Chapter 2

Evolution, Metabolism and Molecular Mechanisms Underlying Extreme Adaptation of *Euryarchaeota* and Its Biotechnological Potential

Victor Castro-Fernandez, Ricardo Zamora, Alejandra Herrera-Morande, Gabriel Vallejos, Felipe Gonzalez-Ordenes and Victoria Guixé

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Abstract

Archaeal organisms harbor many unique genotypic and phenotypic properties, testifying their peculiar evolutionary status. Thus, the so-called extremophiles must be adequately adapted to cope with many extreme environments with regard to metabolic processes, biological functions, genomes, and transcriptomes to overcome the challenges of life. This chapter will illustrate recent progress in the research on extremophiles from the phylum *Euryarchaeota* and compile their evolutive history, metabolic strategies, lipid composition, the structural adaptations of their enzymes to temperature, salinity, and pH and their biotechnological applications. Archaeal organisms have evolved to deal with one or more extreme conditions, and over the evolution, they have accumulated changes in order to optimize protein structure and enzyme activity. The structural basis of these adaptations resulted in the construction of a vast repertoire of macromolecules with particular features not found in other organisms. This repertoire can be explored as an inexhaustible source of biological molecules for industrial or biotechnological applications. We hope that the information compiled herein will open new research lines that will shed light on various aspects of these extremophilic microorganisms. In addition, this information will be a valuable resource for future studies looking for archaeal enzymes with particular properties.

Keywords: archaea, archaea evolution, archaeal lipids, archaea metabolism, biotechnological applications, methanogenesis, *Euryarchaeota*, extremozymes

1. Introduction

Archaea represents the third domain of life. Their peculiar evolutionary status conforms to their unique genotypic and phenotypic properties. Except for methanogenesis, which has not yet been described in bacteria, all central metabolic pathways discovered in archaea also exist in bacteria, although in some cases with important and novel modifications. Archaeal organisms can be either heterotrophs or autotrophs (chemio- or photo-lithoautotroph) and can use a large variety of electron donors and acceptors [1]. One of the most striking features of archaea organisms is their relation with hyperthermophilicity as they are able to colonize ecological niches even above 95°C. This property relies in part on the unique structure of archaeal lipids, which are able to maintain impermeability of the cytoplasmic membrane to ions at such high temperatures. Although archaea are often believed to live in extreme environments, they can also be found in many diverse locations including even moderate environmental conditions.

The phylum *Euryarchaeota*, in archaea, comprises an extremely physiologically diverse group of microorganisms, adapted to the most extreme environments known so far. This phylum includes organisms adapted to different temperature niches (thermophiles, mesophiles, and psychrophiles), pH (acidophiles), and the organisms those grow at elevated salt concentrations (halophiles). On the other hand, the phylum, *Crenarchaeota*, comprises a much more limited phenotypic diversity of cultivable organisms. This chapter focuses on the phylum *Euryarchaeota* and considering the industrial potential of their proteins.

2. The *Euryarchaeota* phylogenetic tree

The evolutionary studies of archaea are important for understanding the origin of life and the course of evolution of the organisms that populate the earth. In the 1970s, Carl Woese's work showed that RNA of the small subunit of the ribosome (SSU rRNA) could be used to define phylogenetic relationships, which allowed the construction of a universal tree of life [2]. From these studies emerged the discovery of archaea, demonstrating that living organisms on earth can be divided into three domains (archaea, eukarya, and bacteria). Initially, two phyla were identified in archaea: *Crenarchaeota* and *Euryarchaeota* [3]. Subsequently, new phyla, namely *Korarchaeota*, *Nanoarchaeota*, and *Thaumarchaeota*, were proposed based on SSU rRNA [2].

The phylum *Euryarchaeota* comprises an extreme physiologically diverse group of microorganisms, adapted to the most extreme environments known. The members constitute the greatest phenotypic diversity among the cultivable species known to date, including halophiles, methanogens, some thermoacidophiles, and some hyperthermophiles. Through SSU rRNA, the following orders of *Euryarchaeota* were identified: *Thermococcales*, *Methanobacteriales*, *Methanococcales*, *Thermoplasmatales*, *Halobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, and *Archaeoglobales*.

However, at the end of the 1990s, the phylogenies based on SSU rRNA were questioned regarding their ability to reconstruct the more ancestral speciation events, given the lack of a phylogenetic signal [4]. The phylogenetic trees, reconstructed with SSU rRNA by maximum

likelihood and evaluated by bootstrap (a method to evaluate the robustness of nodes in a tree), showed that the most ancestral nodes of the phylum *Euryarchaeota* have '60% statistical significance, which makes difficult to have confidence in the phylogenetic relationship between the different orders. Then, by using SSU rRNA, the orders that diverge first and those that diverge last cannot be established [5].

The evolutionary relationships among the archaea orders have been established by the sequencing of several genomes. Petitjean et al. [6] identified 200 protein families, along with 57 ribosomal proteins and 14 RNA polymerase subunits, which represent 273 phylogenetic markers in 129 archaeal genomes. With this conserved core of archaeal genes, they inferred the phylogeny of the nodes of different orders with high robustness (statistical significance >95%). The tree topology obtained with this core of proteins is generally consistent with other topologies such as that obtained by Battistuzzia and Hedgesa [7].

The topology of these phylogenies shows that *Thermococcales* were the first group to diverge (**Figure 1**) in *Euryarchaeota*, approximately 3594 Ma ago, ruling out the possibility that methanogenesis was an ancestral metabolic process in the *Euryarchaeota*. Subsequently, the orders *Methanopyrales*, *Methanobacteriales*, and *Methanococcales* diverged around 3468 Ma ago, and methanogens class I or *Methanomada* appeared [6]. Later on, the order *Thermoplasmatales* diverged (3160 Ma ago), followed by the order *Archaeoglobales* (2799 Ma ago). Finally, methanogens class II diverged (*Methanocellales*, *Methanosarcinales*, and *Methanomicrobiales*), and from this group, the order *Halobacteriales* emerged [6]. However, this hypothesis is debatable as in some topologies, *Halobacteriales* appear prior to the divergence of methanogens class II [7].

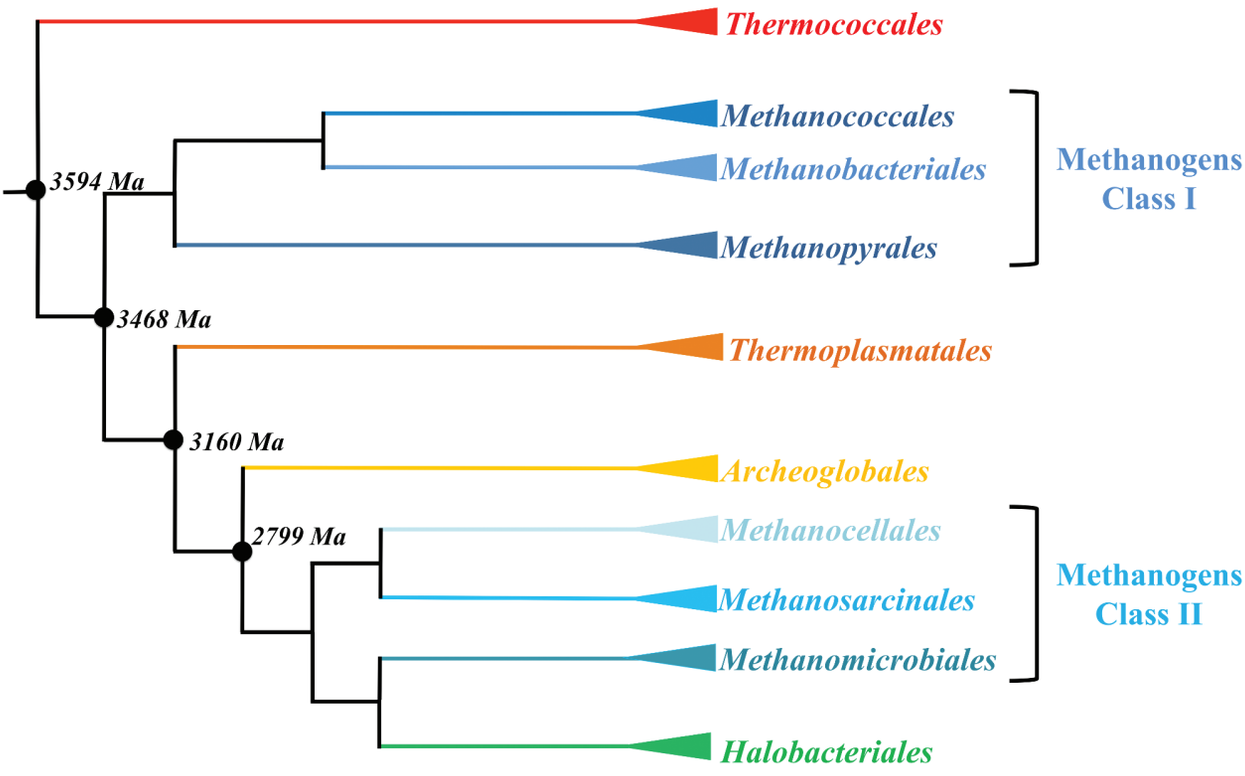

Figure 1. Schematic representation of phylogenetic relationships between the orders of *Euryarchaeota*. The estimated time in millions of years (Ma) for the divergence of some orders is shown according to the time-tree [7].

3. *Euryarchaeota* metabolism

Euryarchaeota organisms show very diverse metabolism. For example, phylogenetically close orders can exhibit very different kinds of metabolism, while important similarities can be observed in distantly related orders. Most of the *Euryarchaeota* are strictly anaerobic, although some of them can grow at low oxygen concentrations. *Euryarchaeota*, like most of the anaerobic organisms studied, lacks the defense mechanisms against oxidative stress (ROS). However, recently, the ability of *P. furiosus* to grow even in the presence of 8% oxygen has been described, which led to postulate the existence of a mechanism through which a part of the electrons destined to H_2 production are diverted to the O_2 reduction [8]. Further, this phylum comprises mainly autotrophic organisms, and some heterotrophs can be found. This trait has been suggested to be an evolutive novelty acquired later [9].

The systematic studies of archaeal metabolism were undertaken soon after the first genome sequence from archaea was obtained. The initial studies contemplated metabolic reconstructions based on the presence of homologous sequences with known activities [10]. In parallel, the *in vitro* studies with cellular cultures that included metabolite and enzymatic measurements in crude extracts began to appear. The most of our current knowledge about their metabolism has been derived from the exhaustive studies performed with the model organisms like *Methanocaldococcus jannaschii* (the first archaeal genome to be sequenced), *Methanococcus maripaludis* [11] (order *Methanococcales*), *Methanosarcina acetivorans* [12] (order *Methanosarcinales*), *Thermococcus kodakarensis*, and *P. furiosus* [13] (both from order *Thermococcales*)—just to mention some of them. In **Figure 2**, a simplified scheme showing the main metabolic pathways of the archaeal life is presented, using *Thermococcales*, *Methanococcales*, and *Methanosarcinales* as examples.

Heterotrophs from *Thermococcales* are the most studied organisms, which can be grown in different conditions using sugars, peptides, or polysaccharides as a carbon source [14, 15]. In these organisms, glycolysis or the Embden-Meyerhof pathway (EM) plays a fundamental role in the production of reduced equivalents and ATP [16]. In this pathway, important modifications to the traditional glycolysis can be observed, such as the phosphorylation of glucose and fructose-6-P is performed by a glucokinase (ADP-GK) and a phosphofructokinase (ADP-PFK) employing ADP instead of ATP as a phosphoryl donor [17, 18], and the canonical G3PDH is replaced by a glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) [19]. In addition, a pyruvate ferredoxin oxidoreductase (POR) that decarboxylases pyruvate to synthesize Acetyl-CoA is also present in these organisms [20]. In both the aforementioned reactions, reduced ferredoxin is produced, which constitutes one of the most important electron carriers. Ferredoxin produced in glycolysis is oxidized later by a membrane hydrogenase, coupled to molecular hydrogen production [21]. During this process, a proton is exported to the extracellular medium, contributing to the proton gradient which in turn allows ATP production carried out by the ATP synthase enzyme [21]. Alternatively, archaea possess a unique Acetyl-CoA synthetase able to synthesize acetate from Acetyl-CoA coupled to ATP production [22]. This reaction, along with pyruvate synthesis by pyruvate kinase, is the only step where phosphorylation of ADP at a substrate level occurs in glycolysis. In these organisms, as well as the autotrophs belonging to *Euryarchaeota*, the presence of an incomplete reductive Kreb's cycle

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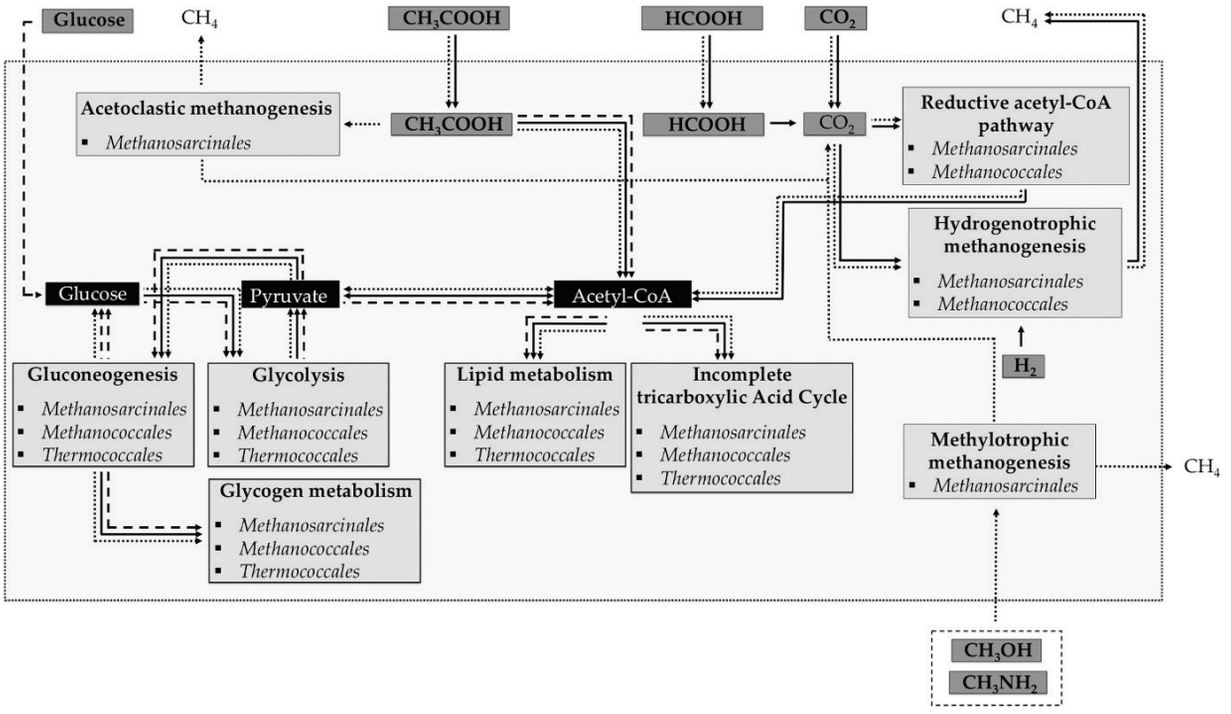

Figure 2. The simplied scheme of *Euryarchaeal* metabolism. *Thermococcales* are used as an example to represent heterotrophic archaea, *Methanococcales* as an example of class I methanogens and *Methanosarcinales* of class II methanogens. Light gray squares represent main metabolic processes mentioned in the text. The genera carrying each process are enclosed in a square. The relevant metabolites that can enter the cell are represented in dark gray squares. Metabolites that serve as intermediaries and connecting different processes are represented by black squares. CH_4, one of the major metabolic final products from methanogenic archaea is shown. The arrows represent connections between metabolites and metabolic processes. The dashed arrows represent the pathways present in *Thermococcales*; the black arrows represent the pathways present in *Methanococcales*, and the dotted arrows represent the pathways present in *Methanosarcinales*.

has been reported and it has been postulated that its function is the production of metabolic intermediates for amino acid synthesis and other anabolic processes [23].

Thermococcales can use elemental sulfur as the final electron acceptor producing sulfhydric acid as a final metabolic product [14]. However, in most cases, the presence of sulfur is not necessary, and alternatively, molecular hydrogen is produced as the final metabolic product [24]. This reaction is mainly carried out by a ferredoxin-dependent membrane hydrogenase. In the presence of elemental sulfur, the expression of this hydrogenase is diminished with the concomitant decrease in H_2 production. Under these conditions, ferredoxin is oxidized by a membrane oxidoreductase coupled to NADPH production and H^+ ions are exported to the extracellular medium. Later, an NADPH-dependent oxidoreductase reduces elemental sulfur, producing sulfhydric acid [24].

In autotrophic organisms of *Euryarchaeota*, such as methanogens class I (*Methanococcales*) and methanogens class II (*Methanosarcinales*), glycolysis also shows certain modifications; of them, most are shared with *Thermococcales* [16]. However, important differences can be observed in *Methanococcales*; since these organisms lack the ADP-GK gene, the ADP-PFK enzyme performs both phosphorylating activities at the same active site [25, 26]. It has been

postulated that in both classes of methanogens, glycolysis is a secondary pathway since during the growth in the presence of a suitable amount of nutrients these organisms maintain active pathways like gluconeogenesis and glycogen synthesis [12, 27]. In the absence of nutrients, glycolysis is activated as a salvage route to produce metabolic intermediates and substrates for methanogenesis. Nonetheless, the role of glycolysis and gluconeogenesis in these organisms is still under an active investigation. Recently, an ADP-dependent PFK-GK with more catalytic efficiency toward glucose synthesis has been described in *M. maripaludis*, but its physiological relevance has not been elucidated [26]. Further, both activities (PFK and GK) are activated by AMP, which might have important consequences for glycolysis regulation [26].

Methanogens are unable to grow in the presence of sugars, peptides or compounds of three or more carbons since they lack specific transporters for these substances [28]. However, they can use CO_2 and other one-carbon compounds, such as formate, as a carbon source. Carbon fixation proceeds via a reductive pathway of Acetyl-CoA (Wood-Ljungdahl pathway) where two molecules of CO_2 are reduced and one molecule of Acetyl-CoA is produced [29]. This reduction is carried out by the oxidation of two equivalents of ferredoxin and one equivalent of coenzyme F420 (a unique coenzyme from methanogenic organisms) [30]. The methanogens, by using the POR enzyme, carry out the carboxylation of Acetyl-CoA to form pyruvate and, in this way, generate the substrates for gluconeogenesis or amino acid synthesis [31].

Methanogenesis is the main metabolic process in autotrophs from *Euryarchaeota*. Class I methanogens produce methane from CO_2 in a pathway called hydrogenotrophic. The electrons required for CO_2 reduction come from the oxidation of H_2 and are transferred to the carriers like ferredoxin and coenzyme F420 and ultimately to CO_2 in successive reactions [32].

In class II methanogens, besides the hydrogenotrophic pathway [33], two variants have also been found: acetoclastic and methylotrophic methanogenesis, both of which have a more recent evolutive origin [34]. In acetoclastic methanogenesis, one molecule of methane and one of CO_2 are generated from one molecule of acetate [35, 36]. Acetate is first converted to Acetyl-CoA by the action of the Acetyl-CoA synthetase enzyme with a concomitant expenditure of ATP. Later, the methyl group of Acetyl-CoA enters methanogenesis and gets reduced to generate methane in a process whose stages are shared with hydrogenotrophic methanogenesis. The electrons required to carry out the process are provided by the oxidation of the carbonyl group of Acetyl-CoA to carbon monoxide and then to CO_2 in a ferredoxin-dependent process.

In the methylotrophic methanogenesis, methane can be produced from the methyl group of several molecules, such as methanol, methylamine, and methanethiol. A total of four methyl groups are metabolized, and three of them enter methanogenesis where they are reduced to yield three methane molecules [35, 37]. The fourth methyl group is oxidized through a process equivalent to hydrogenotrophic methanogenesis, but it occurs in reverse order, yielding CO_2 and providing the electrons needed for the reduction of the other three molecules. The CO_2 produced in this process, as well as the one produced in acetoclastic methanogenesis, can enter in the reductive acetyl-CoA pathway to be destined to biomass generation.

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4. Archaeal membrane lipids

Archaea are generally characterized by the unique structure of their membrane lipids. Their phospholipid composition mainly includes long chains of methylated isoprenoids attached to a glycerol-1-phosphate molecule via an ether bond, which has been suggested to contribute to the survival in extreme environments [38]. Archaeal lipids differ in isoprenoids chain length, composition, configuration, and various modifications at the polar head groups. The two main core structures are C_{20} sn-2, 3-diphytanyl glycerol diether lipid (also known as archaeal) and C_{40} sn-2, 3-diphytanyl diglyceroltetraether also known as glycerol diphytanoyl diglycerol tetraether (GDGT) or caldarchaeol. The archaeal lipids include some lipids with C_{25}isoprenoid chain, which enables the formation of the bilayer membrane. On the other hand, in caldarchaeol, two identical or different glycerol moieties are connected by two C_{40} isoprenoid chains, enabling the formation of monolayer membrane (**Figure 3**). These membranes present a higher degree of compactness compared to the other bilayer

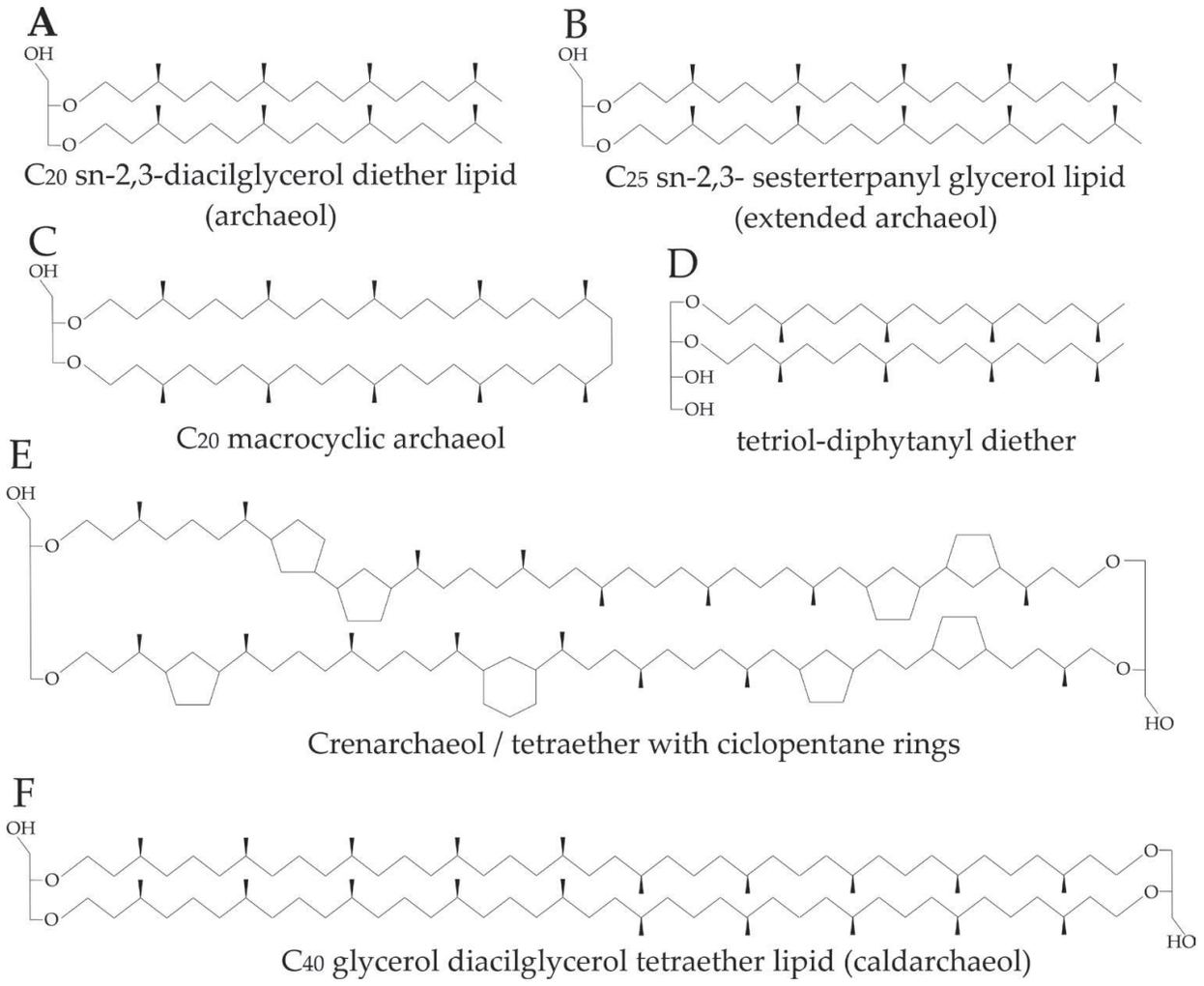

Figure 3. The structures of archaeal membrane lipids. (A) Archaeal C_{20}. (B) Modified archaeal C_{25}. (C) Macrocyclic archaeal. (D) Tetriol-archaeoldiether lipids. (E) Crenarchaeol with cyclopentane and cyclohexane. (F) Caldarchaeol C_{40} (GDGT).

membranes, which precludes external and internal layer fusion at high temperatures [39]. Furthermore, the ether bond typical of archaeal lipids is less susceptible to hydrolysis than the ester bonds present in bacterial lipids, which makes this kind of membrane to be more stable at high temperatures and in acidic environments. Molecular dynamic simulations have confirmed the importance of the monolayer structure in the membrane stability and determined that the presence of cyclic structures, like cyclopentane, increases membrane rigidity, rendering the membrane more resistant to mechanical stresses and high temperatures [40]. In hyperthermophilic archaea, the number of cyclic structures increases with the increase in growth temperature since the interaction between the lipids with cyclopentane is more stable. On the other hand, in psychrophilic *Euryarchaeota* the membrane lipids present unsaturated isoprenoid chains, which offer higher membrane fluidity at low temperatures. This is the case for the membranes from the psychrophilic organism *Methanococcoides burtonii*, where the identified lipids correspond to unsaturated archaeal lipids such as archaeal phosphatidylglycerol, archaeal phosphatidylinositol, hydroxyarchaeol phosphatidylglycerol, and hydroxyarchaeol phosphatidylinositol [41].

As a ubiquitous characteristic, the membrane lipids in *Halobacteria* organisms lack phospholipids with ethanolamine, inositol, and serine groups. Archaetidyl glycerol methyl phosphate (PGP-Me) is the main component of the membrane, which accounts for 50–80% of the total lipids. This particular lipid composition allows that the membranes from *Halobacteria* organisms retain their stability and impermeability in environments up to 4 M NaCl, distinct from the membranes of other *Euryarchaeota* organisms lacking PGP-Me [42].

Archaeal and extended archaeal are the main lipids in the orders *Methanococcales* and *Methanosarcinales*, while the orders *Methanopyrales*, *Thermoplasmatales*, *Archaeoglobales*, and *Methanomicrobiales* contain GDGTs lipids [39]. In the orders, *Thermococcales* and *Methanobacteriales*, both type of lipids, archaeal and GDGT, are present. Furthermore, in hyperthermophilic *Euryarchaeota* such as *Thermococcales* and *Thermoplasmatales*, GDGT with cyclic structures can be found (**Table 1**) [38, 43].

During the lipid synthesis in archaea, the isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP) compounds serve as the building blocks of the isoprenoid chains. There are two pathways for the synthesis of these compounds: one of them corresponds to the mevalonate pathway (MVA), and the other is mevalonate-independent, which is known as C-methyl-D-erythritol-4-phosphate/1-deoxy-D-xylulose-5-phosphate (MEP-DOXP). In the MVA pathway, IPP and DMAPP are formed by the condensation of Acetyl-CoA molecules to give 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is later reduced to mevalonate and then phosphorylated and decarboxylated to form the precursor molecules of the isoprenoid chains [43]. Further, *M. jannaschii* uses a modified MVA pathway, where the conversion of mevalonate-5-phosphate to IP is catalyzed by a phosphomevalonate decarboxylase (MJ0403), and later IP is phosphorylated by the action of the IP kinase (MJ0044) enzyme [44].

Isoprenoid synthesis through the mevalonate-independent pathway was described initially in eukaryotes and later in bacteria, algae, and plants. Both pathways differ in the substrates and enzymes that participate in IPP formation. For example, in the MEP-DOXP pathway,

Euryarchaeota order	Organism characteristic	Organism	Metabolism	Type of lipid	References
Halobacteriales	Psychrophilic Mesophilic	*Halobacterium* sp *Halorubrum lacusprofundi*	Heterotrophic	Archaeal C_{20}*, Archaeal extended C_{25}**, GDGT C_{40} and Archaeal unsaturated (psychrophilic organisms only)	[52, 53]
Methanosarcinales	Thermophilic Mesophilic Psychrophilic	*Methanosarcina* sp *Methanococcoides burtonii*	Autotrophic	Archaeal C_{20}*, GDGT C_{40} and Archaeal unsaturated (psychrophilic organisms only)	[41, 54, 55]
Methanopyrales	Hyperthermophilic	*Methanopyrus kandleri*	Autotrophic	Archaeal C_{20}* with cyclic ring (the degree of cyclization increase with the increase of the T°) and unsaturated Archaeal	[56]
Methanococcales	Hyperthermophilic	*Methanocaldococcus jannaschii*	Autotrophic	Archaeal C_{20} macrocyclic Archaeal***	[44]
Thermococcales	Thermophilic Hyperthermophilic	*Pyrococcus horikoshii Thermococcus* sp	Heterotrophic	Archaeal C_{20}*, Cardarchaeol derivatives and GDGT with up to two cyclic rings	[56]
Methanobacteriales	Mesophilic Thermophilic	*Methanobacterium thermoautotrophicus*	Autotrophic	Archaeal C_{20}* or Archaeal extended C_{25}** and GDGT-0	[39]
Archaeoglobales	Mesophilic Thermophilic	*Archaeoglobus Fulgidus*	Heterotrophic	Archaeal C_{20}*, **Archaeal extended C_{25}** and GDGT with zero to two cyclic rings	[57]
Thermoplasmatales	Mesophilic Thermophilic	*Thermoplasma acidophilum*	Heterotrophic	GDGT with one to three cyclic ring	[56]

Notes: *Archaeal C_{20} = C20 sn-2,3-diacilglycerol diether lipid.

**Archaeal C_{25} = C_{25} sn-2,3-sesterterpanyl.

***Macrocyclic Archaeal = C20 macrocyclic archaeal; GDGT = glycerol diphytanoyl glycerol tetraether; GDGT-0 cyclic ring; GDGT-1-4 cyclic ring; GDGT-5-8 cyclic ring.

Table 1. Main membrane lipids in *Euryarchaeota*.

the initial substrates for IPP formation are pyruvate and glyceraldehyde-3P, while in the MVA pathway are acetyl-CoA and acetoacetyl-CoA. Another difference lies in the last three enzymes of both pathways; in the MEP-DOXP route, they correspond to methylerythritol cyclodiphosphate (MEcPP) synthase and hydroxymethylbutenyl 4-diphosphate (HMBPP) synthase, which are absent in the MVA route. Further, the last enzyme in the MEP-DOXP pathway is an HMBPP reductase, which, in the other pathway, is a mevalonate-5-diphosphate (MVAPP) decarboxylase [45, 46].

5. Structural adaptations of extremophilic proteins

As already mentioned, archaea thrive in many different extremes: heat, cold, acid, base, salinity, pressure, and radiation. These harsh environmental conditions imposed several restrictions to which they had to adapt during the course of their evolution. Considering these extreme environments, archaea can be divided into: halophiles, psychrophiles, thermophiles, acidophiles, and piezophiles. However, it has to be remembered that these branches frequently intersect in interesting ways.

Archaeal organisms have evolved to deal with one or more extreme conditions that have led them to accumulate the changes mostly concerned with protein structure and enzyme activity. These adaptations made them a vast repertoire of macromolecules with particular features not available in their counterparts from other organisms. This constitutes an inexhaustible source of biological molecules for industrial or biotechnological applications.

5.1. Thermophiles and hyperthermophiles

Thermophilic and hyperthermophilic archaea are found mainly in the deep ocean, hydrothermal vents, and hot water near volcanoes. According to their growth temperature, these organisms have been classified as thermophiles or hyperthermophiles. The former are those able to grow at temperatures above 50°C and the latter at 80°C or more [47]. There are diverse cellular mechanisms that make the life possible at these extreme temperatures. The expression of molecular chaperones (HsP) that help in the correct folding of proteins and enzymes accompanied with more rigid membranes and proteins than those found in mesophiles are common characteristics of this kind of organisms [39, 48, 49]. At a genomic level, it is not clear if an increase in the G+C content is indeed a characteristic of thermophilic and hyperthermophilic organisms since some mesophilic organisms do show a higher proportion of these nitrogen bases than their hyperthermophilic counterparts [47, 50]. The high thermal stability, as well as the capacity of the enzymes of these microorganisms to catalyze reactions at very high temperatures, has made them a subject of intense research. Many archaeal thermophilic and hyperthermophilic enzymes employ substrates that are different from those used by their bacterial homologs; even some of them possess novel catalytic activities not found in bacteria, which make the archaeal enzymes a promising source for biotechnological processes.

Thermophilic and hyperthermophilic archaea could be either methanogenic or nonmethanogenic organisms. The nonmethanogenic hyperthermophilic archaea belong to the orders *Thermococcales*, *Thermoplasmatales*, and *Archaeoglobales*, whereas class I methanogenic archaea belong to the thermophilic organisms of the orders *Methanopyrales*, *Methanobacteriales*, and *Methanococcales* and class II methanogens belong to the orders *Methanomicrobiales* and *Methanosarcinales*.

5.1.1. Mechanism of protein adaptation to the heat

In general terms, the enzyme structures of thermophilic and hyperthermophilic archaea have been described as highly rigid and thermostable compared to their mesophilic homologs. The

mechanisms reported to achieve this enhancement in thermostability include an increase in the number of ionic interactions, disulfide bridges, surface charges, higher oligomerization states, and a more compact hydrophobic core [51]. Although to date there are many characterized thermophilic and hyperthermophilic enzymes from the phylum *Euryarchaeota*, it is not possible to compare them with their mesophilic homologs from the same phylum due to the lack of studies regarding the homologous counterpart.

The structure of enzyme triose phosphate isomerase is characterized by TIM barrel fold, and it catalyzes the reversible interconversion of glyceraldehyde-3P and dihydroxyacetone phosphate. In eukaryotes, bacteria, and mesophilic archaea, this enzyme is a dimer, while in thermophilic and hyperthermophilic archaea, it exists in a tetrameric form [58]. The hyperthermophilic triose phosphate isomerase from *P. furiosus* and *Thermococcus onnurineus* was also characterized as tetramers [59]. The increase in the state of oligomerization could be a difference related to an increase in the thermal stability; an interesting but different example of oligomerization state as a strategy to increase thermostability is manifested by an amylase from *P. furiosus*. This hyperthermophilic enzyme, unlike its mesophilic homologs, which are dimers, presents a lower oligomerization state, being described as a highly packed monomer. This highly packed structure, as well as the decrease in the oligomerization state, results in the decrease in the hydrophobic surface exposed to the solvent [60]. In this case, the interactions that favor the compactness of the hydrophobic core of the hyperthermophilic amylase are hydrogen bonds, which play a fundamental role in maintaining the folding at high temperatures [60]. In order to analyze the importance of ionic and noncovalent interactions in the thermostability, Vogt et al. performed a comparative analysis between the mesophilic and thermophilic proteins employing 56 model proteins belonging to 16 families and concluded that in thermophilic proteins, interactions like hydrogen bonds are in a greater number than in mesophilic proteins, which are bestowed by a high number of charged residues constituting a key characteristic to increase thermostability [61]. In addition, it can be noted that thermophilic and hyperthermophilic proteins exhibit a larger polar surface exposed to the solvent in comparison with mesophilic proteins mainly due to the presence of charged residues on the surface and the diminution in the number of noncharged polar residues [62]. The presence of large hydrophobic lateral chains is also a characteristic of thermostable proteins. The comparison of the glutamate dehydrogenase from *P. furiosus* with its bacterial homologs (*Thermotoga marítima*, *Tm*GDH and *Clostridium symbiosum*, *Cs*GDH) shows that this thermostable enzyme presents a greater fraction of charged residues given mainly by arginine residues. In addition, it was established that the ionic-pair strategy follows this trend: *Pf*GDH > *Tm*GDH > *Cs*GDH [63].

A good example of the role of ionic interactions in the adaptation of protein structures to high temperatures is the ionic network present in a triose phosphate isomerase from *P. furiosus* and *T. onnurineus*; this type of ionic network is absent in the psychrophilic homolog from *Methanosarcinales* (*M. burtonii*) [64]. The introduction of ionic interactions in a psychrophilic enzyme significantly increased the thermal stability of a mutant, highlighting the importance of this type of interactions in the increased thermal stability of enzymes from archaea [64]. Even more, when this network of ionic interactions was eliminated from the triose phosphate isomerase from *P. furiosus*, its stability decreased [65].

An example of the use of disulfide bridges for increased stability is the comparison between the archaeal hyperthermophilic enzyme alpha-amylase from *P. furiosus* and its bacterial counterpart from *Bacillus licheniformis*. The study shows that the increased stability of the alpha-amylase from *P. furiosus* was due to a higher content of cysteine residues than the ones observed in the enzyme from *B. licheniformis*. The increase in cysteine residues along with the increase in the number of ionic pairs has been described as the main characteristics responsible for the activity and protein stabilization in this hyperthermophilic organism [66].

5.2. Psychrophiles

Most of the archaeal organisms studied evolved to colonize low-temperature aquatic ecosystems such as those present in Antarctic, Arctic, vast tracts of the deep sea, and also alpine regions. These organisms are called psychrophiles and can be classified in *Stenopsychrophiles* and *Eurypsychrophiles* according to their growth temperature range [67]. This classification is based on two parameters: optimal growth temperature (T_{opt}) and maximal growth temperature (T_{max}). *Stenopsychrophiles* show an upper optimal growth temperature less than ~20°C and a T_{max} not greater than 25°C. On the other hand, *Eurypsychrophiles* tolerate a broader temperature range, presenting T_{opt} above 30°C and T_{max} below 10°C [67]. To date, almost all psychrophilic archaea belong to the phylum *Euryarchaeota* and they are all methanogenic. Psychrophilic and methanogenic archaea can be found in the following archaeal orders: *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales*. *Methanobacterium* sp. is a representative of the order *Methanobacteriales*, while in the order *Methanomicrobiales*, we can find *Methanogenium frigidum*, *Methanogenium marinum*, and *Methanogenium boonei*. In *Methanosarcinales*, the organisms identified correspond to *M. burtonii*, *Methanococcoides alaskaense*, *Methanosarcina baltica*, *Methanosarcina lacustris*, and *Methanolobus psychrophilus*. Psychrophilic archaea and their proteins and enzymes have been a focus of great attention owing to their high potential as biocatalysts in biotechnological applications since a long time.

5.2.1. Mechanism of protein adaptation to the cold

Low temperature imposes several challenges to cellular functions such as replication, transcription, translation, and metabolic reactions crucial for the development of microorganisms. At a cellular level, the common strategies employed to cope low temperatures include, inter alia, cold shock proteins, antifreeze proteins, and an increased membrane fluidity [67]. Besides this, the protein structure should also adapt to a cold environment. Identifying the important features that confer specific thermal properties has been a subject of intense research in the last few years. Even so, to date, very few proteins from psychrophilic archaea have been studied, in contrast to a large number of proteins and enzymes from mesophilic, thermophilic, and hyperthermophilic archaea. The current studies indicate that the main feature of psychrophilic proteins and enzymes is to have a flexible structure, which could offset the energy decrease of the reaction medium, thus facilitating catalysis processes at low temperatures. Heat-induced unfolding experiments for psychrophilic, mesophilic, and thermophilic proteins show distinct stability patterns where the unfolding of the cold-adapted proteins occurs at lower temperatures (T_m) and gradually increases for the other groups [68]. Nonetheless,

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the psychrophilic enzymes do not display unusual or exotic 3D conformations and bear overall folds resembling that of their mesophilic counterparts [69]. The main challenge faced by psychrophilic enzymes is to catalyze reactions at an appropriate rate even when the low temperatures strongly diminish the rates of chemical reactions. Several reports regarding cold-active enzymes have demonstrated that they display a much higher specific activity at low and moderate temperatures as their thermophilic counterparts [70]. This is caused by the destabilization of either the active site or the whole protein, conferring mobility and flexibility to the active site at the temperatures that tend to freeze molecular motions [71]. It is generally accepted that although other molecular traits can contribute to cold activity, the lack of selective pressure on stable proteins, in conjunction with a strong selection of highly active enzymes, is the main factors responsible for cold activity adaptation in natural environments.

The psychrophilic enzymes from archaea evolved to attain more flexible structures by adopting several mechanisms. For example, by reducing the number of charged residues present at both the protein surface and the hydrophobic core (Arg, Lys, Glu). A low content of arginine residues results in a low number of hydrogen bonds that can be formed contributing to structural flexibility. In order to compensate for the loss of charges at the protein surface and avoid aggregation, these proteins present a great proportion of noncharged polar residues such as Gln and Thr, which in turn implies a decrease in stabilizing ion pairs favoring a structural destabilization [72]. Other adaptations include the clustering of glycine residues (providing local mobility), the disappearance of proline residues in loops (providing enhanced chain flexibility between secondary structures), as well as a lower number of ion pairs, aromatic interactions, and hydrogen bonds [73]. Additionally, the hydrophobicity of the protein core and the compactness of the protein interior is usually low [73]. In summary, all aforementioned factors are attenuated in strength and number in the structures of cold-active enzymes.

One remarkable fact about psychrophilic enzymes is that they are inactivated at the temperatures that are well below than the one at which the protein unfolds; this presents a remarkable difference from their mesophilic or thermophilic homologs. This led to the concept of a localized increase in the flexibility at the active site, which is responsible for a high but heat-labile activity, while the other regions of the protein, not involved in catalysis, might not have low stability [74, 75]. The comparison of the experimentally measured activation energy of transition ($\Delta G^{\neq}$) of some cold-active enzymes revealed that this parameter is systematically lower than the mesophilic proteins [74]. It has been proposed that the activation of these enzymes is facilitated by a decrease in the affinity of the enzyme for the substrate (higher level of ES) and by a possibly lower energetic level of $ES^{\neq}$. In many cases, the high activity of these enzymes at low temperatures has been associated with a rather open structure and also to a loss of specificity [73, 74].

The above and the other structural alterations have been reported mainly for psychrophilic enzymes from bacteria and eukarya, and there are not enough studies about psychrophilic archaeal enzymes in order to sustain that the same alternations are also responsible for cold adaptations in these organisms. A general adaptive mechanism proposed for psychrophilic enzymes from bacteria is the optimization of k_{cat} at the expense of K_m [73]. Although this mechanism is generally accepted, it cannot be generalized to archaeal enzymes considering the few

cases studied. In the case of the GTPase of the elongation factor 2 (EF2) from *M. burtonii*, the adaptive mechanism to perform its activity at low temperatures involves a reduction in the K_m value compared to its thermophilic homolog phylogenetically related from *Methanosarcina thermophil*e [76]. This decrease is due to the loss of noncovalent interactions that allow this enzyme to have a greater structural flexibility [76]. The loss of ionic and noncovalent interactions offering an increase in the structural flexibility has also been seen in other psychrophilic archaea enzymes such as the enzyme triphosphate isomerase from *M. burtonii* and in the DNA polymerase from *Cenarchaeum symbiosum* [77, 78].

The biophysical and catalytic features of psychrophilic enzymes present a challenge and offer an interesting model to unravel protein evolution, folding, and dynamics. We hope that these traits along with their tremendous biotechnological potentials will bring further promising advances in the archaeal psychrophilic protein research.

5.3. Halophiles

Hypersaline environments are defined as those containing higher salt concentrations than seawater (>3.5% total dissolved salts). Most hypersaline bodies are thalassohaline, dominated by Na^+, Ca^{2+}, Cl^-, and SO^{4-} ions, generally bearing neutral pH. These bodies derive from the evaporation of seawater and retain the relative proportion of salts in the sea. On the other hand, there is another less common group called athalassohaline of water bodies, dominated, among others, by Ca^{2+}, Mg^{2+}, Cl^-, and Ba^-, with pH conditions ranging from acidic to alkaline, and having a nonmarine origin. Both overall salinity and ionic composition together with the conditions like temperature and nutrient availability determine the existence of highly variable hypersaline environments that can be found extending from Antarctica to alkaline hypersaline soda brines and subterranean evaporite deposits.

Halophilic microorganisms are classically categorized according to their optimal growth at different salt concentrations, and two main groups have been recognized: the extreme halophiles (optimal growth above 15% NaCl) and moderate halophiles (optimal growth 3–15% NaCl).

In archaea, the only halophilic organisms known to date belong to the phylum *Euryarchaeota*. These organisms are distributed into three groups: (1) class *Halobacteria*, whose members are extreme halophiles that require over 3M salinity for growth and structural stability; (2) order *Methanosarcinales*, including extreme and moderate halophilic organisms; and (3) the recently discovered archaeal class, *Nanohaloarchaea*, uncultured to date [79, 80].

5.3.1. Mechanism of protein adaptation to salt

How do these organisms deal with high salinity environments and what adaptations did they incorporate into their molecular machinery and proteins? Both questions have been addressed in multiple studies with the main focus on *Halobacteria* organisms and their molecular machinery and proteins [51]. There are two fundamentally different strategies proposed for halophilic microorganisms explaining how they maintain osmotic pressure in their cytoplasm while growing in a saline medium: the salt-in-cytoplasm and the organic osmolyte

accumulation. It has been demonstrated that, in *Halobacteria*, the intracellular accumulation of inorganic ions, mainly potassium and chloride, to high concentrations is the strategy employed to balance the extracellular osmotic pressure [81] in accordance with the salt-in mechanism. These high intracellular concentrations require unique adaptations of the molecular machinery implying that the proteins must retain their structural and functional integrity under such high salt conditions [80]. Although a perfect model that accurately explains how a protein structure is stabilized at high salt concentrations is still debatable, some specialized features of osmoadaptation have been identified through the determination of the genome sequence of *Halobacteria* organisms [82]. The statistical and bioinformatic analyses of these data, together with previous experimental data [83], have identified a biased amino acid composition known as the "halophilic signature." This signature is characterized by an increase in negative residues Asp and Glu, a reduction in the positively charged residues, Lys but no Arg, the low content of bulky hydrophobic residues like Phe and Ile, and an increase in small hydrophobic residues like Ala and Val [84]. As a result, in addition to preserving the protein folding relative to mesophilic counterparts, halophilic proteins exhibit a low hydrophobic content and a surface with a large negative net charge. This trend has also been confirmed by the means of isoelectric point calculated from the proteome of these organisms (the most acidic proteomes to date) [82].

The classical "solvation-stabilization" model proposed for the understanding of the mechanism behind the adaptation of the halophilic proteins proposes that the stability at high salt concentrations arises from the recruitment of an orderly solvate envelope of high ionic concentration, coordinated through the abundance of carboxylate groups (Asp and Glu) at the protein surface [85]. Despite the studies that have identified an increased stability and solubility of proteins enriched in Asp and Glu residues [86], biophysical [87] and crystallographic data [88] have failed to identify such hydration layers up to the extent proposed. More recently, it has been proposed that the main change in residue composition responsible for "halophilicity" is the diminution of Lys residues [89] due to the decreased solvent accessible area. This idea was confirmed by crystallographic studies, showing that water molecules adopt more structured distributions in the vicinity of Lys residues [90].

On the other hand, *Methanosarcinales* constitutes a heterogeneous group of organisms, consisting of the species with different salt requirements, ranging from moderate to extreme halophiles. These organisms are able to accumulate intracellular organic solutes, and the more halophilic organisms of the group are also able to accumulate inorganic ions (like K^+) in high concentrations [91]. Interestingly, recent phylogenetic studies show that this group of organisms branched off from the same ancestral node as the groups *Halobacteria* and *Nanohaloarchaea* [79]. Nevertheless, no study to date has addressed the possible adaptations on the amino acid composition and structural properties of proteins from *Methanosarcinales*.

5.4. Acidophiles

Acidophilic and acid tolerant organisms can thrive in natural, as well as man-made, acidic environments at pH less than 4.0. These environments are usually present in the combinations with other stressors, like high temperatures, elevated concentrations of heavy metals,

and salinities approaching saturation [92]. As a result, *Euryarchaeota* acidophilic organisms are mostly polyextremophiles, being an example of acidophilic organisms that thrive in high salinities (*Halarchaeum* genera) [93] and in heavy metals along with high temperatures (*Thermoplasmata* class) [94].

Although some members of *Euryarchaeota* live in environments with pH values below 1, it has been demonstrated that most acidophiles maintain their internal pH close to neutrality. In order to adapt to these conditions, acidophilic organisms have evolved different mechanisms, such as a proton impermeable cell membrane, reversed membrane potential, and a cytoplasmic buffering system [95]. However, despite keeping their internal environment close to neutral pH values, these organisms possess macromolecules with adaptations that preserve their structure and function. However, the exact mechanisms underlying these adaptations have not been elucidated to date.

5.4.1. Mechanism of protein adaptation to acid

One striking feature of some acidophilic proteins is their requirement of a low pH (2–5) for their optimal activity, such as alfa-glucosidase and carboxyl esterase [96], even when the internal pH of these organisms is close to neutrality. Nevertheless, not all proteins from *Euryarchaeota* acidophiles have a preference for a low pH for their optimal activity, for example, an ATP-dependent DNA ligase from *Ferroplasma acidarmanus* has its optimal activity at pH 6–7, similar to the DNA ligases from nonacidophilic organisms [51, 97].

A possible explanation for the optimal activity at low pH was proposed through the study of the endo-β-glucanase from the *Crenarchaeota Sulfolobus solfataricus* [98]. This enzyme has an optimum pH of approximately 2.0 and an optimum temperature around 80°C. Through homology modeling, it was determined that its catalytic domain possesses a fold similar to that observed in other mesophilic, acidophilic, and neutral cellulases and its surface displays mostly negative charges. Nonetheless, other β-glucanases from mesophilic homologs, which are optimally active at neutral pH, also display low predicted P.I. values, which suggests that the net charge is not the only factor responsible for the extreme acidic stability [51, 98].

Recent crystallographic studies shed some light on the mechanisms of protein stability and catalytic efficiency at low pH. The strategy of increased negative residues was not present at the same extent in the aspartate racemase from *P. torridus* [99], while in the carboxylesterase from *F. acidiphilum* a highly negatively charged surface around the active site was identified. However, this is not a trend observed for the rest of the protein surface. Further, in this enzyme, it has been demonstrated that the modifications in the hydrogen bond network surrounding the catalytic triad altered the catalytic efficiency and allowed pH preference adjustments from a low pH to a more upward optimum and vice versa [100].

5.5. Piezophiles

Deep-sea hydrothermal vents are another extreme environment colonized by archaea. In these environments, an average pressure of approximately 38 MPa is found that can reach

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even up to 110 MPa, hence imposing a major challenge for life. Organisms that can thrive in such extreme barometric pressure are often termed as piezophiles or barophiles. Several piezophiles have been cultured; however, they require specialized equipment in order to maintain high pressures. Thus, many studies have focused on nonculturing techniques, like genomic analysis. Besides high pressure, hydrothermal vents also have very high temperatures and indeed could be the habitat of hyperthermophiles. However, only a few hyperthermophiles are also piezophiles. To date, the only strictly piezophilic anaerobic hyperthermophilic archaeon reported is *Pyrococcus yayanosii* CH1 [101]. Some reports have indicated that there are no specific pressure-related adaptations required for the enzymes isolated from piezophiles to be stable; however, a hydrostatic pressure asymmetry index (PAI) that reflects the extent to which an amino acid is preferred by piezophiles has been described [102]. Proteomic comparative analysis of *P. furiosus* and *P. abyssi* shows that Asp and Arg are the only two amino acids that can be designated preferentially barophilic, although previous studies designated five (Arg, Ser, Val, Asp, and Gly). On the other hand, only three amino acids (Asn, Lys, and Thr) display a clear preference for nonbarophily [103].

6. Biotechnological applications of extremozymes

The extreme harsh environmental conditions where extremophiles live serve as an enormous source of enzymes with peculiar properties that make them very suitable for industrial or biotechnological applications. The first commercialized enzyme was diastase, available in the market since 1830 in France. Since then, the enzyme market gained importance because they not only reduce the cost of the products but benefit the environment. In 2015, the global market for industrial enzymes reached nearly 4.9 billion and is expected to reach nearly $5.0 billion in 2016 to $6.3 billion in 2021. Food and animal feed industrial enzyme market is expected to grow to $1.9 billion and $1.6 billion in 2021, respectively (BCC Research Biotechnology report 2017). At present, most of the industrially applied enzymes show low activity and stability, which is highly disadvantageous in terms of concomitant high costs (**Table 2**).

There have been continuous efforts for expressing the genes encoding for the enzymes from extremophiles in mesophilic hosts in order to overproduce them and modify their properties to be suitable for commercial applications. In addition, archaeal enzyme expression can be achieved by using extremophilic microorganisms as hosts for autologous gene expression [124]. Integrative and shuttle vectors have been developed for *Methanococcus* species, which allow overexpressing specific enzymes with complex prosthetic groups that are inactive if expressed in *E. coli* [124].

Archaeal compounds also have many applications in the pharmaceutical and alimentary industry. *Haloarchaea* organisms from the order *Halobacterium* (*Haloferax* sp.) produce a peptide called halocin, which is used as an antimicrobial and preservative in food with high salt content. For example, the H6/H7 halocin produced by *Haloferax gibbonsii* affects the Na+/H+ antiporter and then inhibits the membrane ionic gradient of the target cell, provoking cell death by lysis [125, 126]. In addition, compounds like canthaxanthin produced by *Haloferax*

Enzyme	Enzyme characteristics	Organism	Application	References
α-Amylase	Hyperthermophilic	*Thermococcus profundus*	Bread and baking industry, Starch liquefaction and saccharification. Production of glucose, fructose for sweeteners, textile desizing, paper industry	[104]
	Halophilic	*Haloferax mediterranei*		[105]
	Acidophilic	*Picrophilus torridus*		Q6KZM7*
	Psychrophilic	*Methanococcoides burtonii*		Q12YQ1*
Subtilisin	Hyperthermophilic	*Thermococcus kodakaraensis*	Detergents, baking, brewing and amino acid production	[106]
	Halophilic	*Halorubrum litoreum*		M0NQ93*
	Acidophilic	*Thermoplasmatales archaeon*		M7TYK7*
	Psychrophilic	*Methanolobus psychrophilus*		K4M7H8*
Esterase	Hyperthermophilic	*Picrophilus torridus*	Detergent formulations and dairy industry	[107]
	Halophilic	*Haloarcula marismortui*		[108]
	Acidophilic	*Picrophilus torridus*		[107]
DNA polymerase	Hyperthermophilic	*Pyrococcus abyssi*	DNA cloning, sequencing, labeling, mutagenesis, and other purposes	[109]
	Halophilic	*Halobacterium halobium*		[110]
	Acidophilic	*Thermoplasma acidophilum*		Q9HJR0*
	Psychrophilic	*Methanococcoides burtonii*		Q12YC5*
Cellulase	Hyperthermophilic	*Pyrococcus horikoshii*	Pulp and paper, textile, laundry, biofuel production	[111]
	Halophilic	*Halorhabdus utahensis*		[112]
	Acidophilic	*Picrophilus torridus*		Q6KZ15*
	Psychrophilic	*Methanococcoides burtonii*		Q12XZ9*
β-Glycosidase	Hyperthermophilic	*Pyrococcus furiosus*	Polymer degradation, color brightening, color extraction of juice, cotton products, synthesis of sugars	[113]
	Halophilic	*Haloarcula marismortui*		Q5V5G3*
	Acidophilic	*Picrophilus torridus*		[114]
β-Galactosidase	Hyperthermophilic	*Pyrococcus woesei*	Detergent and food industries and for the production of fine chemicals	[115]
	Halophilic	*Haloferax alicante*		[116]
	Acidophilic	*Picrophilus torridus*		[117]
	Psychrophilic	*Halorubrum lacusprofundi*		[118]

Enzyme	Enzyme characteristics	Organism	Application	References
Alcohol dehydrogenase	Hyperthermophilic	*Thermococcus hydrothermalis*	Food, pharmaceutical, and fine chemicals industries	[119]
	Halophilic	*Haloferax volcanii*		[120]
	Acidophilic	*Thermoplasma acidophilum*		[121]
	Psychrophilic	*Halorubrum lacusprofundi*		B9LV78*
Lipase	Hyperthermophilic	*Pyrococcus furiosus*	Detergent formulations and the dairy industry	[122]
	Halophilic	*Natronococcus* sp		[123]
	Acidophilic	*Thermoplasma acidophilum*		Q9HJS7*

*UniProt code.

Table 2. Extremozymes and their applications in industrial and biotechnological processes.

alexandrinus present antioxidant properties that can be used potentially as food supplements to prevent cancer or cardiovascular diseases [127]. Other compounds like exopolysaccharides produced by *Haloferax* have been employed in the food industry as emulsifiers since they are stable at high temperatures. Other interesting biotechnological applications are the use of archaeal lipids for the formation of a new generation of liposomes, known as archaeosomes. Archaeal lipids present a more polar character and have ether bonds which gave them more stability at extreme temperatures, pH, and pressure. In addition, these characteristics provide protection against oxidation, to the action of phospholipases and chemical hydrolysis, providing an advantage over liposomes formed by neutral phospholipids. Owing to their great stability, biocompatibility, and biodegradation, archaeosomes have many uses as vaccine adjuvants and in drug delivery system [128].

There is a huge amount of information available regarding biotechnological applications of extremozymes, and therefore, this chapter made an effort to summarize the applications of these enzymes and compounds in some selected areas. Considering that very few archaeal enzymes have found their way to the market in some applications, we provided the examples of such extremophiles and the corresponding UniProt code for the homologous enzymes present in archaea (**Table 2**). We hope that this kind of information will be extremely valuable for future studies looking for archaeal enzymes with particular properties.

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Author details

Victor Castro-Fernandez*, Ricardo Zamora, Alejandra Herrera-Morande, Gabriel Vallejos, Felipe Gonzalez-Ordenes and Victoria Guixé*

*Address all correspondence to: vguixe@uchile.cl and vcasfe@ug.uchile.cl

Department of Biology, Faculty of Sciences, University of Chile, Santiago, Chile

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