

## MINIREVIEW

# Living with salt: metabolic and phylogenetic diversity of archaea inhabiting saline ecosystems

Adrian-Ştefan Andrei<sup>1</sup>, Horia Leonard Banciu<sup>1</sup> & Aharon Oren<sup>2</sup>

<sup>1</sup>Department of Experimental Biology, Faculty of Biology and Geology, Babeş-Bolyai University, Cluj-Napoca, Romania; and <sup>2</sup>Department of Plant and Environmental Sciences, The Institute of Life Sciences, and the Moshe Shilo Minerva Center for Marine Biogeochemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

**Correspondence:** Horia Leonard Banciu, Faculty of Biology and Geology, Babes-Bolyai University, 5-7 Clinicilor Str., 400006 Cluj-Napoca, Romania. Tel.: +40 264 431691; fax: +40 264 431858; e-mail: Horia.Banciu@ubbcluj.ro

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### Abstract

Archaea that live at high salt concentrations are a phylogenetically diverse group of microorganisms. They include the heterotrophic haloarchaea (class *Halobacteria*) and some methanogenic Archaea, and they inhabit both oxic and anoxic environments. In spite of their common hypersaline environment, halophilic archaea are surprisingly diverse in their nutritional demands, range of carbon sources degraded (including hydrocarbons and aromatic compounds) and metabolic pathways. The recent discovery of a new group of extremely halophilic *Euryarchaeota*, the yet uncultured *Nanohaloarchaea*, shows that the archaeal diversity and metabolic variability in hypersaline environments is higher than hitherto estimated.

## Introduction

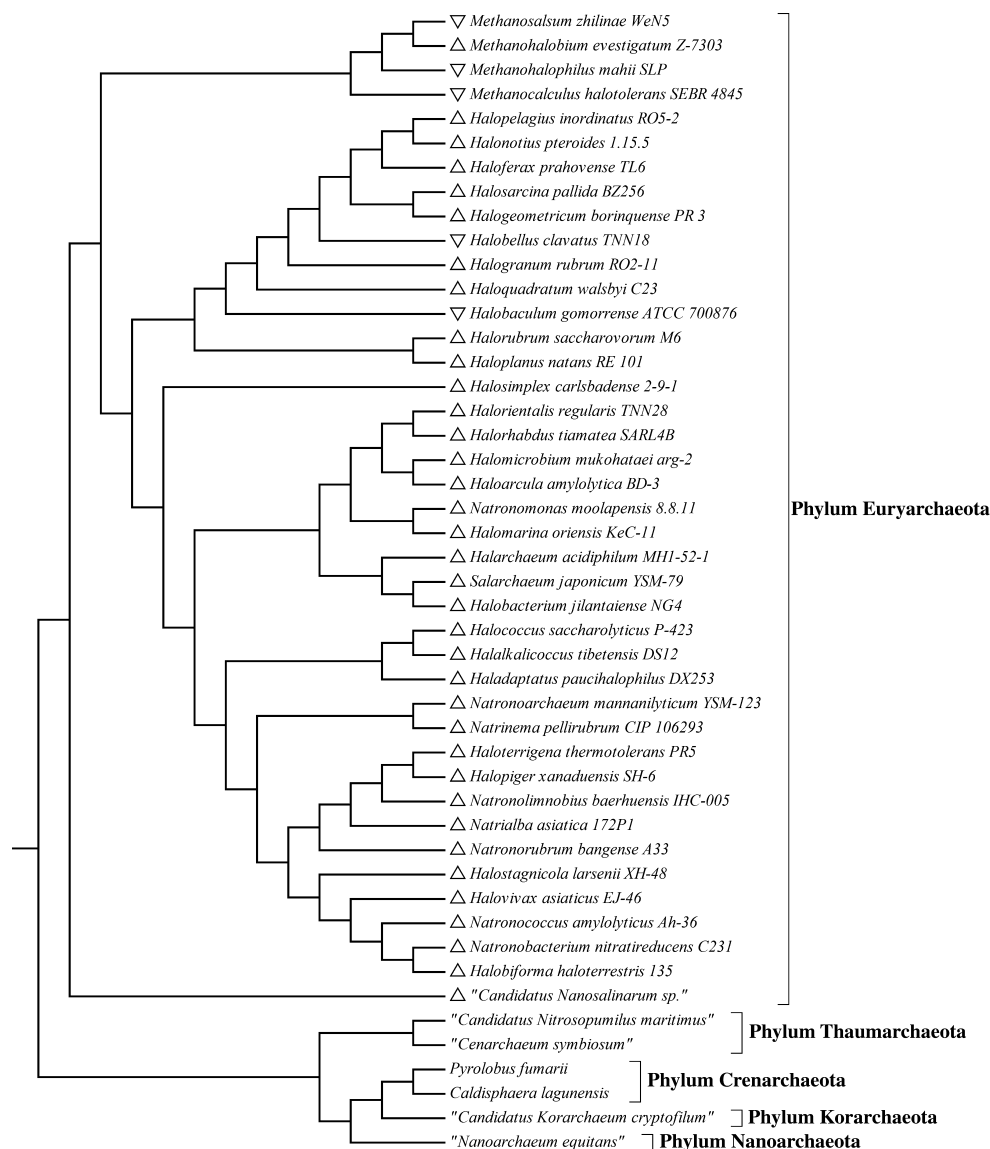
Life on Earth subsists over the whole range of salt concentrations encountered in natural and anthropogenic habitats. It thrives from freshwater environments to hypersaline lakes, solar salterns, and other salt-saturated environments. Hypersaline environments have a cosmopolitan distribution on our planet, and they are represented by aquatic systems, especially salt lakes, as well as saline soils (Rodriguez-Valera, 1988; Oren, 2002a, b; de la Haba *et al.*, 2011). Microorganisms that live in this type of habitats are called halophiles (salt-loving organisms).

The diversity in properties of saline and hypersaline habitats is reflected in the great variety of microorganisms adapted to live under these peculiar conditions. Extreme halophiles are generally defined as organisms that grow optimally in media with a concentration of 150–300 g L<sup>-1</sup> (2.5–5.2 M) NaCl, different from moderate halophiles that grow optimally in media with a concentration of 30–150 g L<sup>-1</sup> (0.5–2.5 M) NaCl. Some nonhalophilic microorganisms are able to tolerate high salt

concentrations and they are characterized as halotolerant or extremely halotolerant organisms (Kushner & Kamekura, 1988; de la Haba *et al.*, 2011).

Halophilic and highly halotolerant species are found in each of the three domains of life: Archaea, Bacteria, and Eukarya. At the highest salt concentrations, halophilic members of the Archaea generally form the main component of the community, and therefore, they deserve a special interest. The Archaea (originally named Archaeobacteria) were proposed as the third domain of life in the late 1970s (Woese & Fox, 1977; Woese *et al.*, 1990). Based on phylogenetic analyses, several phyla/division were proposed within the domain: *Crenarchaeota*, *Euryarchaeota*, *Nanoarchaeota*, *Korarchaeota*, and *Thaumarchaeota* (Cavicchioli, 2011).

The aim of this review is to briefly explore the diversity of the Archaea in hypersaline systems and to assess their metabolic contributions in these environments according to the recent findings in the field. Figure 1 presents a phylogenetic tree of the domain Archaea that includes representative taxa mentioned below.



**Fig. 1.** UPGMA phylogenetic tree based on 16S rRNA partial gene sequences showing the relationships between halophilic archaea and the proposed new class *Nanohaloarchaea*. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA4. The symbols  $\Delta$  and  $\nabla$  correspond to extreme and, respectively, moderate halophiles. Extreme halophiles are considered as growing optimally at 2.5–5.2 M NaCl, while moderate halophiles are considered as growing optimally at 0.5–2.5 M NaCl.

## Phylogenetic diversity of halophilic Archaea

### The class *Halobacteria*

The class *Halobacteria* (Grant *et al.*, 2001) within the phylum *Euryarchaeota* contains the (mostly) aerobic, generally red-pigmented halophiles, often referred to as haloarchaea. The earlier validated name for the class, *Halomebacteria* (Cavalier-Smith, 2002), was rejected by

the International Committee on Systematics of Prokaryotes (Garrity *et al.*, 2011; Oren & Labeda, 2011).

The halophiles of the family *Halobacteriaceae* (Gibbons, 1974), the only family within the *Halobacteriales*, the single order within the *Halobacteria*, are considered the halophiles *par excellence*, because virtually all of them are strictly dependent on high salt concentrations for maintaining growth and cellular integrity. Although scarce reports recorded the presence of *Halobacteriaceae* at relatively low salinities (Rodriguez-Valera *et al.*, 1979;

Munson *et al.*, 1997; Elshahed *et al.*, 2004; Purdy *et al.*, 2004), we consider this phenomenon as the result of their capacity to prevail in localized niches with increased salt concentration, or of their property to maintain viability for a defined time frame. However, the findings of Purdy *et al.* (2004) suggest that representatives of the *Halobacteriaceae* growing at relatively low salinities may be competitive in habitats with salinities at or just above that of seawater. Most species described grow optimally above a concentration of 150 g L<sup>-1</sup> salt and lyse at concentrations below 100 g L<sup>-1</sup> (Oren, 2011b). At the time of writing (November 2011), the family encompassed 129 species, classified based on a polyphasic approach, whose names have been validly published and classified in 36 genera (Oren, 2012).

Aerobic halophilic Archaea thrive in environments with salt concentrations approaching saturation, such as natural brines, alkaline salt lakes, marine solar salterns, and salt rocks of millenary age. They represent the major part of the microbiota of hypersaline soda lakes such as Lake Magadi, Kenya (an extremely alkaline lake), saltern crystallizer ponds, and the Dead Sea (Oren, 2011a). Most representatives are neutrophilic, many are alkaliphilic, and a moderately acidophilic species, *Halarchaeum acidiphilum*, isolated from commercial solar salt does not grow above pH 6.0 (Minegishi *et al.*, 2010).

### Methanogenic halophilic Archaea

Among the groups of methanogenic Archaea within the *Euryarchaeota*, there are a number of halophilic species able to grow at salt concentrations close to saturation. Taxonomically, the methanogens are grouped into five orders. The majority of known halophilic species are classified within the order *Methanosarcinales*, family *Methanosarcinaceae* (Boone *et al.*, 2001; de la Haba *et al.*, 2011). At the time of writing, this family comprised nine genera consisting of 30 species. Moderate and extreme halophiles are found in the genera *Methanohalobium*, *Methanohalophilus*, *Methanosalsum*, and *Methanocalculus* (Ollivier *et al.*, 1998; Boone *et al.*, 2001), all being strict anaerobes. *Methanocalculus halotolerans*, currently included as a genus *incertae sedis* within the order *Methanomicrobiales*, was isolated from an oil well in Alsace, France. It is a moderate halophile that grows optimally at 50 g L<sup>-1</sup> NaCl and produces methane from H<sub>2</sub> + CO<sub>2</sub> and formate (Ollivier *et al.*, 1998).

### Nanohaloarchaea – a new class within the Archaea

Metagenomic studies of the microbial community of the hypersaline (290 g L<sup>-1</sup> salt) Lake Tyrell, Australia,

revealed the existence of a novel major lineage of Archaea. Phylogenetically, the organisms belong to the *Euryarchaeota*, but are not closely related to any of the classes recognized so far; therefore, a new class was proposed: *Nanohaloarchaea* (candidate genera '*Candidatus Nanosalinarum*' and '*Candidatus Nanosalina*'), which appears to be worldwide distributed (Narasimgarao *et al.*, 2012). 16S rRNA gene sequences belonging to this lineage were also reported in several earlier studies (Grant *et al.*, 1999; Baati *et al.*, 2010; Oh *et al.*, 2010).

Based on the genome annotation, these organisms are expected to have a predominantly aerobic heterotrophic lifestyle (Narasimgarao *et al.*, 2012). A similar finding has been reported by Ghai *et al.* (2011) in a 19% salinity layer of a crystallizer pond near Alicante (Spain).

### Nanoarchaeal phylotypes

A low GC euryarchaeote, resembling the novel nanohaloarchaeal organisms described in Lake Tyrell, has been revealed by a single-cell genome approach. 16S rRNA gene sequence analysis showed that the virtual microbe reconstructed from genomic data in Alicante ('*Candidatus Haloredivivus*') is 90% and 88%, respectively, identical with the new candidate genera '*Candidatus Nanosalinarum*' and '*Candidatus Nanosalina*' detected in Lake Tyrell (Ghai *et al.*, 2011).

### Metabolic diversity of halophilic Archaea

#### Aerobic heterotrophic metabolism of haloarchaea

The *Halobacteriaceae* typically lead an aerobic heterotrophic life style. However, in spite of their common requirement for high salt concentrations for growth, their nutritional demands and metabolic pathways are quite diverse. Some species possess complex dietary needs that can be met in culture by including high concentrations of yeast extract or other rich sources of nutrients to their growth medium (e.g. *Halobacterium salinarum*). By contrast, some species grow well on single carbon sources while using ammonia as a nitrogen source. *Haloferax mediterranei* can grow on simple compounds such as acetate, succinate, etc. while supplying its need for nitrogen, sulfur, and other essential elements from inorganic salts. Such simpler growth demands are generally detected in species of the genera *Haloferax* and *Haloarcula* (Oren, 2002b). An even more extreme case is *Halosimplex carlsbadense*, an organism that only grows in defined medium with acetate and glycerol, acetate and pyruvate, or pyruvate alone. Carbohydrates, amino acids,

fats, and proteins do not support its growth (Vreeland *et al.*, 2002). Interestingly, pyruvate is also a preferred substrate of the flat square *Haloquadratum walsbyi* (Burns *et al.*, 2007).

The members of *Halobacteriaceae* use the tricarboxylic acid cycle (TCA) in the process of aerobic degradation of carbon and, if necessary, a combination of the glyoxylate cycle and the respiratory electron transport. Haloarchaeal genomes encode the complete set of enzymes of the TCA cycle (Falb *et al.*, 2008). Furthermore, activity of all enzymes of the cycle was detected in *Hbt. salinarum* (Aitken & Brown, 1969). Field studies on a hypersaline cyanobacterial mat have shown metabolic interactions between haloarchaea and the primary producer *Coleofasciculus (Microcoleus) chthonoplastes*. This cyanobacterium excretes acids of the citrate cycle into the medium, and aerobic halophilic Archaea further utilizes these as the major carbon and energy source (Zvyagintseva *et al.*, 1995). The existence of a functional glyoxylate cycle has been demonstrated in *Haloferax volcanii* (Serrano *et al.*, 1998) and in *Natronococcus occultus* (Kevbrina & Plakunov, 1992). Inquiries effectuated on the 13 complete halophilic genomes present in the HALOWEB data base (DasSarma *et al.*, 2010) did not find any simultaneous positive matches for the glyoxylate cycle key enzymes: isocitrate lyase and malate synthase (with the exception of previous mentioned species *Hfx. volcanii*). A BLASTP (Altschul *et al.*, 1997) search made on NCBI using the amino acid sequences of the *Hfx. volcanii* isocitrate lyase and malate synthase showed that these enzymes are present also in *Haladaptatus paucihalophilus* strain DX253. Recently, a novel pathway for the synthesis of malate from acetyl-CoA was discovered in *Hfx. volcanii* and in *Har. marismortui*, in which acetyl-CoA is oxidized to glyoxylate via methylaspartate as key intermediate (Khomyakova *et al.*, 2011).

Although most halophilic Archaea preferentially use amino acids as carbon and energy source, there are carbohydrate-utilizing species such as *Haloarcula marismortui*, *Halococcus saccharolyticus*, and *Hfx. mediterranei*. These species have the capacity to metabolize pentoses (arabinose, xylulose), hexoses (glucose, fructose), sucrose, and lactose (Rawal *et al.*, 1988; Altekar & Rangaswamy, 1992; Johnsen *et al.*, 2001). Comparative analysis of ten haloarchaeal genomes showed that *Halorhabdus utahensis* and *Haloterrigena turkmenica* encode over forty glycosyl hydrolases each and may break down complex carbohydrates. *Hrb. utahensis* has specialized in growth on carbohydrates and has few amino acid degradation pathways. It uses the nonoxidative pentose phosphate cycle and a transhydrogenase instead of the oxidative pathway, giving it a great deal of flexibility in the metabolism of pentoses (Anderson *et al.*, 2011). *Hrb. utahensis* degrades xylan and can grow on xylose (Wainø & Ingvorsen, 2003). Many species of *Halobacteriaceae* also

produce exoenzymes such as proteases, lipases, DNases, and amylases to degrade organic polymeric substances extracellularly, making small organic molecules available as carbon and energy source.

Studies on glucose and fructose degradation pathways in *Halococcus saccharolyticus* showed that glucose is entirely degraded via an Entner–Doudoroff (ED) type pathway, whereas fructose is almost completely degraded (96%) via an Embden–Meyerhof type pathway and only to a small extent (4%) via an ED pathway (Johnsen *et al.*, 2001). This ED pathway, in which the phosphorylation step is postponed, is also probably used by the other members of the carbohydrate-utilizing group. In this pathway, glucose is oxidized via gluconate to 2-keto-3-deoxygluconate and then phosphorylated to 2-keto-3-deoxy-6-phosphogluconate, which is further split into pyruvate and glyceraldehyde-3-phosphate (Tomlinson *et al.*, 1974). In addition, other steps in common metabolic pathways may have special modifications in the halophilic Archaea, such as the production of acetate by an ADP-forming acetyl-CoA synthetase (Siebers & Schönheit, 2005).

*Halobacterium* does not grow on sugars, but its growth is stimulated by the addition of carbohydrates to the medium (Oren, 2002b), where glucose can be transformed into gluconate (Sonawar *et al.*, 1990). Oxidation of carbohydrates is often incomplete and is usually associated with the production of acids (Hochstein, 1978).

In the presence of glycerol, some species of the genus *Haloferax* and *Haloarcula* produce acetate, pyruvate, and D-lactate (Oren & Gurevich, 1994). Production of D-lactate, acetate, and pyruvate from glycerol by the haloarchaeal communities of the Dead Sea and saltern crystallization ponds has also been observed. In these environments, acetate is poorly utilized (Oren, 1995).

Analysis of the genome of the flat square archaeon *Hqr. walsbyi* showed a few unique features. One of them is the presence of a gene cluster that allows uptake of phosphonates and subsequent cleavage of the carbon–phosphorus bond by a phosphonate lyase. Another is the possible use of dihydroxyacetone as a carbon and energy source after its uptake via a phosphoenol pyruvate-dependent phosphotransferase system (Bolhuis *et al.*, 2006). Growth studies showed that, indeed, *Hqr. walsbyi* could metabolize dihydroxyacetone (Elevi Bardavid & Oren, 2008). Based on the analysis of its genome, this species can also grow on pyruvate and glycerol (Bolhuis *et al.*, 2006). Its apparent inability to take up glycerol, as shown in an analysis of the natural community in a saltern crystallizer pond in Mallorca (Rosselló-Mora *et al.*, 2003) remains unexplained.

A food chain is thus possible, in which glycerol produced as an osmotic solute by the alga *Dunaliella* is converted in part to dihydroxyacetone by extremely

halophilic bacteria of the genus *Salinibacter* (*Bacteroides*). *Haloquadratum* and other members of the *Halobacteriaceae* (Elevi Bardavid & Oren, 2008; Elevi Bardavid *et al.*, 2008) can then take up the dihydroxyacetone and the remainder of the glycerol.

Some representatives of the family can metabolize aliphatic and aromatic hydrocarbons and long-chain fatty acids, such as hexadecanoic acid (Bertrand *et al.*, 1990; Oren, 2006; McGenity, 2010a). Thus, a study of the biodegradation of crude oil and pure hydrocarbons by extreme halophilic Archaea from hypersaline coasts of the Arabian Gulf yielded two strains of *Haloferax*, one of *Halobacterium*, and one of *Halococcus*, which can grow on crude oil vapor as sole carbon and energy source (Al-Mailem *et al.*, 2010). Hydrocarbon-degrading extremely halophilic Archaea were also isolated from a saltern crystallizer pond in the south of France (Tapilatu *et al.*, 2010).

Degradation of aromatic compounds by haloarchaea was first documented by Emerson *et al.* (1994) in *Haloferax* strain D1227 that grew on benzoate, cinnamate, and phenylpropionate. Aerobic degradation of *p*-hydroxybenzoic acid by a *Haloarcula* sp. follows an unusual metabolic pathway (Fairley *et al.*, 2002). More halophilic Archaea growing on benzoic acid, *p*-hydroxybenzoic acid, salicylic acid, and on a mixture of the polycyclic hydrocarbons naphthalene, anthracene, phenanthrene, pyrene and benzo[a]anthracene, with and without 0.05% yeast extract, were isolated from different geographic locations: salt flats in Bolivia, salterns in Chile and Puerto Rico, a sabkha in Saudi Arabia, and the Dead Sea. Most isolates were affiliated with the genus *Haloferax* (Cuadros-Orellana *et al.*, 2006; Bonfá *et al.*, 2011).

Genomic information revealed that the recently discovered nanohaloarchaeal organisms lead an aerobic heterotrophic life style. The presence of lactate dehydrogenase may point to a potential for fermentative metabolism. The genes encoding the enzymes of the Embden–Meyerhof glycolytic pathway were identified, and both the oxidative (based on glucose-6-phosphate dehydrogenase as the key enzyme) and the nonoxidative branches of the pentose phosphate pathway were present. This is the first case in which the complete pentose phosphate pathway was demonstrated in a member of the Archaea (Narasimharao *et al.*, 2012).

### Anaerobic heterotrophic metabolism of the *Halobacteria*

Oxygen has a low solubility in salt-saturated brines, and therefore, it may easily become a limiting factor for the development of halophilic Archaea. Some produce gas vesicles or possess aerotaxis sensors (e.g. HemAT in *Halobacterium*) (Hou *et al.*, 2000) that enable them to reach

the water–air interface, while others have the capacity to grow anaerobically. Variants of anaerobic growth documented within the *Halobacteriaceae* include the use of alternative electron acceptors such as nitrate, dimethylsulfoxide, trimethylamine *N*-oxide or fumarate, fermentation of arginine, and possibly other types of fermentation as well (Oren, 2006).

Considering the low concentrations of nitrate generally encountered in hypersaline brines and the apparent lack of regeneration of nitrate by nitrification at high salt concentrations, the process can be expected to occur only to a limited extent in nature (Oren, 1994). Some halophilic Archaea (e.g. *Har. marismortui*, *Har. vallismortis*, *Hfx. mediterranei*) can grow anaerobically when nitrate is present as the electron acceptor, forming gaseous nitrogen and/or nitrous oxide (Mancinelli & Hochstein, 1986).

The ability to use dimethylsulfoxide and trimethylamine *N*-oxide, or fumarate as electron acceptors for anaerobic growth, is quite widespread among the halophilic Archaea (Oren & Trüper, 1990; Oren, 1991). Additionally, thiosulfate and elemental sulfur have been suggested to act as potential electron acceptors (Tindall & Trüper, 1986; Elshahed *et al.*, 2004). Nonetheless, information on the nature of these processes is scarce (Oren, 2006).

Fermentation of *L*-arginine to citrulline can drive anaerobic growth in *Hbt. salinarum* (Hartmann *et al.*, 1980), but this metabolic pathway does not seem to be widespread among haloarchaea. Thus far, it has only been detected in the genus *Halobacterium* (Oren & Litchfield, 1999; Oren, 2006). When grown anaerobically, species of the mentioned genus are able to ferment arginine via the arginine deiminase pathway (Ruepp & Soppa, 1996). Throughout this pathway, arginine is converted to ornithine and carbamoylphosphate, which is further split into carbon dioxide and ammonia with concomitant ATP production.

Fermentation is probably the preferred mode of life of *Halorhabdus tiamatea*, a nonpigmented, extremely halophilic archaeon isolated from the brine–sediment interface of the Shaban Deep, a hypersaline anoxic basin in the northern Red Sea. This species uses yeast extract and starch as carbon and energy sources and grows anaerobically and under microaerophilic conditions, but aerobic incubation was shown to support only a very poor growth (Antunes *et al.*, 2008). A gene encoding lactate dehydrogenase was found in the *Hrb. tiamatea* genome, and this enzyme might participate in the fermentation pathway (Antunes *et al.*, 2011).

### Photoheterotrophic metabolism of the *Halobacteria*

An entirely different mode of anaerobic growth displayed by some halophilic Archaea is photoheterotrophy,

which consists in the use of light energy absorbed by retinal-based pigments. The light-driven proton pump bacteriorhodopsin can drive anaerobic growth of *Hbt. salinarum* (Hartmann *et al.*, 1980; Oesterhelt, 1982). Many members of the *Halobacteriaceae* and, possibly, the newly described group of *Nanohaloarchaea* (Ghai *et al.*, 2011) possess the necessary genes for the biosynthesis of the bacteriorhodopsin protein and the retinal prosthetic group, but little is known about the relative importance of light as an energy source to drive growth of the halophilic Archaea in their natural environment. Organic substrates serve as carbon sources, still photo-autotrophy has not been demonstrated in the archaeal domain.

### Archaeal methanogenesis in hypersaline systems

Methanogenic Archaea acquires the necessary energy for growth and survival by the stoichiometric conversion of a limited number of substrates to methane gas. The major substrates are  $H_2 + CO_2$ , formate (group 1), acetate (group 3) and, in a lesser extent, compounds such as methanol, trimethylamine, dimethylsulfide (group 2), and some alcohols such as isopropanol.

Methane is a major end product of anaerobic degradation of the biomass only in anoxic environments where the concentration of products such as sulfate, nitrate, Mn (IV), or Fe(III) is low. The presence of these substances in the medium allows other organisms to outcompete methanogens in the competition for electron acceptors, mainly because of thermodynamic reasons. For instance, sulfate-reducing bacteria have the ability to utilize  $H_2$  at lower concentrations than minimum required by methanogens, in the presence of sulfate. Consequently, sulfidogenesis generally prevails in estuarine, marine, and hypersaline sediments where sulfate diffuses from overlying water (McGenity, 2010b). However, increased salinity in many cases supplies higher concentrations of noncompetitive substrates, which derive from compatible solutes synthesized by the environmental microbiota. Such high-salinity-associated solutes include methylated amines and dimethylsulfide.

At high salt concentration, neither the reduction of carbon dioxide by hydrogen nor the acetoclastic reaction was shown to occur. Acetate splitting methanogens appear to be very little salt tolerant. The upper salt concentration for growth of cultures of methanogenic Archaea greatly depends on the substrate used:  $270\text{ g L}^{-1}$  for group 2 methanogens,  $120\text{ g L}^{-1}$  for group 1 methanogens, and  $40\text{ g L}^{-1}$  for group 3 methanogens (Oren, 1999). These salinities should not be considered as the upper limit of activity *in situ*, but to be indicative of the

relative importance of these substrates at different salinities (McGenity, 2010b). The absence of group 1 and group 3 methanogens at high salinity may be governed by the relative energy gain from different methanogenic reactions per mole of substrate (methylophilic  $\gg$  hydrogenotrophic  $\geq$  acetoclastic), especially because halophiles must expend a lot of energy to maintain an osmotically balanced and functional cytoplasm via the biosynthesis and/or uptake of organic compatible solutes, and/or uptake of potassium ions (Oren, 1999). This may further explain the predominance of methylophilic methanogens like *Methanohalophilus* spp. in hypersaline environments. On the other hand, we must approach this interpretation with caution, because standard Gibbs free energy yields are only one determinant of the total metabolic energy yield, and we must take into consideration the rate of substrate flux/consumption.

Trimethylamine is often found in saline systems, where it is formed from glycine betaine or other osmoprotectants used by the resident organisms to equilibrate the cytoplasmic osmolarity to that of the water. This substance is rapidly transformed by methanogens to methane,  $CO_2$ , and ammonia, but it is not easily utilized by sulfidogenic bacteria. Trimethylamine-degrading methanogens from saline environments belong to the family *Methanosarcinaceae*, and all methanogens that have been isolated to date from high-salinity ecosystems use trimethylamine as catabolic substrate (with the exception of *M. halotolerans*, which uses  $H_2 + CO_2$  or formate and has a relatively restricted salt tolerance, and does not grow above  $120\text{ g L}^{-1}$  salt).

### Conclusion

Hypersaline environments harbor surprisingly diverse communities of Archaea, aerobic as well as anaerobic. Only recently, we are starting to obtain a reliable picture of this diversity, which is much greater than the one predicted on the basis of culture methods.

Research on the microbial diversity in hypersaline systems greatly contributes to our understanding of prokaryotic phylogeny, the adaptation of microorganisms to life under extreme conditions, and has biotechnological aspects as well. Although the metabolic diversity displayed by the known halophilic Archaea is much more restricted than that of the halophilic and highly halotolerant representatives of the domain Bacteria, the above survey shows that the range of substrates that can support their growth and the diversity of metabolic pathways used in their degradation is much greater than earlier assumed. The search for novel types of halophiles will expand our understanding of the functioning of hypersaline ecosystems and their biogeochemical cycles.

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