Anaerobic metabolism in *Haloferax* genus: Denitrification as case of study.

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Abstract

A number of species of *Haloferax* genus (halophilic Archaea) are able to grow micro aerobically or even anaerobically using different alternative electron acceptors such as fumarate, nitrate, chlorate, dimethyl sulphoxide, sulphide and/or trimethylamine. This metabolic capability is also shown by other species of the *Halobacteriaceae* and *Haloferacaceae* families (Archaea domain) and it has been mainly tested by physiological studies where cells growth is observed under anaerobic conditions in the presence of the mentioned compounds. This work summarises the main reported features on anaerobic metabolism in the *Haloferax*, one of the better described haloarchaeal genus with significant potential uses in Biotechnology and Bioremediation. Special attention has been paid to denitrification, also called nitrate respiration. This pathway has been studied so far from *Haloferax mediterranei* and *Haloferax denitrificans* mainly from biochemical point of view (purification and characterization of the enzymes catalysing the two first reactions). However, gene expression and gene regulation is far from known at the time of writing this chapter.

ABBREVIATIONS

DDC Diethyldithiocarbamate

DMSO Dimethyl sulphoxide

DTE dithioerythritol

EDTA Ethylenediaminetetraacetic acid

PHA Polyhydroxyalcanoates

PHB Polyhydroxybutyrates

pHMB p-hydroxymercuribenzoate

TMAO Trimethylamine N-oxide

KEY WORDS

Haloarchaea, denitrification, anaerobic metabolism, Nox emissions, bioremediation.

1. INTRODUCTION

In general, microorganisms are metabolically versatile growing in a range of environments. In a basic aerobic microbial model, cells use glucose as a carbon and energy source and oxygen acts as terminal electron acceptor. Thus, glucose becomes oxidized resulting in an electrons flux force and a proton motive force, which are really important to produce ATP and reduced coenzymes such as NADH, NADPH or $FADH_2$. At produced is the vital high-energy molecule that supports growth and synthesis of all the major cellular compounds. Many bacteria and archaea for instance, can grow in environments without oxygen using anaerobic respiration and fermentation. In the anaerobic respiration, a compound is oxidized using something besides oxygen as the terminal electron acceptor and resulting in a proton motive force. Both, aerobic and anaerobic respiration, share the same end goal: the generation of a proton motive force that can be used to synthesise ATP using the ATP synthase.

Archaea, one of the three Life Domains, make up a significant fraction of the microbial biomass on Earth. This Domain is one of the three phylogenetic Domains established for the first time by Woese and co-workers (Woese & Fox, 1977; Woese, Kandler & Wheelis, 1990). This three-Domain model for the deepest branches in evolution is now well grounded by considerable further sequence information and biochemical correlations (Graham, Overbeek, Olsen & Woese, 2000; Woese, 2004).

Archaea were for a long time thought to be restricted to extreme environments, such as those with elevated temperatures, low or really high pH, high salinity or strict anoxia (Valentine, 2007). Thus, the species grouped in this Domain were initially viewed as extremophiles inhabiting hostile environments, such as hot springs and salted lakes. In all of these extreme situations, archaea are found together with bacterial and eukaryal organisms also showing extreme phenotypes adapted to these restrictive conditions.

However, environmental sampling analysis based on rRNA sequences has revealed that archaea are ubiquitous in “normal” ecosystems, including soils, oceans, marshlands, human colon, human oral cavity, and even in human skin. Archaea are particularly numerous in the oceans; thus, archaea in plankton may be one of the most abundant groups of organisms on the planet. From a metabolic point of view, Archaea have evolved a variety of energy metabolisms using organic and/or inorganic electron donors and acceptors. Because of that reason, microorganisms of the Archaea Domain play important roles in the Earth’s global geochemical cycles and greenhouse gas emissions (Offre, Spang & Schleper, 2013). In general, organisms of the Archaea Domain are difficult to culture, which impairs experimental manipulation for many of them. Genetic systems exist for all of them (Leigh, Albers, Atomi & Allers, 2011), but physiological, biochemical and genetic tools have not been developed to an extent similar to *Escherichia coli* (Kletzin, 2007).

Salty environments are dominated by halotolerant and halophilic organisms. Halotolerant organisms do not require salt (mainly NaCl) but their growth is not impaired under saline conditions either; on the contrary, halophiles must have NaCl for growth. In fact, the name “halophile" comes from the Greek word for "salt-loving”. Halophiles can be classified in the three groups according to their NaCl requirements: slight halophiles (2-5% or 0.34-0.85M), moderate halophiles (5-20% or 0.85-3.4M) an extreme halophiles (20-30% or 3.4-5.1M) (Larsen, 1962).

Archaebacterial halophiles, also called Haloarchaea, are extreme or moderated halophilic species inhabiting neutral saline environments such as salt lakes, marine salterns, marshes, saltern crystallizer ponds and the Dead Sea (Grant, Kamekura, McGenity & Ventosa, 2001; Oren, 2002), for instance. In those environments, salt concentrations are around 1.5-4 M, which corresponds to 9-30 % of salts (p/v). NaCl is the predominant salt in these ecosystems and ionic proportions are quite similar to those dissolved salts in seawater. These salted waters/habitats are termed “thalassohalines”, which arise from seawater evaporation

and therefore are dominated by NaCl, such as the crystallizer ponds of coastal solar salterns. In contrast, “athalassohalines” waters/habitats are not of marine origin but from evaporation of fresh water in a system usually dominated by calcium, magnesium and sulphate (Remane & Schleper, 1971).

Hypersaline environments can show neutral (Dead Sea, for instance) or alkaline pH (Big Soda Lake in Nevada). Those ecosystems harbour a large diversity of microorganisms of all three domains: primary producers as the green algae *Dunaliella* (Oren, 2005), aerobic heterotrophic bacteria (mainly belonging to the family *Halomonadaceae*), anaerobic fermentative bacteria (families *Halanaerobiaceae* and *Halobacteroidaceae*), and archaeal microorganisms of the families *Halobacteriaceae* and *Haloferacaceae*. In fact, while cell counts of bacterial and eukaryal species decrease with increasing salt concentrations, haloarchaea become the dominant populations (Kletzin, 2007)

Haloarchaea have also been isolated from fossil halite deposits (Stan-Lotter, 2004), and it has also been reported that those microorganisms can be trapped in salt crystals remaining viable for a long time (Grant, 2004). Haloarchaea are also of interest for astrobiological studies and the search for life on Mars, due to their apparent longevity in dry salty environments (Fendrihan et al., 2006) and their ability to cope with extreme temperatures, pH and radiation. Regarding this extreme capabilities, *Hfx. mediterranei* for instance, was successfully subjected to simulate microgravity (Dornmayr-Pfaffenhuemer, Legat, Schwimbersky, Fendrihan & Stan-Lotter, 2011). On the other hand, during the last 20 years special attention has being paid on potential uses of haloarchaea in Biotechnology and Biomedicine due to the capacity of some species producing secondary metabolites of high interest (carotenoids, enzymes showing catalytic properties useful for some industrial processes, etc.).

This chapter offers a summary about anaerobic metabolism in *Haloferax*, one of the better known haloarchaeal genus. Special attention is paid in nitrate respiration (also called Denitrification) as model of anaerobic respiration in haloarchaea.

2. GENERAL CHARACTERISTICS OF THE *Haloferax* GENUS.

Haloferax is the used name to identify a genus of the *Haloferacaceae* family (Gupta, Naushad & Baker, 2015), one of the two families grouping haloarchaea. This genus was first described by Torreblanca et al. (1986) and currently comprises several well described species and few strains partially characterised. Probably the better known species are those with the following validly published names: *Haloferax volcanii* (Mullakhanbhai & Larsen, 1975), *Haloferax denitrificans* (Tomlinson, Jahnke & Hochstein, 1986), *Haloferax gibbonsii* (Juez, Rodriguez-Valera, Ventosa & Kushner, 1986), *Haloferax mediterranei* (Rodriguez-Valera, Juez & Kushner, 1983), *Haloferax alexandrinus* (Asker & Ohta, 2002), and *Haloferax sulfurifontis* (Elshahed et al., 2004). Other species included in this genus are: *Haloferax lucentense* (formerly *Haloferax alicantei*) (Gutierrez, Kamekura, Holmes, Dyall-Smith & Ventosa, 2002), *Haloferax prahovense* (Enache, Itoh, Kamekura, Teodosiu & Dumitru, 2007), *Haloferax larsenii* (Xu et al., 2007), *Haloferax elongans* (Allen et al., 2008), *Haloferax mucosum* (Allen et al., 2008), *Haloferax chudinovii* (Saralov, Baslerov & Kuznetsov, 2013).

Members of the genus *Haloferax* are characterised by extreme pleomorphism and a relatively low salt requirement compared with other haloarchaea. Thus, species such as *Hfx. mediterranei* are able to grow even at low salt concentration (D'Souza, Altekar & D'Souza, 1997).

Looking cell structure in detail, it is interesting to highlight that most of the outer surfaces of the *Haloferax* species are covered with a hexagonally packed surface called S- layer (Surface-layer), which is mainly constituted by glycoproteins forming a regularly structured array. These glycoprotein subunits join via both N- and O-glycosidic bonds, and are held together by divalent cations (probably magnesium) (Sumper, Berg, Mengele & Strobel, 1990; Mengele & Sumper, 1992). In fact, cell shape and cell wall structure in haloarchaea in general,

and in *Haloferax* in particular, are unusual due to the S-layer. This layer is plenty of pores and can be removed by treating cells with chelating agents, such as EDTA. The S-layer is a common feature of many genera of Archaea.

The main characteristics of species belonging to this genus are: i) cell shape includes irregular rods, cups or disks (1.0-3.0 x 2.0-3.0 μm); ii) stain Gram (-); iii) in general, they show aerobic metabolism but few species are denitrifiers (they use nitrate as terminal electron acceptor under anaerobic conditions); iv) they are chemoheterotrophic microorganisms able to use carbohydrates, alcohols, carboxylic acids, amino acids and nitrogen compounds such as nitrate, nitrite and ammonium as carbon and nitrogen sources; v) acidic compounds are produced from sugars; vi) polyhydroxyalcanoates (PHA) and polyhydroxybutyrates (PHB) are accumulated under certain growth conditions by some *Haloferax* species (Antón, Meseguer & Rodriguez-Valera, 1988; Lillo & Rodríguez-Valera, 1990); vii) polar lipids are characterised by C_{20},C_{20} derivatives of S-DGD-1; viii) some species are bacteriorhodopsin producers; ix) carotenoids such as β-carotene, canthaxanthin, astaxanthin and bacterioruberin are produced at high concentrations by some species under certain conditions (Asker & Ohta, 2002; Rodrigo-Baños, Garbayo, Vilchez, Bonete & Martinez-Espinosa, 2015); x) at least one of the species (*Hfx. mediterranei*) produces gas vasicles (Englert, Horne & Pfeifer, 1990).

Salty environments are highly hostile in terms of life because oxygen is limited and nutrients are scarce. However, haloarchaea have adopted several strategies to sustain metabolism and life under such restricted conditions. Some of the main adaptations are following summarised:

- Cells accumulate molar KCl concentrations to maintain osmotic balance instead of accumulating compatible solutes. Thus, cells are isotonic with their surroundings (salt-in strategy). Haloarchaea contain potent transport systems to expel sodium ions, which are predominant in the medium, from the interior of the cell (Madigan & Oren, 1999). This haloadaptation implies that the whole cellular machinery of the haloarchaea is used to K^+

concentrations around 3-5 M, which requires far-reaching alterations of proteins to enable intracellular enzymatic systems to be active.

- Proteins are rich in acidic amino acids as a consequence of the adaptation mentioned before (Madern, Ebel & Zaccai, 2000). This adaptation allows the proteins to maintain their proper conformation and activity at near-saturating salt concentrations (Oren, 2008). In fact, aspartic and glutamic acid could constitute up to 10% of the overall amino acidic composition. Haloarchaeal proteins have, therefore, become strictly dependent on salt presence (Madern et al., 2000) and most of them denature in solutions containing less that 1-2 M salt (Eisenberg, 1995).

- Modulation of the N-linked glycans decorating the S-layer glycoprotein exits as an adaptive response to salinity changes (Guan, Naparstek, Calo & Eichler, 2012).

- Synthesis of archaeocins (Besse, Peduzzi, Rebuffat & Carré-Mlouka, 2015) to be more competitive in the environment. Halocin, which is a type or archaeocin, is produced by *Hfx. mediterranei* as a molecule to inhibit the growth of other halophilic archaea (Cheung, Danna, O'Connor, Price & Shand, 1997; Naor, Yair & Gophna, 2013). This strategy reports competitive advantages when *Hfx. mediterranei* is colonizing one specific environment.

- Some species such as *Hfx. mediterranei* shows DNA restriction pattern modifications under different salt concentrations (Juez, Rodriguez-Valera, Herrero & Mojica, 1990).

Genetically, the members of *Haloferax* genus usually have one main chromosome and a variable number of plasmids (Soppa et al., 2008). All of them are characterised by high G+C content (around 65%) (Soppa et al., 2008). This feature increases the stability of the genome within a cytoplasm with high ionic strength. Another possible advantage would be that a G+C rich genome decreases the possible targets of insertion sequences (IS) elements, which recognise A + T rich regions (Leigh et al., 2011). IS sequences were studied in *Hfx. volcanii*, where they were located in nonessential regions of the megaplasmids (Lopez-Garcia, Jean, Amils & Charlebois, 1995).

The model for genetic research in *Haloferax* has always been *Hfx. volcanii*: it was the first specie discovered in 1936 by Benjamin Elazari-Volcanii and the first genome fully sequenced. It has managed to develop robust transformation protocols and selection markers for the construction of knock-out cells (Allers, Ngo, Mevarech & Lloyd, 2004). Currently, progress have been made in understanding the genomes of other species such as *Hfx. mediterranei* ATCC33500 (Han et al., 2012), *Hfx. gibbonsii* strain ARA 6 (Pinto, D'Alincourt Carvalho-Assef, Vieira, Clementino & Albano, 2015) or *Hfx. denitrificans* ATCC 35960, *Hfx. mucosum* ATCCBAA-1512, *Hfx. sulfurifontis* ATCC BAA-897 (Lynch et al., 2012).

Lateral gene transfer (LGT) is a process closely associated with the dynamics of the genomes in *Haloferax* species. Although there are not so many studies of this mechanism as in the Bacteria domain, there are evidences demonstrating that it is present in such microorganisms. For example, the UvrABC complex, whose origin is bacterial, is in haloarchaeas such as *Hfx. volcanii,* where it is fully active (Lestini, Duan & Allers, 2010). The LGT process could also explain why there are multiple isoforms of genes in a lot of *Haloferax* species, while in other microorganisms exist only in a single form. In fact, it has been proposed that haloarchaea descended from methanogens that acquire the genes for aerobic respiration from Bacteria (Leigh et al., 2011).

At the time of writing this work, around 650 papers have been published (http://www.ncbi.nlm.nih.gov/pubmed/?term=haloferax) about items related to *Haloferax*'s physiology, molecular metabolism or molecular biology, which is quite scarce information if we compared with the knowledge reported from other microbial groups. In fact, only 13 of the mentioned papers are focused on anaerobiosis in *Haloferax* (http://www.ncbi.nlm.nih.gov/pubmed/?term=haloferax+%26+anaerobic). However, from the details reported for now, it's possible to conclude that haloarchaea exhibit some characteristics close to eukarya and many others close to prokarya, resulting in interesting phenotypes able to be adapted to very restrictive environmental conditions.

3. ANAEROBIC METABOLISM IN THE *Haloferax* GENUS.

Anaerobic metabolism in general, and in particular anaerobic respiration, plays a major role in the global nitrogen, sulphur, and carbon cycles through the reduction of the oxyanions of nitrogen, sulphur, and carbon to more reduced compounds. Climate change, anthropogenic activities as well as seasonal features cause oxygen availability changes and consequently, sequential changes in redox conditions. Environmental redox cycling often has strong effects on natural biogeochemical cycling as well as biodegradation of anthropogenic organic pollutants. In that context, microorganisms, mainly those showing anaerobic metabolism, play an important role.

As mentioned before, haloarchaea are oxygen-respiring heterotrophs that derive from methanogens-strictly anaerobic, hydrogen-dependent autotrophs (Leigh et al., 2011; Nelson-Sathi et al., 2012). Haloarchaeal genomes are known to have acquired, via lateral gene transfer (LGT), several genes from eubacteria.

Extreme or moderate marine origin environments are inhabited by halophiles, and haloarchaea constitute the major populations as mentioned before. The vast majority of these populations include members of the following haloarchaeal genera: *Haloarcula*, *Haloquadratum*, *Halobacterium*, *Haloferax* or even *Natronomonas* when the pH is alkaline. Most of these species generally grow chemo-organotrophically thanks to a respiratory chain that enables them to use oxygen as electron acceptor (Oren, 1991). Nevertheless, some species have facultative anaerobic capabilities (DasSarma & Arora 2002). Due to the low solubility of gasses and other nutrients in salt-saturated brines, oxygen may easily become a limiting factor for reproducing (flourishing) haloarchaea in these environments. In fact, in saline and hypersaline environments inhabited by species of *Haloferax* genus, oxygen is a limiting factor for cell growth. The often high temperatures of their natural habitats, the high salt concentrations as well as the presence of communities of other halophilic archaea and

bacteria that consume oxygen are also responsible for the low availability of it in these environments (Müller & Dassarma, 2005).

Related to this aspect, it is interesting to highlight that a few representative species of the *Haloferax* genus are able to float to the air-water interface thanks to the production of gas vesicles (Oren, 2002; Oren, 2012). Gas vesicles increase the buoyancy of cells and allow them to migrate vertically in the water body from low oxygen conditions to regions with optimal conditions to sustain microaerobic or aerobic growth (Pfeifer, 2015). Haloarchaeal gas vesicles consist of mainly one protein called GvpA, but their formation occurs along a complex pathway involving 14 different *gvp* genes, some of which regulate the process (Zimmermann & Pfeifer, 2004). Gas vesicles synthesis depends on environmental factors, such as light, temperature, salt concentration and oxygen supply. Thus, the production of these vesicles is inhibited under anaerobic conditions at least in *Hfx. mediterranei* and *Hfx. volcanii* (Hechler & Pfeifer, 2009), and glucose also inhibits the formation of gas vesicles in *Hfx. volcanii* transformants (Hechler, Frech & Pfeifer, 2008).

Apart from gas vesicle strategy, as a mechanisms to optimise cell location in an oxic or microaerobic environment, there are many other strategies supporting haloarchaeal growth under anaerobic conditions: denitrification (Mancinelli & Hochstein, 1986; Martínez-Espinosa et al., 2007; Bonete, Martinez-Espinosa, Pire, Zafrilla & Richardson, 2008), arginine fermentation (Ruepp & Soppa, 1996), and use of the bacteriorhodopsin (a light-driven proton pump) (Dassarma et al., 2001; Papke, Douady, Doolittle & Rodriguez-Valera, 2003; Sharma et al., 2007). However, the last strategy has not been developed by *Hfx. mediterranei* for instance, which lacks energy-generating retinal-based, light-driven ion pumps such as bacteriorhodopsin and halorhodopsin (Oren & Hallsworth, 2014).

In general terms, *Haloferax* growth under those microaerobic or even strict anaerobic conditions is possible because oxygen is replaced by other final electron acceptors such us nitrate, nitrite (Lledó, Martinez-Espinosa, Marhuenda-Egea & Bonete, 2004; Bonete et al.,

2008; Nájera-Fernández, Zafrilla, Bonete & Martinez-Espinosa, 2012; Esclapez, Zafrilla, Martinez-Espinosa & Bonete, 2013) (per)chlorate (Oren, Elevi-Bardavid & Mana, 2014; Martínez-Espinosa, Richardson & Bonete, 2015), sulphur or sulphide (Elshahed et al., 2004), arsenate (Rascovan, Maldonado, Vazquez & Eugenia Farias, 2015), dimethyl sulfoxide (DMSO), trimethylamine N-oxide (TMAO) and fumarate (Oren & Trüper, 1990; Oren, 1991; Oren, 1999; Müller & DasSarma, 2005).

Biochemical characterisation of these pathways as well as the enzymes involved in is still scarce, with the exception of denitrification. What is clear is that Rieske like proteins and cytochromes play an important role to sustain bioenergetics not only under aerobic but also under anaerobic conditions in haloarchaea (Baymann, Schoepp-Cothenet, Lebrun, van Lis & Nitscke, 2012). More details about these processes are summarised below:

3.1. Denitrification.

Denitrification is probably the most studied anaerobic metabolic pathway in *Haloferax* genus (mainly in *Hfx. mediterranei* and *Hfx. denitrificans*). It is based on the use of nitrate (NO_3^-) as final electron acceptor, which is further reduced to gaseous products: nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2). Generally, organisms able to perform denitrification are classified as complete or incomplete denitrifiers: in the first case, NO_3^- (nitrate) is completely reduced to N_2 (dinitrogen); in the second case, NO_3^- is reduced partially to NO (nitric oxide) or N_2O (nitrous oxide) (NO_x gases). The release of these gases (NOx) to the atmosphere is harmful since they are responsible of the destruction of the ozone layer and contribute to the greenhouse effect (Ravishankara, Daniel & Portmann, 2009; Thomson, Giannopoulos, Pretty, Baggs, & Richardson, 2012).

Some enzymes involved in denitrification have been purified and characterised from *Haloferax* species (see section 4). However, there are no detailed physiological studies on denitrification and NOx production in species of *Haloferax* genus, thus revealing the depth

process. With the information available today, it appears that some organisms such as *Hfx. mediterranei* are complete denitrifiers (Bonete et al., 2008), other such as *Hfx. volcanii* are incomplete and in some cases (*Hfx. denitrificans)* are complete or incomplete depending on the initial amount of NO_3^- (Tindall, Tomlinson, & Hochstein, 1989). Nevertheless, it is clear that denitrification is a form of anaerobic respiration really significant in haloarchaea, and probably the best characterised anaerobic pathway for now from this kind of microorganisms.

Denitrification occurs in many environments including soils, oceans, and freshwaters and it is usually carried out by facultative anaerobes growing under microaerophilic or anoxic conditions (Zumft, 1997). Denitrifying organisms include various bacteria, some archaea, and even eukaryotes (Cabello, Roldán & Moreno-Vivián, 2004). Only a few cultured archaea are capable of denitrification; *Haloferax* genus plays an important role in that sense grouping several species able to perform partial or even complete denitrification as mentioned before (*Hfx. mediterranei*, *Hfx. volcanii* and *Hfx. denitrificans*, for instance).

The predominance of anaerobic metabolism in Archaea and the biogeochemical significance of archaeal denitrification have been little investigated (for review Offre et al., 2013). There are few studies focused on the metabolism of halophilic archaea, however most of them analyse this subject through systematic metabolic reconstruction and comparative analysis of available genomes (Falb et al., 2008). At the time of writing this chapter, when systems biology approaches have been used to construct predictive models of gene expression and metabolism in Bacteria and Eukarya, only few studies summarise details about the status of genomics, functional genomics, and molecular genetics of haloarchaea (Soppa et al, 2008). This situation could be due to the fact that not too many haloarchaeal genomes are completely sequenced and assembled.

It also remains unclear the role of denitrification in haloarchaea in terms of its ecological relevance. In saline and hypersaline habitats, nitrate is rarely found at high concentrations mainly because of the lack of autotrophic nitrification. So, why these

microorganisms have been maintained over time those denitrification genes? Are they an adaptive advantage? These are open questions that should be addressed in the next future.

3.2. Perchlorate and chlorate reduction.

Microbial reduction of chlorine oxyanions can be found in diverse habitats and different environmental conditions (temperature, salinities, pH) (Nilsson, Rova & Smedja Bäcklund, 2013). This metabolic process commonly involves the enzymes perchlorate reductase (Pcr), chlorate reductase (Clr) and chlorite dismutase (Cld). The final products are oxygen and chloride (Cl^-). Horizontal gene transfer seems to play an important role for the acquisition of functional genes. Novel and efficient Clds were isolated from microorganisms incapable of growing on chlorine oxyanions (Liebensteiner, Oosterkamp & Stams, 2015).

One of the latest anaerobic pathways studied in haloarchaea is the anaerobic respiration of perchlorate and chlorate. It has been found that *Hfx. mediterranei* can grow in anaerobic environment using (per) chlorate as final electron acceptors (Martínez-Espinosa et al., 2015). *Hfx. mediterranei* genome analysis revealed that there are not genes coding for the enzymes involved in the (per) chlorate reduction. So, compounds such as chlorate or perchlorate may be reduced through the respiratory nitrate reductase enzyme (Martínez-Espinosa et al., 2015). This is the reason why cells can grow with (per) chlorate as terminal electron acceptor, but only if they have previously been exposed to nitrate. These results revealed that it is necessary a pre-induction of respiratory nitrate reductase to support the anaerobic growth with (per) chlorate. What it has been suggested for now is that haloarchaea is able to reduce (per)chlorate using the respiratory nitrate reductase located at the positive side of the membrane (pNar) for perchlorate reduction and lack a functional Cld. Chlorite is possibly eliminated by alternative (abiotic) reactions (Martínez-Espinosa et al., 2015).

3.3. Dimethyl sulphoxide, trimethylamine *N*-oxide and fumarate as final electron acceptors.

Some members of the *Haloferax* genus can reduce dimethyl sulphoxide (DMSO), trimethylamine *N*-oxide (TMAO) (Oren & Trüper, 1990) and fumarate using them as final electron acceptors (Oren, 1991). These results come from physiological experiments where cells are grown in the presence of TMAO or DMSO, but there is no information about the regulation of those metabolic pathways or the enzymes catalysing those reactions. On the one hand, the reduction of the first two compounds is usually coupled, producing dimethylsufide and trimethylamine as final products. *Hfx. mediterranei* can reduce both while *Haloferax volcanii* only can use DMSO. Other members of the *Haloferax* group like *Hfx. gibbonsii* do not grow in anaerobic conditions with addition of DMSO or TMAO (Oren & Trüper, 1990). Although the bases of the DMSO and TMAO respiratory systems have not been described for any member or the domain Archaea, some studies revealed that the genetic machineries involved in two pathways are closely related in haloarchaea (Müller & DasSarma, 2005). On the other hand, the use of fumarate as terminal electron acceptor producing succinate has been described for *Hfx. volcanii* and *Hfx. denitrificans*, but not for *Hfx. mediterranei* and *Hfx. gibbonsii* (Oren, 1991). The ability to reduce fumarate is not correlated with the ability to reduce nitrate, (per) chlorate, DMSO or TMAO.

The presence of the named compounds is minority in environments with high salt concentrations. Their ecological role is still uncertain in the context of the habitat where *Haloferax* species live. In this field, not only physiological but also genetic and biochemical studies are needed.

4. ENZYMES INVOLVED IN ANAEROBIC METABOLISM IN *Haloferax* GENUS: DENITRIFICATION AS STUDY OF CASE.

Denitrification pathway, carried out under anoxic conditions, is a key process involved in the nitrogen cycle of the Earth. In the complete pathway, the nitrate is reduced to N_2 by the

action of four metalloenzymes: respiratory nitrate reductase, respiratory nitrite reductase, nitric oxide reductase and nitrous oxide reductase. Physiological, biochemical and genetic data have provided a detailed process for this pathway in the *Bacteria* Domain (Zumft, 1997). However, the biochemical and genomic data related to denitrification process in extremophiles, and specifically in *Haloferax* genus, are still scarce. Although during the last years the number of available genomes of *Haloferax* genus has increased allowing the identification of denitrification genes, the biochemical studies related to this pathway are basically restricted to the purification and characterisation of respiratory nitrate and nitrite reductases from *Hfx. mediterranei, Hfx. denitrificans* and *Hfx. volcanii*.

In view of this, the present section describes the biochemical characteristic of the denitrifying enzymes from *Haloferax* microorganisms.

4.1. Respiratory nitrate reductases in *Haloferax* genus

Denitrifying microorganisms contain nitrate reductase as the terminal enzyme of the nitrate respiration (Zumft, 1997). According to the structural and catalytic characteristics, dissimilatory nitrate reductases can be classified into two groups: periplasmic nitrate reductase (Nap) and membrane-bound nitrate reductase (Nar). The Nap enzymes are mainly found in Gram negative bacteria and they are involved in different processes depending on the organism in which are found (Gavira, Roldan, Castillo & Moreno-Vivian, 2002; Ellington, 2003). Generally, Nap enzymes are heterodimers composed of a catalytic subunit (NapA) and a cytochrome *c* (NapB) which receives electrons from NapC, a membrane cytochrome *c* (Richardson, Berks, Russell, Spiro, & Taylor, 2001). On the other hand, Nar enzymes are distributed more widely in the nitrate-respiring microorganisms and they are the responsible for the generation of metabolic energy using nitrate as a terminal electron acceptor. Not surprisingly, they are negatively regulated by oxygen, induced by the presence of nitrate and unaffected by ammonium. In general, Nar complex is a heterotrimer composed of: a catalytic

subunit (NarG) that binds a bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor for nitrate reduction, an electron-transfer subunit with four iron-sulphur centres (NarH) as well as a di- *b*-heme integral membrane quinol dehydrogenase subunit (NarI). The NarG and NarH are membrane-extrinsic domain whereas the NarI is a hydrophobic membrane protein which connects the NarGH complex to the membrane (Richardson et al., 2001; Cabello et al., 2004; Martínez-Espinosa, Richardson, Butt & Bonete, 2006).

At the time of writing, all the purified and characterised nitrate reductases from haloarchaea, belonging to *Haloferax* genus, are membrane-bound Nar enzymes (Table 1). In general, the characteristics of these enzymes showed marked resemblance with the bacterial NarGH complex, underscoring the fact that there was a relevant difference related to the subcellular localization between the halophilic and bacterial enzymes (Yoshimatsu, Iwasaki, & Fujiwara 2002; Martinez-Espinosa et al., 2007).

In *Haloferax* genus, purification of respiratory Nar enzymes has been reported from three halophilic microorganisms (Table 1), being the best studied Nar enzyme that one which belongs to *Hfx. mediterranei* specie.

The first respiratory nitrate reductase purified and characterised from the *Haloferax* genus was the *Hfx. denitrificans* membrane-bound Nar (Table 1). This enzyme is a heterodimer with a K_m for nitrate of 0.2 mM. The enzyme is able to reduce not only nitrate but also chlorate, the electron donor is methyl viologen (MV) and is inhibited by azide and cyanide. Azide and cyanide are inhibitors with respect to nitrate. The first one acts directly in the molybdenum containing site or the Nar, probably by metal chelation. The second one blocks electron transfer in oxygen respiration and acts as a noncompetitive inhibitor of nitrate reduction. Curiously, unlike other halophilic enzymes, this nitrate reductase is stable in the absence of salt and its activity decreases with increasing salt concentration. Besides, it was suggested that the enzyme contains molybdenum because tungstate represses nitrate reductase synthesis (Hochstein & Lang, 1991).

Bickel-Sandkotter & Ufer described the properties of respiratory Nar from *Hfx. volcanii* in 1995 (Table 1), whose activity was induced by the addition of nitrate as nitrogen source to the culture media and anaerobic conditions. This enzyme was also located on the surface of the cytoplasma membrane. It was described as a trimeric protein, whose putative subunits showed a molecular masses of approximatly 100, 61 and 31 kDa. The kinetic constant of respiratory Nar from *Hfx. volcanii* was determined using methyl viologen and dithionite in saturating conditions and high NaCl concentration (1.75 M). The K_m calculated for nitrate was 0.36 mM, being similar to the date calculated for other dissimilatory Nar. Like the *Hfx. denitrificans* Nar, this enzyme showed optimal activity in the absence of NaCl. Moreover, *Hfx. volcanii* Nar reached its optimum activity in a buffer with medium pH of 7.5 and high temperatures up to 80 ºC, and it was inhibited in the presence of cyanide, azide and a relatively high concentrations of thiocyanate (Bickel-Sandkotter & Ufer, 1995).

In *Hfx. mediterranei* two different non-assimilatory nitrate reductases have been purified and characterised: a dissimilatory nitrate reductase described by Alvarez-Ossorio, Muriana, de la Rosa & Relimpio 1992, and Nar characterised by Lledó et al. 2004. The expression of the first one was induced not only by means of the presence of nitrate as nitrogen source but also by the switch to anaerobic conditions. This enzyme was purified in five steps (ammonium sulphate precipitation followed by Sepharose DL-4B, calcium phosphate, DEAE-Sephacel and Sephacryl S-200 chromatographies), being its estimated molecular weight 170,000 Da. Unlike previously described dissimilatory nitrate reductases, the activity of this enzyme was salt dependent, showing its optimal activity at 89 ºC in 3.2 M NaCl. Its kinetic parameters depend also on salt concentration in the assay. In fact, the K_m for nitrate changed from 2.5 to 6.7 mM when the salt concentration increased from 0.8 to 3.4 M. This halophilic enzyme was strongly inhibited in the presence of p-hydroxymercuribenzoate (pHMB), dithioerythritol (DTE), azide and cyanide while the cyanate, potassium chlorate or EDTA produced a partial inhibition. The electron donor studies revealed that methyl and

benzyl viologen are the best for this enzyme while FMA o FAD were ineffective electron donors (Alvarez-Ossorio et al., 1992). According to its molecular mass and enzymatic properties, Lledó et al. (2004) proposed that the enzyme purified by Alvarez-Ossorio allows the dissipation of reducing power for redox balancing. The *Hfx. mediterranei* Nar was purified by means of three chromatographic steps: two DEAE-Sepharose CL-6B and Sephacryl S-300 chromatographies. The enzyme was described as a heterodimer with a K_m for nitrate of 0.82 mM, which is in the range of the values determined from other nitrate reductases (Zumft, 1997). Like other nitrate reductases, cyanide and azide were strong inhibitors of this enzyme. Other compounds as dithiothreitol (DTT) and EDTA were also tested, but they were not effective inhibitors since only decreased partially the activity. The *Hfx. mediterranei* Nar did not exhibit a strong dependence on temperature at the different NaCl concentrations assayed (0-3.8 M NaCl), showing the maximum activity at 70 ºC for all NaCl concentrations. Hence, this halophilic enzyme also presented a remarkable thermophilicity although the Nar activity did not show a dependence on salt concentration, as was described for *Hfx. denitrificans* Nar (Hochstein & Lang, 1991) and *Hfx. volcanii* Nar (Bickel-Sandkotter & Ufer, 1995). Not all nitrate reductases activities found in halophilic archaea exhibit similar dependence (Alvarez-Ossorio et al., 1992; Yoshimatsu, Sakurai & Fujiwara 2000). Even though most proteins for haloarchaea are stable and active at high ionic strength, there are some that are either active or stable in the absence of salt. The origin of haloarchaeal enzymes which does not require salt is unclear, but it has been proposed that Nar could be acquired by the extreme halophiles from a eubacterial source (Hochstein & Lang, 1991). The absorption spectrum of the *Hfx. mediterranei* Nar showed a broad band around 400 to 415 nm indicating that this enzyme has Fe-S clusters as other Nar purified from denitrifying microorganisms (Lledó et al., 2004).

Classically, it has been considered that the subunits NarG and NarH are located in the cytoplasm and associate with NarI at the membrane potencial-negative cytoplasmic face of the cytoplasmic membrane. This data indicates that the nitrate reduction must produce it on the

inside of this membrane. This arrangement is conserved in Gram-negative bacteria and indeed, for many years, it was assumed that this orientation would be conserved among prokaryotes in general. However, the presence of a typical twin-arginine signal in *Hfx. mediterranei* NarG and another halophilic microorganisms suggests that nitrate reductases from *Archaea* could be translocated across the membrane by Tat export pathway. Later, the analysis of N-terminal region of the archaeal nitrate reductases revealed the conservation of a twin-arginine motif (Martinez-Espinosa et al., 2007). This data underlines the fact that NarG protein could be strongly attached to the membrane fraction and requires detergent solubilisation to release it (Lledó et al. 2004). In order to study the location of the NarG, amino acid sequence analysis and bioinformatic studies were carried out with *Hfx. mediterranei* (Martinez-Espinosa et al., 2007). The results obtained revealed that the electron donation to the active site of an enzyme is on the outside, rather than inside, of the cytoplasmic membrane. These experiments have not yet been reported for the other archaeal Nars with Tat sequences thus far identified. Nonetheless, the available data support the fact that the active site of these archaeal Nar systems is indeed on the outside of the cytoplasmic membrane (Martinez-Espinosa et al., 2007).

Hence, according to the subunit composition and subcellular location in *Hfx. mediterranei*, it can suggest that archaeal Nars are a new type of enzymes with the active site facing the outside and connected to the membrane by cytrochrome *b*. This location of the catalytic site of archaeal NarG has important implications because to be energy-conserving require the coupling of this process to a proton-motive complex, instead of the typical redox-loop mechanism, the NarI subunit described in bacteria. On the other hand, it appears that an active nitrate-uptake system would not be required for respiratory nitrate reduction in archaea, consequently the energetic yield of the nitrate reduction process increases (Martinez-Espinosa et al., 2007; Bonete et al., 2008).

The last advances related to the knowledge of respiratory Nar has been carried out in *Hfx. mediterranei* (Martínez-Espinosa et al., 2015), where it has been tested the capacity of the whole cells and pure NarGH to reduce different substrates as chlorate, perchlorate, bromate, iodate and selenate. The results demonstrated that not only the whole *Hfx. mediterranei* cells but also pure NarGH were able to reduce chlorate, bromate and perchlorate, but no reduction activity was observed with iodate or selenate. Therefore, it is clear that the same microorganism is able to reduce nitrate and chlorate thanks to the nitrate reductase under microaerobic or anaerobic conditions. Undoubtedly, due to most of the waste water samples containing nitrate also include chlorate and other oxyanions, these results are crucial for waste water bioremediation aims. Although the removal procedure is not really fast (4.8 mM chlorate after 150 h incubation) the removed concentration using microorganisms is one of the highest described up to now (Bardiya & Bae, 2005; van Ginkel, van Haperen, & van der Togt, 2005). In addition, one of the advantages of using *Hfx. mediterranei* cells as well as its NarGH is that nitrate reduction is not inhibited by the presence of chlorate or perchlorate at high ionic strength. These results are of great interest for bioremediation processes based on the use of haloarchaea, as it has been explained above, or even to improve the knowledge of biological chlorate reduction in early Earth or Martian environments (Martínez-Espinosa et al., 2015).

4.2. Respiratory nitrite reductases in *Haloferax* genus

One of the most important steps in denitrification pathway involves the reduction of nitrite to nitric oxide by the respiratory nitrite reductase (NiR), a key enzyme used to distinguish between nitrate reducers and denitrifiers. This reaction represents the return of nitrite to the gaseous state leading to a significant loss of fixed nitrogen from the terrestrial environment. According to structural features and prosthetic metal, the respiratory nitrite reductases have been classified in two types: cytochrome cd_1-nitrite reductase (encoded by *nirS*) and Cu-containing dissimilatory nitrite reductase (encoded by *nirK*). The cd_1-nitrite

reductase is homodimeric and contains hemes *c* and d_1 as prosthetic cofactors, whereas Cu-nitrite reductase (Cu-NiR) is homotrimeric and contains two Cu atoms per subunit molecule. Cu-NiR enzymes can be easily distinguished according to their spectra and its sensitivity to diethyldithiocarbamate (DDC) (Shapleigh & Payne, 1985). The two NiR types are functionally and physiologically equivalent, but while cd_1-nitrite reductase predominates in denitrifying bacteria, Cu-nitrite reductase is present in a greater variety of physiological groups and bacteria from various habitats (Zumft, 1997; Heylen et al. 2006).

The first evidence related to the activity of respiratory nitrite reductase in *Haloferax* genus was reported in *Hfx. denitrificans,* where it was described that the reduction of nitrite to nitric oxide by their membranes was inhibited by DDC. These results suggested that the Cu-NiR was involved in that reaction (Tomlinson & Hochstein, 1988). It was in 1996 when the first extremophilic respiratory nitrite reductase from *Hfx. denitrificans* was purified and characterised (Table 1) from soluble and membrane fractions (Inatomi & Hochstein, 1996). Electrophoretic analysis of the purified protein revealed the presence of two peptides of 64 and 51 kDa. The molecular mass of this protein was solved by gel filtration chromatography, suggesting that the enzyme was a dimer with 127 kDa. The authors proposed that the small band present in polyacrylamide gel was the result of a degradation of the larger subunit, although nowadays it is known that these data are inaccurate. Although the protein showed its maximum activity in the presence of 4 M NaCl (Table 1), in the absence of salt the enzyme did not loss activity. Its absorption spectrum was characterised by maxima located at 462, 594 and 682 nm, which disappeared after the addition of dithionite. These data indicated that the halophilic NiR belongs to the green Cu-NiR. The inhibition of Cu-NiR in the presence of low concentrations of DDC supported that this enzyme was a Cu-NiR. Even though the membrane-bound Cu-NiR was not totally purified, its characteristics were similar to those of the enzyme purified from the soluble fraction (Inatomi & Hochstein, 1996).

The last advances in the study of respiratory nitrite reductases in extremophilic microorganisms, in general, and in *Haloferax* genus, specifically, have been carried out in *Hfx. mediterranei* (Table 1) (Esclapez et al., 2013). This halophilic respiratory nitrite reductase was expressed in the halophilic host *Hfx. volcanii*. The enzymatic activity of the recombinant protein was detected in cytoplasmic fraction and membranes as well as in the culture media. The enzymes isolated from cytoplasmic fraction and culture media were purified and characterised. The cytoplasmic NiR was described as a trimeric protein, which presented its maximum activity in the presence of 2 M of salt (NaCl or KCl), and around 70 ºC. The presence of four significant regions in its structure were established from bioinformatics analysis, which are:

- Probable Tat motif. Consequently, that region could act as the Tat motif for the protein to be exported via Tat system.

- Possible cutting targets recognised for proteases in positions 27 and 34 from the N-terminal end. The presence of this sequence is associated with the Tat signals because of the mature protein exportation through the cytoplasmic membrane requires the removal of the signal peptide.

- Type 1 copper centre constituted by His129, Cys170, His178 and Met183.

- Type 2 copper centre constituted by Asp132, His134 and His 169.

On the other hand, two different maxima absorption at 453 nm and 587 nm were identified in the UV-vis spectrum suggesting that the enzyme belongs to the green Cu-NiR group. In order to elucidate the composition of the native enzyme, a native PAGE of pure enzyme followed by activity NiR staining showed that the intracellular Cu-NiR is composed of at least five different isoforms of the enzyme. The SDS-PAGE of each of the five bands revealed that each one presents a different combination of two isoforms with 44.3 and 39.8 kDa. The

smaller form was the predominant isoform protein in this cellular fraction (Figure 1A). According to the two cleavage sites present in *Hfx. mediterranei* Cu-NiR sequence, it is logical to think that the expression of recombinant protein could conclude with the maturation of the initial polypeptide through a cut in one of the two targets present at its N-terminal end. Then, the two possible isoforms could combine to form a pool of active trimers. This maturation mechanism could also explain why it is possible to observe two bands with different masses to NiR purification carried out in *Hfx. denitrificans*. On the other hand, the extracellular pool of recombinant NiR was also purified and characterised. The results obtained with this fraction were similar to those obtained with the intracellular Cu-NiR fraction. However, the comparison of the isoform expression pattern of both samples in the SDS-PAGE revealed a significant difference. In the intracellular fraction, the 39.8 kDa isoform was predominant and the 44.3 kDa isoform appeared slightly, whereas in the extracellular fractions the 44.3 kDa isoform was the predominant or even the only one (Figure 1B). According to these electrophoretic analysis, the halophilic Cu-NiR could be involved in a maturation process and exportation via the Tat system. To elucidate the nature of the two isoforms the first eight amino acids of each one were sequenced. The results showed that the 44.3 kDa isoform is obtained due to the cleavage between the 33rd and 34th residues. Therefore, this isoform could be exported via the Tat system, being cleaved by the twin arginine signal sequence after its translocation to extracellular medium. The sequence of the small isoform started in the 52nd position, but no cutting target was predicted around this location. Consequently, it seems more likely that this isoform could be obtained as a result of an alternative translation mechanism (Hering, Brenneis, Beer, Suess & Soppa, 2009) or mRNA processing rather than as a cleavage process. Once the two possible transcripts are translated, a combination of the two isoforms to form the trimer occurs between them. This process originates the pool of possible isoforms found both in the cytoplasmic and extracellular fractions. Finally, the Tat system of *Hfx. volcanii* could promote the exportation of recombinant Cu-NiR active trimers whenever

any of the three contain the signal peptide. In the process of exportation through the membrane, the signal peptides of the large isoform are cleaved. Therefore, outside the cell it can find a mixture of the cleaved and signal-avoided NiR, prevailing over the large isoform. Otherwise only the trimmers remain inside the cell exclusively composed by untargeted peptides that not are able to cross the membrane and go outside the cell.

This difference between targeted and non-targeted peptides could be a mechanism for regulating the system and final Cu-NiR location. The location of recombinant Cu-NiR outside the cell agrees with the results related with the extracellular location of membrane-associated NarGH from *Hfx. mediterranei* detailed above (Martinez-Espinosa et al., 2007). For this reason, there is considerable evidences to propose that the complete reduction of nitrate could take place through an extracellular enzymatic complex which is part of the machinery associated with the outer face of the cytoplasmic membrane whereas the rest of soluble enzymes and metabolites are embedded in the porous S-layer. This unusual respiratory structure offers advantages to these microorganisms in oxygen-poor environments such as hypersaline ecosystems. With this modification, the presence of NO_3^- transporters is not needed and the electron acceptor can be reduced directly in the growth media improving the efficiency of the process. Finally, the mobilization of the proteins involved in NO_3^- respiration appears to be regulated by the Tat system so that they are folded and loaded with metallic cofactors inside the cell before being exported out of the cell where they will take part in their physiological role.

4.3. Nitric oxide reductases in *Haloferax* genus

Nitric oxide is the product of the reaction catalysed by respiratory nitrite reductase. This compound is toxic for the cells and for that reason it is immediately reduced to N_2O by nitric oxide reductases (Nor). The toxicity of NO is due to its reactivity with transition metal proteins and oxygen and its capacity to produce adducts with amines and thiols of fluctuating

stability. In fact, knockout mutation for Nor enzymes results lethal for the microorganisms (Zumft, 1997). There are various difficulties that complicate the biochemical analysis of this type of enzyme as for example:

-Nor enzymes are membrane-bound proteins which requires a detergent for solubilisation.

- NO reactivity with cellular components complicates the enzymatic purification.

- Nor enzymes must be isolated in anaerobic conditions.

- Nor enzymes could be part of other protein complexes.

Nonetheless, different enzymes with Nor activities have been described up to now, among which are the following (Nakara, Tanimoto, Hatano, Usuda & Shoun, 1993; Cabello et al., 2004; Bonete et al. 2008):

- Denitrifying fungi: Nor enzymes are soluble and monomeric. They belong to the cytochrome P-450 family. Its expression is induced by the presence of nitrate or nitrite under anaerobic conditions.

- Denitrifying bacteria: Nor enzymes are heterodimer and constitute membrane complex of a cytochrome *c* (encoded by *norC*) and a cytochrome *b* with 12 transmembrane regions (encoded by *norB*). These enzymes are known as cNor.

- Other bacteria: Nor enzymes are monomeric with 14 transmembrane regions. These enzymes are called qNor due to its quinol-oxidizing activity. qNor enzyme is similar to NorB subunit, although it contains an N-terminal extension, with a quinone-binding site, absent in NorB.

Despite the fact that there is only one study related to the characterisation of Nor in extremophilic microorganisms thus far, gas formation from nitrite has been reported for two microorganisms belonging to *Haloferax* genus, which are *Hfx. denitrificans* and *Hfx. mediterranei* (Zumft & Kroneck, 2006). Regarding to the genetic analysis, *Hfx. volcanii* and *Hfx.*

mediterranei contain in their genomes a copy of a *norB* gene (see section 5). However, at the time of writing this review, no Nor enzyme has been characterised and purified neither in microorganisms belonging to *Haloferax* genus nor in extreme halophilic microorganisms.

4.4. Nitrous oxide reductases in *Haloferax* genus

Conversion of N_2O to N_2 is the last step of denitrification pathway, and represents a respiratory process in its own right. This reaction is of high environmental importance because it closes the N-cycle. For that reason, no wonder that this pathway is found in a broad spectrum of microorganisms, ranging from psycrophiles to hyperthermophiles or from halophiles to barophiles. Therefore, extreme habitats and N_2O use are compatible, being its reduction catalysed by nitrous oxide reductases (Nos). However, N_2O is less toxic that NO or nitrite and the vast majority of microorganisms could manage without converting N_2O into N_2, performing a partial denitrification.

The nitrous oxide reductases are structurally complex enzymes with different copper centres. Two different novel Cu centres have been described in bacteria: the mixed-valent dinuclear Cu_A species at the electron entry site of the enzyme, and the tetranuclear Cu_z centre as the first catalytically active Cu-S complex described. Furthermore, the synthesis of this type of enzymes is very complicated, being accessory proteins (Cu chaperone and ABC transporters) involved in the biogenesis of the catalytic centre. This is the reason why there are few studies related with this enzyme. The presence of other important bioelements as Mo, Mn, Co, Ni or Zn appears not be required for Nos activity. Nevertheless, calcium could play an important function in the stabilization of the protein structure, which is critical for catalysis. Related to Nos inhibition studies, it is known that the acetylene acts as non-competitive inhibitor, although its mechanism of action is still unknown (Zumft & Kroneck, 2006).

Total denitrification pathway has been studied in a high number of bacteria, which contain nitrous oxide reductases encoded by the *nosZ* gene. These bacterial enzymes are

located in the periplasm and they are multicopper homodimers whose electron donor is cytochrome *c* or pesudoazurin (Zumft, 1997). Putative *nosZ* gene has been identified in *Hfx. mediterranei* and *Hfx. denitrificans,* but no Nos enzyme has been purified and characterised from these microorganisms yet, due to the difficulty of working with this type of enzymes and the scarce information available related to the denitrification enzymes in extreme halophilic microorganisms. Despite this, preliminary assays carried out with *Hfx. mediterranei* have revealed that this enzyme is expressed in anaerobic conditions and it is located in its membranes.

5. GENES CODING FOR THE ENZYMES SUSTAINING DENITRIFICATION.

From the previous section it is possible to conclude that some studies about biochemical characterisation of denitrifying enzymes have been reported from *Haloferax* species. However, no details have been published so far from genes coding these enzymes (with the exception of *nar* operon from *Hfx. mediterranei*) (Lledó et al., 2004) or their regulation.

A general analysis of the *Haloferax* genome sequences already published has been designed and performed in our research group. The aim of this analysis was to look for genes coding for enzymes and proteins involved in denitrification. This analysis has been tedious due to two main reasons: i) most of the sequences are in contig format (only few genomes are completely sequenced, assembled and annotated), ii) the nomenclature used to identify the genes is confuse and not conserved. To perform this study the following tools have been used: DNA and amino acid sequences were analysed using the available database on the NCBI (National Center for Biotechnology Information) server (http://www.ncbi.nlm.nih.gov/protein; http://www.ncbi.nlm.nih.gov/bioproject/; http://www.ncbi.nlm.nih.gov/gene) and the Ensembl genome annotation system (http://ensemblgenomes.org/).

Figures 2-4 summarised the organization of the respiratory nitrate reductase, respiratory nitrite reductase, nitric oxide reductase and nitrous oxide reductase gene clusters in several species of the *Haloferax* genus: *Hfx. mediterranei* (which is the model organisms in our group to study nitrogen metabolism), *Hfx. volcanii*, *Hfx. denitrificans*, *Hfx. larsenii, Hfx. elongans, Hfx. lucentense, Hfx. alexandrinus, Hfx. gibbonsii, Hfx. prahovense* and *Hfx. sulfurifontis.* The gene cluster organization in *Hfx. larsenii*, *Hfx. elongans*, *Hfx. lucentense*, *Hfx. alexandrinus*, *Hfx. prahovense and Hfx. sulfurifontis*, was obtained from genome sequencing projects in which the DNA sequences are in diferent contings. As the ORFs have been automatic annotated, in some of them the nomenclature used to identify the genes is confuse. The homolgy of these ORFs was comprobed using the Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Because all the previous reason, the bioinformatic analysis is dificult and in some cases could contain gaps due to the information lack in the sequenced genomes.

Regarding the respiratory nitrate reductase gene cluster (Figure 2), the organization is almost the same in the 7 compared species. The three ORFs encoding NarJ and the two adjacent hypothetical proteins cannot be found in *Hfx. alexandrinus*. It is notorious that in the genome of *Hfx. prahovense* nitrate reductase operon was not identify, althougt the nitrite reductase, and de nitric oxide reductase gene clusters are present. The nitrate reductase cluster is encoded in a plasmid in *Hfx. mediterranei* and in *Hfx volcanii,* but it is impossible to know their localization in the other species. It is possible to think that the ability of some species for nitrate respiration has been acquired by lateral transference of the plasmid encoded genes. This cluster contains genes coding for the catalytic and the electron transfer subunit (*narG* and *NarH*, respectively), a gene (*mobA*) involved in the MOCO cofactor synthesis, genes coding for proteins involved in the electron transfer (*narB* and *narC*), and a gene coding for chaperone like protein (*narJ*), which is presumably involved in maturation and assembly of the αβγ complex (Palmer, Santini, Lobbi-Nivol, Eaves, Boxer & Giordano, 1996; Liu

& DeMoss, 1997; Blasco et al., 1998). The hypothetical protein codified by the gene following *narH* belongs to the DMSO reductase family type II enzyme, heme b subunit, which can be identified as the homologue to the *narI* gene coding for the gamma subunit in bacteria. *mrp* codes for a Mrp protein; this protein belongs to the conserved protein domain family MRP like (Multiple Resistance and pH adaptation), a homologue of the Fer4 NifH superfamily, which is found in bacteria as a membrane-spanning protein and functions as a Na^+/H^+ antiporter. Like the other members of the superfamily, it contains an ATP-binding domain. The N-terminal presents also homology with a FeS assembly SUF system protein, and thus, could be involved in cofactor biosynthesis or electron trasport. The gene *acrR* encodes a DNA binding protein putative transcriptional regulator, *boa* codes for bacterio-opsin activator-like protein and *prp* encodes a protein phosphatase. These three elements could be implied in a regulation mechanism of nitrate respiration but physiological and biochemical studies should be done to properly elucidate this role. Finally, *tnp* codes for a transposase. The Nar operon has been very well described from *Hfx. mediterranei* (Lledó et al., 2004) and other haloarchaea such as *Haloarcula marismortui* (Yoshimatsu et al., 2002), however, the its regulation has not been explored yet.

Genes coding for respiratory nitrite reductase and nitric oxide reductase are closely located and constituting a gene cluster summarised in Figure 3 for several species. These gene are encoded in the chromosome of *Hfx. mediterranei* and in *Hfx. volcanii*. The first important feature to be highlighted when comparing between species is that the gene coding for respiratory nitrite reductase copper containing protein (*nirK*) is close to *hcy,* a gene coding for a halocyanin precursor-like protein. Halocyanin is small blue copper protein with a molecular mass of about 15 kDa which serves as a mobile electron carrier.

Although more work must be done to elucidate de role of this protein in the context of the haloarchaeal denitrification, it is possible to conclude from the bibliography that it may be involved in electron transfer during nitrite and/or nitric oxide reduction (Brischwein, Scharf,

Engelhard & Mäntele, 1993; Scharf & Engelhard, 1993; Mattar et al., 1994). There is also a gene coding for multicopper oxidase (*mco*) which oxidise their substrate by accepting electrons at a mononuclear copper centre and transferring them to a trinuclear copper centre. This protein as well as halocyanin could be involved in the reaction catalysed in vivo by NirK. This cluster also contains several genes related to nitric oxide reductase: *norB*, coding for cytochrome b subunit of nitric oxide reductase; *norZ*, which encodes Nitric oxide reductase, also called NorZ apoprotein. Other genes code for proteins related to electron transfer or cofactor biosynthesis. Thus, *cox* codes for cytochrome c oxidase subunit I; while, *pqqE* encodes Coenzyme PQQ synthesis protein, which is a protein involved in the pathway pyrroloquinoline quinone biosynthesis (Cofactor biosynthesis). Other genes included in these cluster are: *ycfA,* which codes for YcfA family protein (most of these proteins are hypothetical proteins of unknown function); *lip* which encodes a lipoprotein; *arsR*: Putative transcriptional regulator, ArsR family; *gdhA1,* coding for glutamate dehydrogenase (NAD(P)+); *hth,* encoding HTH DNA binding domain and *mtt,* coding for Methyltransferase type 12.

Figure 4 summarised the organization of nitrous oxide reductase cluster. In this case, only the information from three species has been included because it was impossible to identify genes coding for neither the enzyme nor their accessory proteins from other species. In this cluster, the following genes coding for nitrous oxide reductase and its accessory proteins are identify: *nosL*, a gen coding for Cu(I) protein of the nitrous oxide; *nosY*, which codes for an ABC-type transport system involved in multi-copper enzyme maturation permease component; and *nosZ,* a gene coding for nitrous-oxide reductase. The rest of the gees included in this cluster code for proteins involved in electron transfer (i.e. *pcy:* copper-binding plastocyanin like protein) as well as proteins involved in protein maturation can be found. *Hfx. larsenii* and *Hfx. denitrificans* clusters also contain the gen *trxR* which codes for a thioredoxin reductase. This protein is ubiquitous and it is involved in the defence against

oxidative damage due to oxygen metabolism, and redox signalling using molecules like hydrogen peroxide and nitric oxide.

In *Hfx. denitrificans* as well as in *Hfx. larsenii* genes coding for NirK are closely located to genes encoding Nos. The genomes of the mentioned species are not completely sequenced, assembly and annotated so it is impossible to identify genes localization (chromosome, plasmids) or even operons. *Hfx. mediterranei* genome offers a more details analysis. IN that case, genes coding for NarGH and Nos are located in one of the three plasmids (pHM300), whilst genes encoding Nirk and Nor are located in the main chromosome. This fact supports the theory of lateral gene transfer as an important tool in haloarchaea resulting in several phenotypes better adapted to the environment they are inhabiting. Nar genes constitute an operon. However, *nir* and *nor* genes are close and probably constitute an operon (molecular biology studies must be done to address this hypothesis). On the other hand, *nos* genes in *Hfx. mediterranei* could constitute another operon which should be analysed in terms of regulation in the next future.

6. POTENTIAL USES OF THE DENITRIFICATION CARRIED OUT BY *Haloferax* IN BIOTECHNOLOGY.

Nowadays many industrial processes such as food processing, oil production or handling of pharmaceuticals generate compounds containing nitrogen in different forms (Lefebvre & Moletta, 2006). Organic and inorganic nitrogen are harmful to the environment and lead to many health problems (Li et al., 2013). High concentrations of nitrates and nitrites in water are very toxic to humans, fauna and flora (Philips, Laanbroek & Verstraete, 2002). Furthermore, the presence of this anion in the bloodstream transforms the haemoglobin to methaemoglobin irreversibly, hindering the release of oxygen to tissues (Van Leeuwen, 2000) and causing respiratory problems in aquatic and terrestrial animals, including humans (Philips et al., 2002; Nájera-Fernández et al., 2012).

Although most biochemical, genetic and physiological studies are needed about denitrification process in members of *Haloferax* genus, specially focused in the last steps of this process (reduction of nitric oxide and nitrous oxide), the acquired knowledge since today permits to apply some species such *Hfx. mediterranei* in different biotechnological processes like wastewater treatments or the development of biosensors. In that sense, not only denitrification as pathway but also isolated enzymes involved in denitrification could be used.

6.1. Wastewater treatments by *Haloferax* members.

Traditional wastewater treatments are based on two essential procedures for nitrogen removal: firstly, autotrophic nitrification (oxidation of ammonium to nitrate by nitrifying bacteria under aerobic conditions); secondly, anoxic denitrification (which converts nitrate and nitrite to N_2 gas by denitrifying bacteria under anaerobic conditions) (Zhu et al., 2012).

The most studied microorganisms capable of heterotrophic nitrification and aerobic denitrification are *Paracoccus* (Blaszczyk, 1993), *Thioalkalivibrio* spp. (Sorokin & Kuenen, 2005), *Bacillus licheniformis* (Takenaka et al., 2007), *Halomonas* spp. (Boltianskaia et al., 2007) and *Pseudomonas stutzeri* (Miyahara et al., 2010). These strains can oxidize ammonium to nitrite and, simultaneously, can reduce nitrates and nitrites to N_2 by aerobic denitrification (Guo et al., 2013).

However, this approach is very costly and time consuming because nitrifying bacteria do not grow quickly and require different conditions (Chen et al., 2014). Moreover, in the last years there is an additional problem in urban and industrial wastewater: the increase of salinity.

The fish and seafood processing industry, leather and petroleum industry and the manufacturing of chemicals such as pesticides, herbicides and explosives generates effluents containing complex mixtures of salts and nitrate or nitrite (Nájera-Fernández et al., 2012; Zhang, Zhang & Quan, 2012). Moreover, the use of seawater as a substitute for fresh water in

the municipal sanitation for some human activities such as the maintenance of public urinals, produces saline waste entering in the wastewater circuit and increasing its salinity (Duan, Fang, Su, Chen & Lin, 2015).

Thus in this context and due the fact that the bioactivity of denitrification significantly decreased when salt was above 2% w/v (Guo et al., 2013), traditional denitrifying bacteria used in wastewater treatments may suffer a loss of enzymatic activities and the unbalance of osmotic stress across the cell wall resulting in plasmolysis (Nájera-Fernández et al., 2012; Zhang et al., 2012).

One solution could be some strains from *Halomonas* (Shapovalova, Khijniak, Tourova, Muyzer & Sorokin, 2008), which are actively denitrifiers under highly halophilic conditions (salt concentrations of 4 M Na^+), but the problem is that they grow and denitrify only aerobically; due to the low dissolved oxygen concentrations in industrial wastewater, these aerobic halophilic species are not suitable for use in the treatment of industrial salt wastewater (Shapovalova et al., 2008).

Therefore, it is very important to use halophilic microorganisms tolerant to a wide range of salinities, resistant to salt shock (Duan et al., 2015) and anoxic conditions. In this sense, organisms belonging to the *Haloferax* genera are perfect candidates for been used in wastewater treatments.

6.1.1. The use of Haloferax mediterranei in wastewater treatments.

Hfx. mediterranei is an extreme halophilic archaeon able to grow on an unusually large range of concentrations of NaCl (1.0 to 5.2 M) (Torreblanca et al., 1986). It has been used as model in saline wastewater treatments or brines bioremediation because of its resistance to very high nitrate and nitrite concentrations (Bonete et al., 2008; Martínez-Espinosa, Lledó, Marhuenda-Egea, Díaz & Bonete, 2009) and tolerance to a broad range of salinities (D'Souza & Altekar, 1997).

In a recent study, Nájera-Fernández et al. grew *Hfx. mediterranei* at 42ºC using brines either prepared in the laboratory or collected from wastewater plant treatments with 30, 40 and 50 mM KNO_2. Given that the maximum tolerance of the majority of microorganisms studied to date ranges from 2 to 5 mM NO_2^-, the concentrations in this experiment can be considered very high.

The results showed that *Hfx. mediterranei* consumed 100% of nitrate and nitrite present in media with 30 and 40 mM KNO_2^- and 60% of the nitrate and 75% of the nitrite in medium with 50 mM KNO_2^-. Using nitrogen species by this haloarchaea to grow occurred both in oxic and anoxic conditions thanks to assimilatory nitrate and nitrite reductases in the first case and to respiratory nitrate and nitrite reductases when oxygen is depleted.

In addition to the advantages of using *Haloferax s*pecies in removing nitrogenous compounds from wastewater, recently it has been discovered that *Hfx. mediterranei* is able to reduce perchlorate and chlorate in anaerobic conditions through respiratory nitrate reductase (NarGH) lacking genes coding for (per)chlorate reductases, as previously mentioned (Martínez-Espinosa et al., 2015). These anions, which have very harmful effects on health, are present in sewage because of many human activities such as the manufacture of propellants, explosives or bleaching paper.

The ability of NarGH to use (per)chlorate as final electron acceptor in the absence of oxygen is due the location of its active site facing the membrane potential positive face (pNars) (Martinez-Espinosa et al., 2007; Martínez-Espinosa et al., 2015). It permits to reduce these anions to chlorite with any intracellular damage. Moreover, this enzyme is capable to reduce bromate too.

Hfx. mediterranei could be a good model to remove perchlorate, chlorate and bromate in brines and wastewater even in the presence of low salt concentrations (Martínez-Espinosa et al., 2015). Due that many wastewater contain both nitrates and chlorates (Wolterink et al., 2003), the processes based on bioremediation by *Hfx. mediterranei* could replace or even

improve protocols where perchlorate and nitrate are removed from brines by ion exchange techniques (Lehman, Badruzzaman, Adham, Roberts & Clifford, 2008).

Despite the advantages of using denitrifiers to remove toxic anions from sewage, there are some negative effects related to denitrification during biological wastewater treatments such as the emission of nitrous oxide, which is involved in global warming. Although the release of this gas from wastewater treatments is relatively small (3% of the estimated total anthropogenic N_2O emission), but is a significant factor (26%) in the greenhouse gas footprint of the total water chain (Kampschreur, Temmink, Kleerebezem, Jetten & van Loosdrecht, 2009).

The emission of N_2O can be due to several factors: first, the use of partial denitrifiers (reducing nitrates and nitrites to nitrous oxide because of the lack of nitrous oxide reductase); second, due to some abiotic conditions such as pH (Čuhel et al., 2010; Liu, Mørkved, Frostegård, & Bakken, 2010; Sánchez-Andrea, Rojas-Ojeda, Amils, & Sanz, 2012; Huang, Long, Chapman & Yao, 2014), salinity or temperature which can inactive this enzyme; third, because of some operational parameters in wastewater treatments such as low COD/N ratio in the denitrification stage or the increase of nitrite concentrations (Kampschreur et al., 2009).

A little is known about the relation between the use of *Haloferax* species in wastewater treatments and nitrous oxide emission. In case of *Hfx. mediterranei*, although nitrous oxide reductase has not been purified and characterised yet, it is known that it is a complete denitrifier haloarchaea because of the reduction of N_2O to N_2 observed in anaerobic conditions after the addition of KNO_3 as terminal electron acceptor (Bonete et al., 2008). Concerning other *Hfx.* species such as *Hfx. volcanii*, it is not yet known if it is complete or incomplete denitrifier organism. Despite of this knowledge, there are not studies focused on the nitrous oxide emission during wastewater treatments using members of the *Haloferax* genus.

6.2. Biosensors based on denitrification enzymes.

In the last years, a lot of legislative actions for environmental pollution control have been grown due the social concern in this area (Rodriguez-Mozaz, Lopez De Alda & Barceló, 2006). Special attention has received water quality control in terms of nitrates and nitrites concentrations because of their negative effects on human health. The most important governmental agencies have promulgated rules and directives to restrict the level of these ions in drinking water and food products (Gabriela Almeida, Serra, Silveira & Moura, 2010).

In order to comply with the new controls, it is needed efficient measures of nitrates and nitrites. Their determination using traditional methods such as chromatography, spectrophotometry or polarography is expensive and they have susceptibility to matrix interferences, require pre-treatments and long-time of analysis (Cosnier, Da Silva, Shan & Gorgy, 2008; Gabriela Almeida et al., 2010; Mohd Zuki, Suhaity Azmi, & Ling Ling, 2014).

Therefore, it becomes necessary to develop systems which can detect a lot of compounds in environmental samples as quickly and as cheaply as possible (Rodriguez-Mozaz et al., 2006). In these terms, biosensors have become one of the most important devices. They are capable of providing quantitative or semi-quantitative analytical information with the help of two elements: a biological recognition component which detects the analyte connected to a transducing element which transforms the measure in a (semi)quantitative signal (Rodriguez-Mozaz et al., 2006; Mohd Zuki et al., 2014).

Biosensors can be classified into various groups (Fig. X), according either to their two principal components (Rodriguez-Mozaz et al., 2006; Slonczewski, Coker & DasSarma, 2010): on the basis of the transducing element, they can be named as electrochemical, optical, piezoelectric or thermal sensors; according to the bio recognition principle, biosensors are classified into antibodies and antigens, enzymatic, non-enzymatic, whole-cell and nucleic acids biosensors (Marazuela & Moreno-Bondi, 2002; Rodriguez-Mozaz et al., 2006).

In the context of nitrates and nitrites biosensors, there have been two main lines of development: on the one hand, using whole cells and detecting some products of their nitrogen metabolism; on the other hand, systems based on the immobilisation of denitrification enzymes in a matrix.

In case of using whole cells, bacteria are placed in a reaction chamber where the reduction NO_3^- to N_2O occurs and it is measured by a specific nitrous oxide micro-electrode (Larsen, Kjær & Revsbech, 1997). A new improvement utilizes a whole cell fluorescence system which is based on recombinant Escherichia coli bacteria without interference of phosphate, chloride and nitrite (Rodriguez-Mozaz et al., 2006).

But the most important nitrates and nitrites biosensors are based on the immobilisation of nitrate and nitrite reductases from bacteria (*Paracoccus denitrificans*, *Paracoccus pantotrophus*, *Alcaligenes faecalis*, *Desulfovibrio desulfuricans*, etc.) in different materials improving their stability and half-life (Mohd Zuki et al., 2014). The most common methods used to immobilize enzymes are covalent binding and cross-linking (Mohd Zuki et al., 2014). Proteins should be placed on surfaces that do not alter their structure and functions: a good example is the use of inorganic clay nanoparticles as hydrophilic additives in combination with polypyrrole films (Cosnier et al., 2008).

The majority of proposals use redox mediators (viologen derivatives) to shuttle electrons from the protein redox centres to the transducing elements (ammeters or voltmeters). Very recently, new approaches are based on the direct electron transfer between the enzymes and the electrode material (Gabriela Almeida et al., 2010) to simplify the structure of biosensor.

In the context of the genus *Haloferax*, biosensors could be developed based on nitrate and nitrite reductases from *Hfx. mediterranei* because these enzymes are purified and characterised. These biosensors would have several advantages over bacterial biosensors described since today: firstly, nitrate and nitrite reductases from this haloarchaea tolerate high

salt concentrations, allowing their use in salt water and brines; secondly, their Km values are quite low so the affinity for their substrates is very high (Km of nitrite reductase of *Hfx. mediterranei* is 4.04 +/- 0.33 mM which is almost 100-fold greater than for other enzymes NirK characterized) (Esclapez et al., 2013). Despite all advantages, it has not yet developed any prototype of biosensor for *Haloferax.*

7. CONCLUSIONS AND FUTURE PERSPECTIVES.

Although knowledge about anaerobic metabolism in haloarchaea is improving, more effort should be done to understand how those microorganisms, and in particular, *Haloferax* genus, switch on the biological traits required to be alive in these open and hostile habitats characterised by high salt concentrations and nutrients and oxygen limitation. Currently, huge amount of open questions remain unaddressed about haloarchaea anaerobic metabolism:

- the key signals inducing anaerobic metabolism must be identify, (oxygen depletion alone or oxygen depletion concomitant with the presence of specific compounds such us nitrate, chlorate, sulphur, redox balance, etc.);

- the molecular mechanisms involved in anaerobic pathways have to be characterised; No information about regulators involved in anaerobic metabolism induction or inhibition has been reported yet.

-the importance of NOx emissions as a consequence of haloarchaeal denitrification should be quantify in situ and in vitro.

- Molecules acting as oxygen sensors in haloarchaea must be identify and characterised. Same for nitrate, nitrite, nitric oxide, chlorate, sulphur and nitrous oxide.

- The presence of oxygen alternative terminal electron acceptors is minority in environments with high salt concentrations. Thus, their ecological role is still uncertain in the context of the habitat where *Haloferax* species live.

Another aspect that remains partially know is the capacity of the denitrification from those haloarchaea initially characterised as denitrifiers. This aspect, although the last one in this sections, is probably the most important one. When a new taxon is isolated, several microbial and biochemical analysis are performed to characterised the phenotype. Usually, a new isolated is considered denitrifier when nitrate reduction is observed under anoxic conditions. However, this capability does not directly show denitrification activity (denitrification implies complete nitrate reduction to dinitrogen). As a result of this partial characterisation, more work is currently required to properly characterise whether or not one single *Haloferax* species, initially characterised as a denitrifiers is in fact a complete or a partial denitrifier.

New insights on gene expression and their regulation as well as protein characterisation will contribute to quantify how important those species are in terms of behaviour as 'microbial weeds', NOx gasses emissions, nitrogen compounds recycling, potential biotechnology applications, etc.

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