15 9
	Searles Lake, California	*Halarsenatibacter silvermanii*	20 to saturated	saturated

[a]The following species have been isolated from ordinary, nonsaline soil samples taken in Japan: *Alkalibacillus silvisoli* 5–25% (10–15%), *Geomicrobium halophilum* 5–25% (10–15%), and *Halalkalibacillus halophilus* 5.0–25% (10–15%)

2.2 Halophilic Bacteria

Table 1B is a summary of saline and hypersaline environments and the halophilic Bacteria isolated from these sites. The range of salt concentrations, mostly NaCl, or a mixture of salt in some cases, that permitted growth and optimum concentration are also indicated. The species listed in Table 1B belong to the following classes: Cyanobacteria of the phylum Cyanobacteria, Alphaproteobacteria and Gammaproteobacteria of the phylum Proteobacteria, Clostridia and “Bacilli” of the phylum

Firmicutes, Actinobacteria of the phylum Actinobacteria, and Flavobacteria of the phylum Bacteroidetes. References for each species are not given in the list because of the huge number which would have to be cited. Readers are recommended to consult the very useful web site "List of Prokaryotic names with Standing in Nomenclature," maintained by J. P. Euzéby at http://www.bacterio.cict.fr/for relevant papers.

2.3 Romanian hypersaline environments

Hypersaline environments are widely distributed also in Romania, either in solid form or in liquid form: salt lakes and salt mines located in Prahova county, the Techirghiol lake nearby to Black Sea coast, the Balta Albă lake in Buzău county, etc. (Fig. 1). Some of these environments have been well described some time ago (Broşteanu 1901) and today also constitute an attractive research area either for

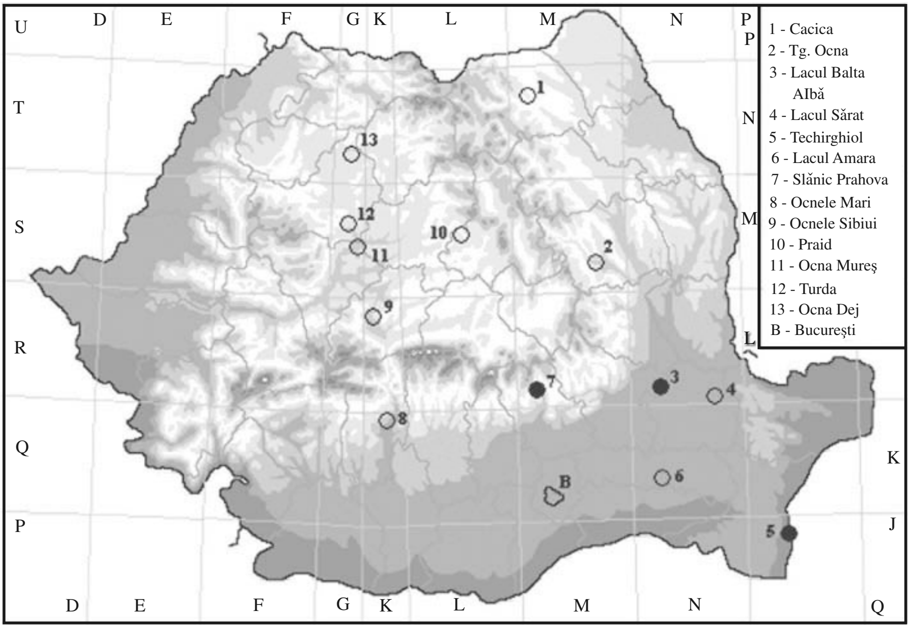

Fig. 1. Geographical positions of major hypersaline environments in Romania. The filled points represent the examined areas described in the text. **1**, salt mine at Cacica; **2**, salt mine at Targu; **3**, salt lake (Lacul Balta Alba); **4**, hypersaline salt lake (Lacul Sarat); **5**, salty therapeutical mud lake at Techirghiol (near the coast of the Black Sea); **6**, haloalkaline lake (Lacul Amara); **7**, lakes and salt mine of Slănic (Slănic Prahova); **8**, salt mine at Ocnele Mari (Ocnele Mari); **9**, lakes and salt mine at Sibiu (Ocnele Sibiu); **10**, salt mine at Praid (Praid); **11**, salt mine at Mures (Ocna Mures); **12**, salt mine at Turda (Turda); **13**, salt mine at Dej (Ocna Dej); B, Bucharest, capital of Romania

geologists or for biologists (Sencu 1968; Faghi et al. 1999; Teodosiu et al. 1999; Har et al. 2006).

Salt exploitation in the Romanian Carpathian area has been conducted by various methods from antiquity until today (Drăgănescu and Drăgănescu 2001), due to the presence of lots of salt massifs with characteristics that supported their continued use such as surface proximity, superior purity of NaCl, or large reserves. In some places, the massifs are located at the surface as small salt mountains, for example at Slănic, Praid, Sărata Monteoru (Fig. 1). In the Slănic Prahova area for example, salt exploitation started in 1685 by using bell type exploitation technology. After the eighteenth century, some areas of exploitation were abandoned, resulting in various man-made salt lakes with different depths (varying from 3 until to 40 m, see Table 2) and widths, along to the left or right side of the Slănic valley (Drăgănescu 1990). On the other hand, in Telega, where similar technologies were used, salt extraction started before 1685 and relinquished exploitation areas

Table 2. Chemical features of examined lakes and colony forming units (c.f.u.), as determined on agar plates containing 12.5% NaCl and 16% $MgCl_2 \cdot 6H_2O$, from surface water samples

Lake	Maximum depth (m)	pH	Density	Chloride content (g/l)	c.f.u./ml
Red Bath	3	7.9	1.06	74.9	2500
Green Bath	40	9.0	1.10	138.5	1050
Shepherd Bath	7.25	8.7	1.07	97.4	2400
Bride Cave	32	8.3	1.20	254.6	750
Techirghiol	9	7.0	–	60	–
Telega (Palada)	36	8.3	1.15	161	1100
White Bath	–	8.6	1.10	44	890

Table 3. Characteristics of salt lakes from Telega (Gâştescu 1965)

Lake	Height above the sea (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 (Telega)	416	1416	30	36

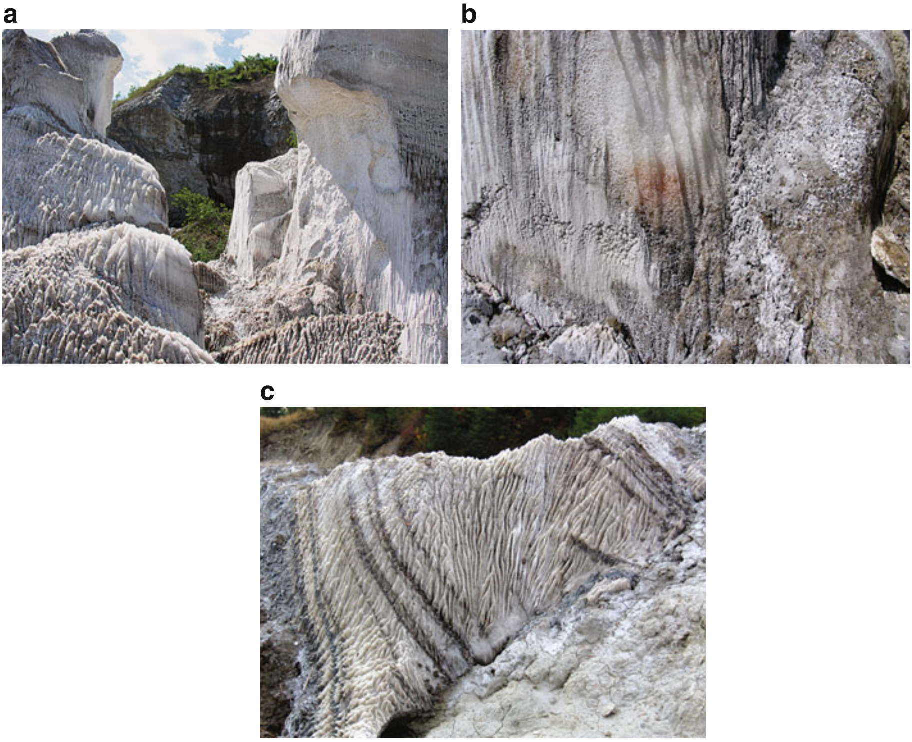

Fig. 2. Salt massifs in the Slănic Prahova (**a**, **b**) and Praid (**c**) areas. The red color may be possible due to the presence of microorganisms or their remnants inside of the salt massifs in Slănic (**b**)

resulted in various man-made salt lakes, as shown in Table 3 (Gâştescu 1971; Enache et al. 2008a).

The salt deposit in Slănic Prahova formed in the Neogene period (24 My ago) and is located at around 100 km north of Bucharest in a sub-Carpathian hillock area (Figs. 1 and 2). This salt deposit has various lengths and thicknesses as described previously (Drăgănescu 1990; Enache et al. 2008a). Investigations for the presence of halophilic microorganisms were conducted in several salt lakes which formed in the relinquished salt mine exploitations in this salt deposit (see Sect. 2.4). These lakes are known today as Bride Cave, Red Bath, Green Bath, and Shepherd Bath.

The salt deposit of Telega (Doftana-Telega), which formed also in the Neogene period, is located at a similar distance and position from Bucharest such as the Slănic deposit, and is a mixture of crystals with colors varying from white to gray and swarthy, having a surface of 2.1 km^2. Various salt lakes which resulted in the mouth of an abandoned salt mine in this deposit are detailed in Table 3.

Another hypersaline environment with large reserves located at Ocnele Mari (Vâlcea county) has been exploited by dissolving salt, and the brine was channeled directly into a petrochemical plant by pipelines for the production of various chemicals ($NaOH$, Na_2CO_3, HCl, Cl_2, $NaOCl$, etc.). Consequently, a large subterranean void resulted which nowadays is under control to preserve collapsing. A huge man-made salt lake, estimated as larger than 50 ha, is expected to be present. This man-made hypersaline environment will constitute a further subject for halophilic research.

Another examined salt lake is Techirghiol lake, located near the Black Sea coast. This lake is characterized by variable concentrations of salt, ranging from negligible to 60 g/l. The lake is relatively well known for its biological communities and saline regimes, and has been well investigated over time, because of either the important therapeutical properties of the mud present in the lake or the dynamics of moderately halophilic microbial communities.

Another hypersaline environment, recently investigated by our team, is represented by the Balta Albă salt lake, located near the town of Râmnicu Sărat. The area is characterized by the presence of various salty (not extremely) soils. Various hypotheses have been elaborated on the origin of a salt lake in this area, arguing for either an ancient origin or the consequence of intense evaporation processes which took place in this area. Some geological data supported the latter hypothesis (Gâştescu 1965). The salinity of this lake was 40 g/l in our surface sample water.

2.4 Halophiles from salty environments in Romania

The chloride concentration of water samples taken from these salt lakes ranged from 44 to 254.6 g/l (Table 2) and pH values between 7.0 and 9.0. A number of these environments have been found to contain halophilic microorganisms able to grow in the presence of high concentrations of $NaCl$ and $MgCl_2 \cdot 6H_2O$. The numbers of colonies ranged from 750 c.f.u./ml in water samples from Bride Cave until to around 2,500 c.f.u./ml in Red Bath (Table 1). In other lakes, the colony numbers were relatively low, when compared to those reported for various salt lakes with similar salt concentrations (Enache et al. 2008b). Investigations have been conducted (Enache et al. 1999b, 2000a, b) and a new species *Haloferax prahovense* was proposed recently (Enache et al. 2007a).

The diversity of halophiles, mainly in crystallizer ponds of solar salterns, remains one of the important research targets, although the constant NaCl concentrations of these artificial hypersaline environments over the years could be considered as a selective pressure for halophilic microorganisms. Due to various climatic and other natural conditions, different salt concentrations could be found in the salt lakes.

Although various molecular techniques were applied to understand the ecological principles of salt lakes, the microorganisms which could be isolated and cultivated in the laboratory appear to play the most important role in the ecological economy of these lakes.

The number of colony forming units decreased with increasing chloride and sodium concentrations in the examined salt lakes, but the colonies assigned to be Archaea had no apparent correlation with the concentrations of these two ions (Enache et al. 2008b). The predominant presence of *Haloferax* species in these lakes (Enache et al. 2008a, b) suggests that members of this genus play an important role in the ecology of salt lakes, even though the largest numbers of species, which have been identified in hypersaline environments, are of the genus *Halorubrum*. On the other hand, the microbiota of subterranean rock salt from the Slănic area are characterized by the presence of some *Halorubrum* and *Haloarcula* species, and some isolates appear to be closely related to *Hbt. noricense*, a strain isolated from an ancient evaporite formed in the Permo-Triassic period (Gruber et al. 2004). The *Haloferax* members, observable in hypersaline lakes located at the surface of the Slănic salt deposit, were identified also in subterranean rock salt, but with decreasing numbers (unpublished data).

3 Biotechnological potential

The physiological and biochemical features specific for halophilic Archaea and Bacteria as well as their capacity to produce biopolymers, enzymes, osmoprotectors of industrial interest, with properties superior to those synthesized by nonhalophilic species, make them a promising group of unlimited biotechnological potential (Rodriguez-Valera 1992). In the following sections, we would like to describe the potential for biotechnological application of halophilic microorganisms, including those isolated from Romanian hypersaline environments. Readers are also advised to consult previous general reviews (Rodriguez-Valera 1992; Ventosa et al. 1998; Horikoshi 1999; Mellado and Ventosa 2003; Ventosa 2004; Borgne et al. 2008).

3.1 Bioremediation

Biodegradation of organic pollutants by halophilic Bacteria and Archaea has been reviewed by Borgne et al. (2008). A few moderately halophilic bacteria were found to show the capacity, to various degrees, of decontaminating the pesticide dichlorvos ($C_4H_7O_4Cl_2P$) from saline environments (Oncescu et al. 2007). These strains, which were isolated from Shepherd Bath, Telega (Palada), and Techirghiol lakes, were tentatively assigned as *Halomonas* species according to their preliminary biochemical and physiological characterization. The kinetics of dichlorvos degradation appeared to follow the mechanism of "compatible solutes", used by large numbers of organisms to cope with osmotic stress generated by the presence of high concentrations of salt. Considerable efforts are still necessary in order to estimate the true potential of these halophilic microorganisms to be applied in environmental processes and in the remediation of contaminated hypersaline ecosystems. This effort should also be focused on basic research to understand the overall degradation mechanism, to identify the enzymes involved in the degradation process and the metabolic regulation.

3.2 Nanobiotechnology

A novel approach involving halophilic microorganisms appears to be their potential for nanobiotechnology. The interaction between some silica and titanium nanotubes with moderately halophilic microorganisms, for example *Virgibacillus halodenitrificans*, has recently been reported (Merciu et al. 2009). Variation in methods for the preparation of nanotubes (hydrothermal method or sol–gel method in the presence of templates) and different chemical treatments (thermal treatment and acid washing) after synthesis appear to be correlated with a putative antibacterial effect of these nano-materials toward various halophilic strains. Although a bacteriocin (halocin)-like attack mechanism is likely, further investigation will be necessary concerning the interaction between nano-materials and halophiles.

3.3 S-layers

Crystalline cell surface layers are commonly observed cell envelope structures of several Bacteria and Archaea, and they have numerous applications in biotechnology and nanotechnology. The extremely halophilic Archaea lack a peptidoglycan component in their cell wall and contain simple S-layers external to the cell membrane (Trachtenberg et al. 2000; Eichler 2003). S-layers consist of identical protein or glycoprotein subunits and completely cover the cell surface during all stages of growth and division. Most S-layers are 5–15 nm in thickness and possess pores of identical size and morphology in the 2–8 nm range (Sleytr and Sara 1997; Schuster et al. 2005).

Isolated S-layer subunits of various microorganisms have the intrinsic ability to self-assemble into highly defined monomolecular arrays either in suspension, at air/water interfaces or liquid/surface interfaces, including lipid films, liposomes, and solid supports such as silicon wafers (Schuster and Sleytr 2000; Schuster et al. 2005). The relative simplicity, regularity, and symmetry within the monolayer plane of the S-layer make it an attractive subject for nanobiotechnological studies with targets for medical applications (Sleytr et al. 1999; Trachtenberg et al. 2000).

Our investigations on S-layers were carried out using a halophilic archaeon, *Haloferax* sp. strain GR 2 (deposited as JCM 13922), isolated from the Bride Cave Lake in Prahova county. Preliminary investigations related to the binding of S-layer to some porous silicon substrates were performed. The biochemical characterizations by protein content and chemical treatment had demonstrated the presence of S-layer in the isolated strain. Transmission electron microscopic examination of the isolated S-layer showed the existence of the monomolecular crystalline lattice with a highly ordered arrangement in the dense form, while in relaxed form after treatment with 4 M urea (Dumitru et al. 2007). The S-layer proteins attached to both hydrophilic and hydrophobic surfaces of all plates of porous silicon, which were investigated, but it seemed that the hydrophobic surfaces were more favorable. Thus, the treatment of silicon plates with hexamethyldisilazane, which promotes the hydrophobicity and organic character of the porous silicon surface, increased the amount of attached S-layer protein (Sleytr et al. 1999; Dumitru et al. 2007; Kleps et al. 2009).

3.4 Extracellular enzymes

A great deal of information on eukaryotic and bacterial halophilic enzymes is currently available, for examples, on amylases, lipases, nucleases, nucleotidases, and proteases (Ventosa et al. 2005). For the production of Thai fish sauce (*nam-pla*), a condiment similar to *Garum* or *Liquamen* of the ancient Roman society, prepared from fish in concentrated brine, proteases of haloarchaea which are present in solar salt, plays an important role in the degradation of fish protein into amino acids (Thongthai et al. 1992). Further studies on haloarchaeal enzymes are expected to contribute to the elucidation of the properties of these extracellular enzymes.

Amylases were produced by some strains isolated from Bride Cave and Techirghiol lakes (Enache and Faghi 1999, 2009). The enzyme of *Hfx.* sp. GR1 purified by ethanol precipitation and differential chromatography showed maximum activity at pH 6.5 and 50°C in the presence of 3.5 M NaCl, and lost its activity below 1.5 M NaCl (Enache et al. 2001). Amylases produced by strains of *Haloferax* and *Halorubrum* isolated from Techirghiol lake, a low salt environment, showed higher activity with increasing concentration of $MgCl_2$ in the presence of the relatively low 2.1 M NaCl, but activity decreased with increasing Mg concentrations at the higher concentration of 3.4 M NaCl (Enache et al. 2009).

Extracellular lipase activity was detected in some strains isolated from lakes Shepherd Bath, Green Bath, Red Bath, and Bride Cave. Among them, the enzyme produced by *Hfx.* sp. GR1 was influenced by NaCl concentrations in the growth media and had a maximum activity at 3 M NaCl. The activity was lost at NaCl concentrations below 2.5 M (Enache et al. 2004a).

The molecular mechanisms of the adaptation of enzyme proteins to high salt have been described in detail (Vellieux et al. 2007, Yamamura et al. 2009).

3.5 Halocins

The halocins, proteinaceous antibiotics, which are haloarchaeal equivalents of bacteriocins, were first discovered by Rodriguez-Valera et al. (1982). The wide variety of activity spectra detected for halocins (H1, H4, H6, S8, C8, etc.) may imply that a great number of different halocins are produced and probably show various mechanisms of action (Torreblanca et al. 1994; O'Connor and Shand 2002). Halocins were also detected in some strains isolated from Romanian salt lakes, and they showed a variety of action spectra (Enache et al. 1999a). When compared as halocin producers and targets, some strains showed identical patterns, supporting the tight clustering of strains in the phylogenetic tree reconstructed from 16S rRNA gene sequences (Enache et al. 2004b, 2008b).

Halocin 6 (H6) is a protein of 32 kDa produced by *Hfx. gibbonsii* SH7 and blocks the Na^{+}/H^{+} antiporter in sensitive strains. Quite interestingly, H6 has been shown to inhibit in vitro the Na^{+}/H^{+} exchanger of mammalian cells and to exert in vivo a cardio-protective effects against ischemia and reperfusion injury (Lequerica et al. 2006).

3.6 Exopolysaccharides

Halophilic microorganisms are able to synthesize extracellular polysaccharide (EPS), which are biopolymers combining, in an excellent manner, the rheological properties (high viscosity and pseudoplasticity) with a remarkable resistance to extreme salinity, temperature, and pH values, conditions which are encountered in several industrial processes and which make usage of biopolymers produced by nonhalophilic microorganisms impossible (Rodriguez-Valera 1992; Ventosa et al. 1998). The physical and chemical properties of the EPS produced by halophilic microorganisms enable their utilization in the food, textile, and dye industries, also for the production of pharmaceuticals and cosmetic products, in the oil extracting industry as well as for processes for the removal of toxic compounds.

The biosynthetic activity for EPS was detected in some *Haloferax* strains isolated from Telega (Palada) lake such as *Hfx. prahovense* and *Hfx.* sp. TL5. The optimal conditions for EPS production by *Hfx. prahovense* were the same as those resulting in highest cell growth. The maximum EPS yield (0.475 g%) was obtained in medium with 3% glucose as single carbon source at 2 M NaCl, under stirring at 200 rpm, at 37°C, after 7 days of incubation. The strain produced EPS also in media with galactose, lactose, maltose, sucrose, or fructose as carbon source. Higher salt concentrations (5 M) and higher temperature (45°C) had an inhibitory effect on both growth and EPS synthesis. Synthesis of EPS started during the early exponential growth phase, increased concomitantly with a rise in the number of viable cells, and then decreased after 7 days of cultivation. The monomer composition of the EPS from *Hfx. prahovense* was similar to the composition of EPS synthesized by halophilic Archaea of the genus *Haloferax* (Anton et al. 1988). The polymer of *Hfx. prahovense* was a heteropolysaccharide containing mainly glucose, fructose, galactose, and mannose as was observed by TLC. Differential scanning calorimetry revealed that the polymer was stable up to 207°C; the chemical composition observed by TLC was confirmed by FTIR investigations. FTIR also showed the presence of uronic acids and sulfate in the polymer (Popescu et al. unpublished results). A similar highly thermostable EPS was isolated and characterized from cultures of some moderately halophilic bacteria isolated from Shepherd Bath (Cojoc et al. 2009).

3.7 Resistance to heavy metals

Several halophilic Archaea (Dumitru et al. 2002) and Bacteria (Enache et al. 2000c) isolated in Romania were shown to be resistant to heavy metals. The data suggested that moderately halophilic Bacteria exhibited a higher tolerance to metallic ions as compared to halophilic Archaea. The investigated haloarchaeal strains were susceptible to Zn and Hg, but moderately resistant to Cr and Ni, being classified as tolerant according to the criteria proposed by Nieto (1991).

The metal tolerance level of our isolate *Haloferax* sp. TL5 (assigned as a strain of *Hfx. prahovense*, see Enache et al. 2008a) was compared with that of *Hfx. mediterranei*. Strain *Hfx.* sp. TL5 showed a similar behavior to the collection strain *Hfx. mediterranei*; both strains tolerated 5.0 mM Cr and 2.5 mM Ni and Pb. Strain *Hfx.* sp. TL5 had a higher susceptibility for Zn ions, compared with *Hfx. mediterranei*.

We also measured the capacity of these strains to reduce the concentration of several heavy metal ions from media. Both strains showed the capacity to reduce the concentration of Pb, Cr, Zn, and Ni ions from media with high salinity (Popescu and Dumitru 2009). The two strains produced higher cell densities when grown in media with metal ions and 2–2.5% glucose than in media without glucose. This suggests that EPS synthesized in the presence of glucose may protect the cells against the toxicity of heavy metals. The two *Haloferax* strains showed the same capacity to reduce the concentration of Pb ion; for example, the initial concentration of 331 mg Pb/l was reduced to 5 mg/l after 10 days of cultivation. *Hfx.* sp. TL5 has a higher biosorption capacity of Cr and Ni ions from medium with or without glucose than *Hfx. mediterranei*.

Hfx. mediterranei presented a higher removal activity of Zn ion from media with or without glucose than *Hfx.* sp. TL5 (Popescu and Dumitru 2009). The results revealed that the synthesis of EPS enhanced the reduction activity of Cr, Zn, and Ni by the haloarchaeal strains which were investigated. The anionic nature of EPS synthesized by *Haloferax* strains, based on their high sulfate and uronic-acid contents (Rodriguez-Valera 1992; Mellado and Ventosa 2003), is similar to that of EPS synthesized by other halophilic microorganisms and may be responsible for the capacity of these strains to bind and remove heavy metals from solutions with high NaCl concentrations. This property would make these biopolymers a viable alternative to the more aggressive physical and chemical methods, and they could be used as bioadsorbents in polluted hypersaline environments.

3.8 Therapeutical value

The salt lakes in Romania have been used also for various economical, recreational, and therapeutical purposes. The therapeutical use of the mud from salt lakes started in 1840 (Bulgăreanu 1993). Although attributed to the accumulation of sapropelic material, the mechanisms of mud formation and their microbiota are poorly understood. A few attempts in our laboratory to elucidate the mechanisms, using the mud from Techirghiol lake, were fruitless. An industry has been developed for the exploitation of mud mainly from Balta Albă and Techirghiol lakes.

In Middle Europe and Eastern Europe, the advantageous effect of some natural salt caves on lung diseases has been known since the nineteenth century; possibly, salt-miners knew it far earlier based on the observation that injured animals went to caves for recovering. The beneficial effect of salt (speleotherapy) was reported by the Polish doctor F. Bochkowsky in 1843. Speleotherapy is based on the in-patient treatment in salt caves possessing a specific microclimate. The effects of salt mine treatment on health in the village of Solotvino in the Carpathian Mountains have been investigated by Russian scientists (Simyonka 1989). Several salt caves are used for speleotherapy in Middle Europe and Eastern Europe as follows: Salzgrotten, Saliseum/Vienna (Austria); Slanic, Turda, and Praid (Romania); Wieliczka (Poland); Nakhlichevan (Azerbaidjan); Chon-Tous (Kirghizstan); Cave Berezniki in Perm (Russia); Solotvino (Ukraine). Halotherapy is a form of speleotherapy, a science aimed to create somewhat similar conditions in a microclimatic environment as in salt caves. In the 1980s, Russia was the pioneer in creating the first salt chambers. These are specially prepared rooms with walls and basements covered with halite, a crystal form of salt (Chervinskaya et al. 1995; Hedman et al. 2006; Nica et al. 2007).

4 Concluding remarks

Research in the field of halophilic microbiology attracted huge interest during the last decade, yielding more than 18 new genera (from 2000 to the present) in the archaeal family *Halobacteriaceae* and numerous bacterial genera (Table 1). Many of them have their origin in man-made "hypersaline" environments developed for various purposes, e.g. commercial solar salt and salted food. These sources have a connection to marine environments, which apparently are not "hypersaline." We would like to remind the readers of the fact that a relatively small number of the validly published halophilic species of Archaea and Bacteria come from truly hypersaline lakes (such as the Dead Sea or the Great Salt Lake). Another fact is that no rigid evidence of the existence of viable haloarchaeal cells in seawater has been presented, except perhaps for halococci (Rodriguez-Valera et al. 1979). Taking into account these facts, future research will be necessary to give an answer to the question of how the halophilic microorganisms originated during the early stages in the evolution of life and how they diversified and were distributed throughout the world, in the past and present (Oren 2004). Although the molecular clock of organisms isolated from inclusion bodies of ancient salt crystals is difficult to be correlated with the geological time of evaporation, the question of longevity of halophilic Archaea and Bacteria continues to be a tremendous fascination to all microbiologists (Grant et al. 1998; Fendrihan et al. 2009a, b; Park et al. 2009).

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