In the light of the haloarchaea metabolism

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1. Introduction.

Nitrogen (N), carbon (C), hydrogen (H) and oxygen (O) are the major elements of all organisms and are the components of the most important biological macromolecules: proteins, lipids, carbohydrates and nucleic acids. The reactions performed in different ways to allow the conversion of chemical species into other compounds, make up the biological metabolic pathways which are not well known in halophilic archaea. Using *Haloferax mediterranei*, *Haloferax volcanii* and *Halobacterium salinarum* as haloarchaea models the main metabolic pathways have been characterized in the last few years.

Halophilic archaea (haloarchaea) grow in hypersaline (3–5 M NaCl) environments, including hypersaline lakes and marine salterns [1]. Acetate could have a role in the nutrition of natural communities of halophilic Archaea, as it is produced from glycerol (the main carbon source in nature) in hypersaline lakes by some species of halophiles [2]. With regard to carbon metabolism it has been characterized the glyoxylate cycle, a metabolic pathway by which organisms can synthesize carbohydrates from C2 compounds, in *Hfx. volcanii* [3].

It has been demonstrated that nitrate, nitrite or ammonium can be used by some halophilic archaea for growth in presence of oxygen. This pathway is known as “assimilatory nitrate reduction” and it was characterized first in *Hfx. mediterranei*. However, under anoxic conditions, nitrate and nitrite are the final electron acceptor in a respiratory process denominated denitrification.

In our research group we have characterized some of the enzymes involved in different metabolic pathways.

2. Carbon metabolism in haloarchaea

Organisms of the three domains of life need polysaccharides as a carbon source to support their heterotrophic growth. In general, the utilization of polysaccharides involves their extracellular hydrolysis, the intake of oligosaccharides by specific transporters and their intracellular hydrolysis to generate hexoses and pentoses. Subsequently, these monosaccharides are oxidized via a well-conserved set of central metabolic pathways.

The glycolytic pathway and the citric acid cycle are the main energy producing pathways in cells. Although these pathways show a great variability between organisms, many of the core enzymes of both glycolysis and the citric acid cycle seem to be conserved in the majority of organisms. Glycolysis is responsible for sugars metabolism, in particular glucose, and its conservation in the evolution of the organisms suggests that glucose is a major source of energy for many organisms. Glucose is found in nature primarily in a polymeric state as cellulose, starch and glycogen.

2.1. Amylases.
Starch is an important source of energy especially for microorganisms. It is composed exclusively of D-glucose units that are linked by α-1,4- or α-1,6-glycosidic bonds. The two components of starch are amylose (15–25%), a linear polymer consisting of α-1,4-linked glucopyranose residues, and amylopectin (75–85%), a branched polymer containing, in addition to α-1,4 glycosidic linkages, α-1,6-linked branch points occurring every 17–26 glucose units. Selective hydrolysis of glycosidic bonds is therefore crucial for energy uptake, and other biological functions like cell wall expansion and degradation, and turnover of signalling molecules. Enormous amounts of glucose are available to organisms that can hydrolyse glucose polymers efficiently, that show the significance that glucose polymers have for life [4].

Because of the complex structure of starch, cells require an appropriate combination of intracellular and extracellular enzymes for its conversion to oligosaccharides and smaller sugars, such as glucose and maltose. There are basically four groups of starch-converting enzymes [5]:

- **Endoamylases**: these enzymes are able to cleave α-1,4-glycosidic bonds in the inner part of the amylose or amylpectin chain. A well studied endoamylase is α-amylase (EC 3.2.1.1) which end products reaction are oligosaccharides with a α-configuration and α-limit dextrins.
- **Exoamylases**: there are enzymes that only cleave α-1,4 glycosidic bonds such as β-amylase (EC 3.2.1.2) or cleave both α-1,4 and α-1,6 glycosidic bonds like amyloglucosidase or glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20). Exoamylases act on the external part of amylose or amylpectin and thus produce only glucose (glucoamylase and α-glucosidase), or maltose and β-limit dextrin (β-amylase).
- **Debranching enzymes**: to this group belong enzymes that exclusively hydrolyze α-1,6 glycosidic bonds, like isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41). Its degradation products are maltotriose and linear oligosaccharides.
- **Transfereases**: these starch-converting enzymes cleave an α-1,4 glycosidic bond of the donor molecule and transfer part of this donor to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC 2.4.1.19) form a new α-1,4 glycosidic bond while branching enzyme (EC 2.4.1.18) forms a new α-1,6 glycosidic bond. Cyclodextrin glycosyltransferase produces a series of non-reducing cyclic dextrins, α-, β- and γ-cyclodextrins, that contain six, seven and eight glucose units respectively. The branching enzyme produces cyclic glucans from amylase and amylpectin.

Most of the starch-converting enzymes belong to the α-amylase family or family 13 glycosyl hydrolases according to the classification of Henrissat [6]. Family GH13, is the largest sequence-based family of glycosyl hydrolases. This family groups together a great number of enzymes with different activities but all of them share the following characteristics:

- They act on α-glycosidic bonds hydrolyzing them (i) to produce α-anomeric mono- or oligosaccharides (hydrolysis); (ii) to form α-1,4 or 1,6 glycosidic linkages (transglycosylation); (iii) or a combination of both activities.
- They adopt a (β/α)8 barrel structure that contains the catalytic site residues.
- They share four highly conserved regions in their primary sequence which contain the amino acids that form the catalytic site, and some amino acids that are essential for the stability of the (β/α)8 barrel [7].

Structurally, the GH13 enzymes are multidomain proteins characterized by a conserved structural core composed of three domains often designated as domains A, B and C. Domain A is the catalytic domain in the form of a (β/α)8 barrel, a barrel of eight parallel β-strands surrounded by eight helices, the so-called domain A [8]. Domain B is a loop of variable length between the third β-strand and third helix of the (β/α)8 barrel. This loop has an irregular structure that varies from enzyme to enzyme. The active site is found in a cleft between domains A and B where a triad of catalytic residues performs catalysis. Domain C is a C-terminal extension following the catalytic domain characterized by a Greek key structure. This domain is made up of β-strands and is thought to stabilise the catalytic domain by shielding hydrophobic residues of domain A from the solvent. It has also been suggested that domain C
may aid in substrate binding. In addition to this conserved core, some members of family GH13 have additional β-sheet domains after domain C, domains D and E. While no role has been assigned to domain D, domain E is believed to be important for binding sugar or granular starch.

Many starch-degrading proteins have been identified in various organisms, but relatively few studies are focused on archaeal α-amylases. In recent studies it has been purified and biochemically characterised a haloarchaeal α-amylase from *Hfx. mediterranei* by our research group [9]. The behaviour of this enzyme is very close to that reported for the amylases from *Hbt. salinarum* [10], and *Natronococcus amylolyticus* [11], and the moderately halophilic bacteria *Halomonas meridiana* [12].

The enzymatic hydrolysis and modification of polysaccharides is of great interest in the field of biotechnology (food, chemical, and pharmaceutical industries) [13]. α-Amylases are used in the starch liquefaction process that converts starch into fructose and glucose syrups. They are also used as a partial replacement for the expensive malt in the brewing industry, to improve flour in the baking industry, and to produce modified starches for the paper industry. In addition to this, they are used to remove starch in the manufacture of textiles and as additives to detergents for both washing machines and automated dishwashers.

2.2 Glucose dehydrogenase

Different pathways are involved in the catabolism of glucose. The classical Embden-Meyerhof (EM) pathway, or glycolysis, is well-conserved in bacteria and eukaryotes. In archaea, the best study about EM pathway has been carried out in the thermophilic microorganism *Pyrococcus furiosus*. These studies have revealed a modified EM pathway which presents novel enzymes involved in the catabolism of glucose. In addition, a new regulation site for energy metabolism and a unique mode of ATP regeneration have been postulated [14-16]. The Entner-Doudoroff (ED) is also widely distributed in nature. In bacteria consists of nine enzymes, and starts with the phosphorylation and oxidation of glucose. The main difference between both pathways is found at the number of ATP obtained per glucose molecule, two ATP molecules are generated in the EM whereas the ED pathway only yields a single ATP [17].
In some archaea, glucose is metabolized by way of modifications of the ED pathway (Figure 1). The first one is the non-phosphorylated ED pathway, which was discovered in extreme thermoacidophiles like *Thermoplasma acidophilum* and *Sulfolobus solfataricus* [18,19]. The second one was found in halophilic archaea (and some bacteria) and it is the modified ED pathway, in which the phosphorylation step is postponed. Glucose is oxidized to gluconate via NADP⁺-dependent glucose dehydrogenase (Figure 1). Evidence for the operation of this pathway has been demonstrated for species of *Halobacterium*, *Haloferax* and *Halococcus* [20].

Extremely halophilic archaea are considered a rather homogeneous group of heterotrophic microorganisms predominantly using amino acids as their source of carbon and energy. However, it has been shown that some halophilic archaea are able to use not only amino acids but different sugars as well. *Hfx. mediterranei* for example grows in a minimal medium containing glucose as the only source of carbon [21] using a modified ED pathway. The first step of this pathway is catalyzed by NAD(P)⁺-dependent glucose dehydrogenase (GlcDH). This protein is a dimeric enzyme with a molecular weight of 39 kDa per subunit. It shows dual cofactor specificity, although it displays a marked preference for NADP⁺ over NAD⁺. The protein requires a zinc ion for catalysis and sequence analysis revealed that this enzyme belongs to the zinc-dependent medium-chain alcohol dehydrogenase superfamily [22].

![Diagram of the catabolism pathway of glucose](image)

**Figure 1:** Catabolism pathway of glucose. Entner-Doudoroff pathway is represented as continuous line, modified ED pathway of halophilic archaea is shown in broken line and non-phosphorylated ED pathway of thermophilic microorganisms is represented as dotted line.
activity of halophilic GlcDH was dependent on the temperature, showing its maximum between 40 and 50 °C. The thermophilic nature of this kind of proteins has been noted in most of the halophilic enzymes, for instance Haloarcula marismortui malate dehydrogenase. Structural studies of this enzyme have revealed that several of the structural features conferring halophilicity are the same as those contributing to the stability of thermophilic enzymes [23].

To study their structural properties and to extend the understanding of the molecular basis of salt tolerance responsible for halophilic adaptation, glucose dehydrogenase from Hfx. mediterranei has been overexpressed [24] and its structure has been solved at 1.6 Å. Analysis of protein solvent interactions reveals a highly ordered multilayered solvation shell that can be seen to be organized into one domain network covering much of the exposed surface accessible area to an extent not seen in almost any other protein structure solved [25]. These studies are important not only to establish the molecular basis of adaptation at high ionic strength mediums of this kind of proteins, but also to amplify the information available about the halophilic enzymes involved in the catabolism of glucose.

A study of the metabolic adaptations of Hfx. volcanii by transcriptome analysis using shot-gun DNA microarrays shown that after glucose induction transcript levels of glucose dehydrogenase and 2-keto-3-desoxy-6-phosphogluconate (KDPG) aldolase were found to be increased [26]. Also induced transcript levels were found in a gene encoding a protein with similarity to 12 transmembrane helix transport proteins, which was proposed to be a glucose transporter, whereas transcript levels of several enzymes involved in glucose biosynthesis are repressed. This study also reveals that the citric acid cycle, the electron transport chain and ATP synthesis are down regulated after the shift to glucose medium. A similar down regulation of some genes of the citric acid cycle and of respiration has been postulated in Haloarcula marismortui [27].

It has been shown in various halophilic archaea that during exponential growth on glucose significant amounts of acetate were formed [28]. The formation of acetate from acetyl-CoA is catalyzed by an ADP-forming acetyl-CoA synthetase (ACD). This unusual synthetase was found in all acetate forming archaea, including anaerobic hyperthermophiles. In Har. marismortui during growth on glucose and on glucose/acetate both ACD and glucose dehydrogenase activity increased parallel to phases of glucose consumption and acetate formation and both activities decreased during growth on acetate or peptides. Acetate formation in Haloarcula appears to be restricted to sugar metabolism under conditions when the rate of glycolysis exceeds that of subsequent pathways.

2.3 Isocitrate DH

The citric acid cycle is the common mode of oxidative degradation in eukaryotes and prokaryotes. This cycle, which is alternatively known as the tricarboxylic acid (TCA) cycle and the Krebs cycle, accounts for the major portion of carbohydrate, fatty acid, and amino acid oxidation and generates numerous biosynthetic precursors (Figure 2). The citric acid cycle is therefore amphibolic, that is, it operates both catabolically and anabolically.

The eight enzymes of the citric acid cycle catalyse a series of well-known organic reactions that cumulatively oxidize an acetyl group to two CO₂ molecules with the concomitant generation of three NADHs, one FADH₂ and one GTP. Archaea metabolize pyruvate (which is produced by hexose catabolism), via the citric acid cycle. However, there are differences in the enzymes used. For example, archaea catalyse pyruvate to acetyl CoA via pyruvate oxidoreductase, having ferredoxin as electron acceptor in halophiles and thermophiles and a deazin derivative, F₄₂₀ as the electron acceptor in methanogens [29]. This enzyme is also found in anaerobic eubacteria which might suggest that the oxidoreductase existed before the divergence of the archaeal, eubacterial and the ancestral eukaryotic line of descent. The pyruvate dehydrogenase (PDH) multi-enzyme complex, characteristic of respiratory eubacteria and eukaryotes, is absent from archaea; however, thermophiles and halophiles contain dihydrolipoamide dehydrogenase which is normally the third component in the PDH complex [30]. Therefore, it is likely that the PDH complex may have evolved soon after development of oxidative phosphorylation since the complete complex has only been detected in respiratory organisms [31].
The type of citric acid cycle seen in archaea depends on the class to which they belong. For example, halophiles metabolize pyruvate via an oxidative citric acid cycle. Sulphur-dependent thermophiles generally fix carbon dioxide via a reductive citric acid cycle when growing autotrophically and use an oxidative cycle when heterotrophic [32]. In methanogens there is no complete citric acid cycle; instead there are two different routes in which 2-oxoglutarate is formed [33]. One uses an incomplete reductive pathway where 2-oxoglutarate is synthesized from oxaloacetate, and the other uses an incomplete route where 2-oxoglutarate is formed via citrate. It is possible that the type of citric acid cycle present in an archaea will determine enzyme function and, hence, reflect any unique features.

Isocitrate dehydrogenase (EC 1.1.1.41 or EC 1.1.1.42) (ICDH), catalyses the oxidative decarboxylation of isocitrate:

\[
\text{Isocitrate} + \text{NAD}(\text{P})^+ \rightarrow 2\text{-oxoglutarate} + \text{NAD}(\text{P})\text{H} + \text{H}^+ + \text{CO}_2
\]

A number of isoenzymes of isocitrate dehydrogenase, distinguished by their cofactor requirement and subcellular localization, have been identified. In Eukarya, ICDH exists in two forms: an NAD-linked enzyme found only in the mitochondrion and displaying allosteric properties, and a non-allosteric NADP-ICDH that is found in both the mitochondrion and cytoplasm [34,35]. Most Bacteria possess only an NADP-ICDH [36], although NAD-linked [37] and dual-cofactor-specific (NAD and NADP) bacterial enzymes are known [38,39].

The purification and characterisation of ICDH from the extremely halophilic Archaeon, *Hfx. volcanii* have been determined. It is a dimer; strictly NADP-dependent; and its thermal stability has been studied at 0.5 M or 3 M KCl, obtaining an activation energy for the thermal inactivation process of 360 kJ mol\(^{-1}\) and 610 kJ mol\(^{-1}\), respectively [39]. Also, the cloning of the gene encoding this ICDH from *Hfx. volcanii* has been described [40]. The DNA sequence has been determined, and the derived amino acid sequence has been compared with known ICDH sequences. The protein deduced from the nucleotide sequence is composed of 419 amino acids with a molecular mass of 45 837 Da. The molecular weight of halophilic enzymes should be treated with some caution in SDS-PAGE because they electrophorese more slowly because of their highly negative intrinsic charges. For halophilic ICDH, this subunit molecular mass was 62 000 Da. Also, the enzyme shows 12 \(\alpha\)-helical and 12 \(\beta\)-sheet conformations in the polypeptide. Other typical studies of halophilic enzymes have been investigated with ICDH. The salt-dependent stability of the recombinant dimeric ICDH from the halophilic Archaeon *Hfx. volcanii* has been studied in various conditions [41]. The enzyme stability is found to be mainly sensitive to cations and very little (or not) sensitive to anions. Divalent cations induce a strong shift of the active/inactive transition towards low salt concentration.

Dehydrogenases discriminate between nicotinamide coenzymes through interactions established between the protein and the 2’-phosphate of NADP+ and the 2’- and 3’-hydroxyls of NAD+ [42]. In NAD binding site, the introduction of positively charged residues changes the preference of an NAD-dependent enzyme to neutralize the negatively charged 2’-phosphate of NADP+. In the halophilic ICDH, the objective was to switch the coenzyme specificity by altering five conserved amino acids by site-directed mutagenesis (Arg291, Lys343, Tyr344, Val350 and Tyr390). The five mutants of ICDH were overexpressed in *E. coli* as inclusion bodies [43] and each recombinant ICDH protein was refolded and purified, and its kinetic parameters were determined. Coenzyme specificity did not switch until all five amino acids were substituted.

### 2.4 Isocitrate lyase and malate synthase

In general, the Krebs cycle functions similarly in bacteria and eukaryotic systems, but major differences are found among bacteria. One difference is that in obligate aerobes, L-malate may be oxidized directly by molecular \(\text{O}_2\) via an electron transport chain. In other bacteria, only some Krebs cycle intermediate reactions occur because 2-oxoglutarate dehydrogenase is missing.
A modification of the Krebs cycle is the commonly called “glyoxylate cycle”, which has been detected in the three domains of living organisms. It is a metabolic pathway by which organisms can synthesize carbohydrates from C2 compounds. This cycle functions similarly to the Krebs cycle but lacks many of the Krebs cycle enzyme reactions (Figure 2). The glyoxylate cycle involves five enzymes, three of which also participate in the citric acid cycle: citrate synthase, aconitase, and malate dehydrogenase. The two other enzymes, isocitrate lyase (ICL) and malate synthase (MS), are unique to this cycle.

Isocitrate lyase (EC 4.1.3.1) catalyzes the cleavage of D-isocitrate to glyoxylate and succinate; and malate synthase (EC 4.1.3.2) catalyzes the formation of L-malate from glyoxylate and acetyl CoA. The net function of glyoxylate cycle is the formation of a C4 compound from two acetyl CoA (C2) molecules in each cycle. One primary function of the glyoxylate cycle is to replenish the tricarboxylic and dicarboxylic acid intermediates that are normally provided by the Krebs cycle. A pathway whose primary purpose is to replenish such intermediate compounds is called anaplerotic.

In microorganisms growing on acetate, isocitrate can be metabolized either by the citric acid cycle or by the glyoxylate bypass [44]. In Escherichia coli, this branch point is regulated by reversible phosphorylation and inactivation of NADP+-isocitrate dehydrogenase, but the regulation of isocitrate flux in eukaryotic microorganisms remains to be established. The growth of E. coli on acetate requires the operation of the glyoxylate bypass in which ICL must compete with ICDH for isocitrate. However, ICDH has a much higher affinity for isocitrate than does ICL. Inactivation of a large part of the ICDH allows intracellular isocitrate to rise to a concentration at which ICL can operate, and as soon as the glyoxylate bypass is not required, ICDH is rapidly reactivated [45].

Acetate could have a role in the nutrition of natural communities of halophilic Archaea, as it is produced from glycerol (the main carbon source in nature) in hypersaline lakes by some species of halophiles. Hfx. volcanii is a halophilic archaean able to grow in minimal medium with acetate as the sole carbon source. ICL activity was detected in this organism when it was grown on a medium with acetate as the main carbon source [46]. We demonstrated that the activities of glyoxylate cycle key enzymes are necessary in Hfx. volcanii to synthesize precursors for carbohydrates only when the carbon source is a C2 compound, such as acetate [3]. Those activities are not needed when the carbon source is a compound with three or more carbons, such as lactate. No activity could be detected when lactate was the carbon source, but high activities were measured when acetate was.

We have isolated and sequenced the ace (acetate assimilation) gene operon, comprising the glyoxylate cycle key enzymes isocitrate lyase and malate synthase genes (icl or aceA and ms or aceB), from the
halophilic archaeon *Hfx. volcanii*. This is the first time that these genes are sequenced in an organism from the domain Archaea. Phylogenetic analysis of the sequenced genes revealed that isocitrate lyase shows a significant identity with isocitrate lyases from Eukarya and Bacteria, but it is not more closely related to eukaryal or bacterial enzymes, and that malate synthase from *Hfx. volcanii* has very little identity with any other known protein. This enzyme forms a new class of malate synthases. Afterwards, transcriptional analysis has showed that both genes are cotranscribed into a single mRNA molecule. The genes were transcribed only when the acetate was the carbon source, indicating transcriptional regulation [47]. Moreover, there are two sets of palindromic sequences in the promoter region; they are possibly involved in binding of transcriptional regulator(s) (repressor or, activator proteins). This is an interesting point because there are only a few studies on transcriptional regulation in Archaea, which are not enough to form a global idea of how the process take place.

3. Nitrogen metabolism

Nitrogen (N) is a major element of all organisms and it accounts for 6% approximately of their dry mass on average. In the nature, N can be found in different redox states from +5 (as nitrate) to -3 (as ammonia). Redox reactions are performed in different ways by several organisms, and the reactions in total make up the biological N-cycle which is summarised in Figure 3. All of these reactions are performed by bacteria, cyanobacteria, archaea and some specialized fungi. However, there is only one example of a higher life form performing such a reaction; the remarkable exceptions are assimilatory nitrate reduction and nitrogen fixation, which also occurs in plants.

![Figure 3](image_url)

Figure 3. NO$_3^-$ is used as nitrogen source for growth under aerobic conditions thanks to the assimilatory NO$_3^-$ reduction, while it acts as an electron acceptor to eliminate excess of reductant power through dissimilatory NO$_3^-$ reduction. Dissimilatory NO$_3^-$ reduction, NO$_3^-$ respiration or denitrification are used equivalently in the literature because in all cases, N is finally excreted as gas, however, dissimilatory pathway makes reference to non-assimilatory reaction not directly coupled to generation of proton-motive force. In some Enterobacteriaceae member, NO$_2^-$ is reduced to NH$_4^+$ which is then excreted; this process is known as NO$_2^-/NO_3^-$ ammonification. Specialised organisms are able to oxidize either NH$_4^+$ or NO$_2^-$ by using a pathway called nitrification, while other organisms such as some planctomycetes oxidize NH$_4^+$ and utilize NO$_2^-$ as respiratory electron acceptor in a pathway named anammox. Finally, (di)nitrogen fixation allows several bacteria and archaea to reduce N$_2$ to NH$_4^+$ to provide N-requirements.
3.1 Assimilatory nitrate reduction

Nitrate assimilation is a main process of the N-cycle and is carried out by higher plants, fungi, algae, many bacteria, cyanobacteria and some archaea. Thanks to this pathway, NO$_3^-$ is used as N source for growth under oxygenic conditions. More than 2 x 10$^{13}$ kg of N per year are removed from the environment by NO$_3^-$ assimilation. This metabolic pathway has been studied extensively in higher plants, fungi and algae from genetic, physiological and biochemical points of view. However, physiological and biochemical studies have been only performed in a few bacterial and archaea members. In fact, in halophilic archaea this pathway has been only described from *Hfx. mediterranei*, a denitrifier haloarchaea that has been chosen as a model to analysed N-cycle in our laboratory [48].

Assimilation of nitrate involves three pathway-specific steps: uptake, reduction to nitrite and further reduction to ammonium. External nitrite can also be taken up and reduced directly to ammonium (Figure 4).

![Assimilatory nitrate reduction in *Hfx. mediterranei*. All the enzymes presented in the figure has been purified and characterised except glutamate synthase.](image)

With regard to the first step, NO$_3^-$ is incorporated into the cells by high-affinity transport. These transporters have been characterised so far in bacteria such as *Klebsiella*, *Bacillus* and *Azotobacter*. There are different NO$_3^-$ uptake systems: ABC-type transporters and MFS-type permeases. However, there is no any analysis of this kind of transporters in halophilic archaea. The assimilatory nitrate operon described in our research group has revealed that the *nasB* gene probably encodes a NO$_3^-$ transporter of NarK group, which is proton-motive force dependent transporter [49]. When NO$_3^-$ is in the cytoplasm, it is reduced to NO$_2^-$ by assimilatory nitrate reductase (Nas, EC 1.6.6.2), a cytoplasmic molybdoenzyme that catalyse a two-electron reduction. This enzyme is structurally and functionally different from the dissimilatory or respiratory nitrate reductases.

Nas are traditionally classified into two classes: one class depends upon ferredoxin (Fd) or flavodoxin as reductant, while the other depends upon NADH. Fd-Nas have been characterised as monomeric enzymes containing one 4Fe-4S cluster and molybdenum cofactor as redox centres, while NADH enzymes are heterodimers in most of the described cases. Nas from *Hfx. mediterranei* is a monomeric enzyme with remarkable thermohalophilicity character as it is expected for a halophilic enzyme [50]. This enzyme takes electrons from ferredoxin [51], which is a small protein containing a 2Fe-2S cluster. 2Fe-2S cluster ferredoxins and flavodoxins are very low negative redox potential electron donors ($E_m \approx -300$ mV), so electrons are transferred from ferredoxin to the 4Fe-4S cluster of Nas ($E_m \approx -190$ mV) and then from this redox centre to de Mo-cofactor (MoCo) ($E_m \approx -150$ mV); in this cofactor takes place the substrate reduction. The MoCo in bacteria consists of a unique pterin complexed with Mo, forming Mo-MPT, and modified by the addition of a guanosine to form Mo-MGD. We have sequenced the *mobA*
gene from Hfx. mediterranei and its product is implied in Mo-MGD synthesis, for this reason, Hfx. mediterranei Nas could be included in the Nas bacterial group [49].

Nitrite produced by Nas is reduced to ammonium by assimilatory nitrite reductase (NiR, EC 1.7.7.1) which is also a monomeric enzyme that catalyses a six-electron reduction. This enzyme is structurally and functionally different from the dissimilatory or respiratory nitrite reductases. According to their electron donor specificity, two types of NiRs have been described: Fd-dependent NiRs and NAD(P)H-dependent NiRs. The last type of NiR is usually present in fungi and most heterotrophic bacteria. NiR from Hfx. mediterranei is an Fd-dependent enzyme that contains siroheme and a 4Fe-4S cluster as redox centres. In this case, electrons flow from ferredoxin to the 4Fe-4S cluster of NiR and then from this centre to the siroheme, where the NO$_2^-$ takes place. At the moment, this is the only assimilatory nitrite reductase characterised from a halophilic archaea [49, 52] (Figure 4). The resulting ammonium is finally metabolized through central pathways such as those where glutamine synthetase-glutamate synthase (GS-GOGAT) or glutamate dehydrogenase (GDH) play the main role (see below).

NO$_3^-$ assimilation in general is regulated by NO$_3^-$ and/or NO$_2^-$ induction (pathway-specific control) and NH$_4^+$ repression (general N control). Nevertheless, the regulatory proteins and systems involved in both controls are quite different depending on the organism. Related to this point, recently we have demonstrated that in Hfx. mediterranei, ammonium exerts a negative effect on nitrate assimilation as it has been described from most of the organisms [53], but this negative effect was reversed in presence of L-methionine-D,L-sulfoximine (MSX), an irreversible inhibitor of glutamine synthetase activity [54], so assimilation of NH$_4^+$, rather than NH$_4^+$ per se, has a negative effect on assimilatory nitrate and nitrite reduction in Hfx. mediterranei.

3.2 Ammonium assimilation.

3.2.1 Glutamate dehydrogenases.

L-glutamate dehydrogenases (EC 1.4.1.2-4, GDH) are important enzymes in any cell due to the pivotal position occupied by glutamate and 2-oxoglutarate in the central metabolism of nitrogen and carbon compounds, being implicated in the two major pathways (GS-GOGAT and GDH metabolic routes). They catalyse the interconversion between 2-oxoglutarate and L-glutamate reversibly using NAD(P)H or both as cofactors and playing a key role since they provide a link between carbon and nitrogen metabolism [55].

$$2\text{-oxoglutarate} + \text{NAD(P)H} + \text{NH}_4^+ + H^+ \leftrightarrow \text{L-glutamate} + \text{NADP}^+ + H_2O$$

GDHs are evolutionarily conserved in the three domains, Archaea, Bacteria and Eukarya. These enzymes are classified into three groups according to their coenzyme specificity: NAD or NADP-specific and NAD(P)-non specific dependent GDH. Generally, in prokaryotic organisms, NAD-GDH displays a catabolic role; meanwhile NADP-GDH is a biosynthetic enzyme involved in ammonia assimilation. Dual NAD(P)-GDHs are found in animals and higher plants, but NADP-GDHs are mostly involved in ammonia assimilation in bacteria, fungi and algae [56-59]. Due to their ubiquitous presence in all the domains, we can find very interesting GDHs adapted to each medium including extreme ones occupied by thermophiles, halophiles, acidophiles, etc.

Hyperthermophilic GDHs from the archaea Pyrococcus furiosus, Thermococcus litoralis, Thermococcus profundus and Sulfolobus solfataricus are hexameric, as in mesophilic bacteria, and show a high temperature optimum for activity [60-64]. However, the coenzyme specificity of GDH from these hyperthermophiles resembles that of the eukaryotes rather than bacteria, since the enzyme uses both NADH and NADPH [64]. Halophilic GDHs also displayed a high thermostability. This thermostophilic nature of halophilic enzymes has been noted in several systems [22,40,65]. Dym et al. [65] pointed out, taking into account the crystal structure of Har. marismortui malate dehydrogenase, several of the
Structural features concerning halophilicity are the same as those which contribute to the stability of thermophilic enzymes. The amino acid composition for halophilic GDHs showed a higher cumulative amount of acidic amino acids than basic amino acids, similar to other halophilic proteins [22, 40, 65]. *Hfx. Mediterranei* has at least two different GDHs. The NADP-GDH (EC 1.4.1.4) is an hexameric enzyme composed of monomers with a molecular mass of 55 kDa and its activity was markedly dependent on the salt concentration, being optimal at 1.5 M NaCl or KCl at 60 °C and pH 8.5 [58]. It also displayed a high thermostability, requiring temperatures above 60 °C for measurable rates of thermodenaturation. Another GDH, NAD-dependent (EC 1.4.1.2), displays a different behaviour that may be related to the different and essential roles that GDHs should play in the metabolism of organisms [61, 55], including extreme halophilic Archaea; although it still “raising questions” as to the role of the distinct GDH proteins, as recently reported for *Hbt. salinarum*, which had at least four putative GDH genes in its genome [66].

Bone and col. [57, 58, 67, 68] have isolated and characterised glutamate dehydrogenases from *Hbt. salinarum, Hfx. Mediterranei*, and *Salinibacter ruber*. These enzymes do not present dual-coenzyme specificity, they are specific for either NAD or NADP. *Hbt. salinarum* (formerly *Hbt. halobium*) has two different GDHs. A catabolic role is assigned to the NAD-GDH whereas the NADP-GDH may catalyse in vivo the formation of glutamate from ammonium and 2-oxoglutarate [57, 67]. Both of them displayed a very high optimum temperature (70 °C). GDHs from *Hbt. salinarum* are also remarkably resistant to thermal denaturation, reflecting the thermophilic character of these enzymes, a characteristic also found in other dehydrogenases from haloarchaea [22, 40, 58, 70]. An important difference between these two GDHs is the regulation of catalytic activity. NAD-GDH is stimulated in vitro by L-amino acids [22, 67]. Besides, a variety of metabolites act as inhibitors of the oxidative deamination of L-glutamate by NAD-GDH from *Hbt. salinarum*, such as TCA metabolites [71]. Other halophilic organisms also have two or more GDHs, which differ in coenzyme specificity [72].

Protein sequence analysis of thermophilic GDHs indicates that the enzymes from *P. furiosus* and *Pyrococcus horikoshii* are more closely related to the mesophilic bacterial GDH than to the thermophilic archaeon *S. solfataricus* or to the halophilic archaeon *Hbt. salinarum* [64], suggesting that lateral gene transfer between bacterial and archaeal *gdhA* genes may have occurred during evolution.

### 3.2.2 Glutamine synthetase/ Glutamate synthase

The ammonia assimilation pathway that implies glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1), also well-known as route GS/GOGAT, is of crucial importance since the products L-glutamine and L-glutamate play a key role as nitrogen donors in the biosynthetic reactions. The GS/GOGAT pathway is particularly important because it allows ammonia assimilation into L-Glu at low intracellular ammonia concentrations and it seems that efficiently substitutes the other glutamate biosynthetic reaction (that of glutamate dehydrogenase, GDH, EC 1.4.1.2) in these conditions. Nevertheless, in some bacteria it has been found that GDH is active under low ammonium conditions [73]. Glutamine synthetase, the first enzyme of the GS/GOGAT pathway, was first purified and characterised from plants, but in the last 20 years, it has been extensively studied at both biochemical and molecular levels in bacteria [74], cyanobacteria, yeast, mammals and some methanogenic and hyperthermophilic archaea [75]. Three types of GS have been described based on the molecular size and the number of subunits. GS-I is a dodecameric protein with subunit ranging between 44 and 60 kDa, and is usually found among several groups of bacteria and archaea [76, 77]. GS-II is common in eukaryotes and a few soil-dwelling bacteria as an octameric enzyme composed of subunits ranging between 35 and 50 kDa [78]. Finally, GSIII has been characterised from cyanobacteria and two anaerobic bacteria as a hexameric enzyme composed of a 75 kDa subunits [79].

Although GS from Bacteria and Eukarya has been studied so far, only a few analysis have been carried out with GS from members of Archaea, and just two of them correspond to a GS from haloarchaea [80, 81]. GSs from *Hfx. Mediterranei* and *Hbt. salinarum* are octamers belonging to the GS type II. However, a few GSs described from methanogenic or hyperthermophilic archaea are dodecamers of...
about 600 kDa, as it has been expected from bacterial GS. So, GS from haloarchaea exhibits typical properties of GS from eukaryotes and soil bacteria species. This fact supports the hypothesis that some members of Archaea are quite likely to eukaryotic organisms because both of them share similar properties at physiological and metabolic levels [82]. The results obtained from Hfx. mediterranei suggest that GS from this haloarchaea could allow the assimilation of the ammonium produced by assimilatory nitrite reductase [81,83], while GDH would allow the assimilation of ammonium when this nitrogen source is present in the culture media at high concentration.

The typical bacterial glutamate synthase is NADPH dependent and it has two dissimilar enzyme subunits $\alpha$ and $\beta$ that forms the $\alpha\beta$ active protomer that contains one FAD, one FMN, and three different [Fe-S] centres: one [3Fe-4S] cluster and two [4Fe-4S] centres [84]. The plant type glutamate synthase is dependent on reduced ferredoxin as physiological electron donor and it is formed by a single polypeptide chain similar in sequence and cofactor content (one FMN, and one [3Fe-4S] cluster) to de $\alpha$ subunit of the bacterial enzyme. The eukaryotic pyridine nucleotide dependent glutamate synthase has been purified from yeast, lupin seeds and nodules, alfalfa (Medicago sativa) and B. mori. It uses NADH as reductant and it is a single polypeptide chain formed by an N-terminal region similar to the bacterial $\alpha$ and a C-terminal region similar to the bacterial $\beta$ subunit [84].

Whether Archaea contain a bacterial type, a plant type or a forth class stills needs to be verified. Methanococcus jannaschii genome sequencing first revealed the presence of one ORF encoding a 490 residues polypeptide that appears to be formed by an N-terminal domain containing the Cys-signature typical of two bacterial ferredoxins [4Fe–4S] clusters followed by a polypeptide mapping on the synthase domain of NADPH-GltS $\alpha$ subunit [84]. A similar ORF has been found in Archeoglobus fulgidus and appears to be conserved in other Archaea and in Thermatogales as the result of lateral gene transfer. It has also been proposed that a fifth type of glutamate synthase may exist. An ORF encoding a 50 kDa protein of Pyrococcus sp. KOD1 with significant sequence similarity to NADPH-GltS $\beta$ subunit has been found and it was assigned the gene name of gltA [84]. The analysis of halophilic archaea genome sequenced revealed that in Har.marismortui, Natronomonas pharaonis and Haloquadratum walsbyi there are an open reading frame of about 1500 residues similar in sequence to the plant type ferredoxin dependent glutamate synthase and to the bacterial NADPH glutamate synthase $\alpha$ subunit, but there are not evidence of the presence of an open reading frame corresponding to a polypeptide similar to the bacterial NADPH glutamate synthase $\beta$ subunit. In Hfx. mediterranei we have measured activity of glutamate synthase with methylviologen as reducing agent in extracts from cells grown in ammonium starvation (data not published) and the sequencing of the gene is carried on showing high homology with the halophilic archaea glutamate synthase sequenced but, at the moment, the classification of Archaea glutamate synthase and particularly of halophilic archaea glutamate synthase stills needs to be verified by characterizing the enzyme from organisms of this class. These preliminary studies shed light on the possibility of an active GS/GOGAT cycle in Hfx. mediterranei under ammonium starvation.

4. Nitrate respiration

Although haloarchaea generally grow heterotrophically under aerobic conditions in hypersaline environments, they possess facultative anaerobic capabilities [85,86]. These anaerobic capabilities are important since the high salt concentrations and elevated temperatures the organisms encounter, together with high cell densities promoted by aerobic growth and flotation, reduce the availability of molecular oxygen. Although haloarchaeal microorganisms frequently encounter microaerobic or even anoxic conditions, detailed knowledge regarding the extent of haloarchaeal anaerobic growth is still only beginning to emerge.

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