Chapter 9
Recent Advances in the Nitrogen Metabolism in Haloarchaea and Its Biotechnological Applications

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1 Introduction

In the biosphere, nitrate assimilation is the significant pathway for turning inorganic nitrogen into organic forms. This pathway requires the presence of two assimilative enzymes, nitrate reductase and nitrite reductase. First, NO_3^- is transported into the cells by high-affinity transporters and next reduced to NH_4^+ by two sequential reactions catalysed by assimilative nitrate reductase (Nas; EC 1.6.6.2) and assimilative nitrite reductase (Nir; EC 1.7.7.1). The resulting ammonium is combined with carbon skeletons by the glutamine synthetase/glutamate synthase pathway (GS-GOGAT; EC 6.3.1.2, EC 1.4.7.1, respectively) or via glutamate dehydrogenase (GDH; EC 1.4.1.2). The GS/GOGAT pathway is especially important as it allows ammonia assimilation into L-glutamate (Glu) at low intracellular ammonia concentrations and it efficiently substitutes the other glutamate biosynthetic reaction (GDH) in these conditions (Bonete et al. 2008).

Genes encoding the main proteins involved in nitrate and ammonium assimilation have been found in the genomes of the two major Archaea subgroups: Crenarchaeota and Euryarchaeota (Feng et al. 2012). On the other hand, studies of haloarchaea communities from soil revealed that the haloarchaeal assimilatory nitrate-reducing community seems to be important in salty and/or alkaline environments (Alcántara-Hernández et al. 2009). However, biochemical characterisation of haloarchaeal enzymes involved in NO_3^-, NO_2^- and NH_4^+ assimilation is insufficient and it has been performed mainly in *Haloferax mediterranei* at the time of writing this

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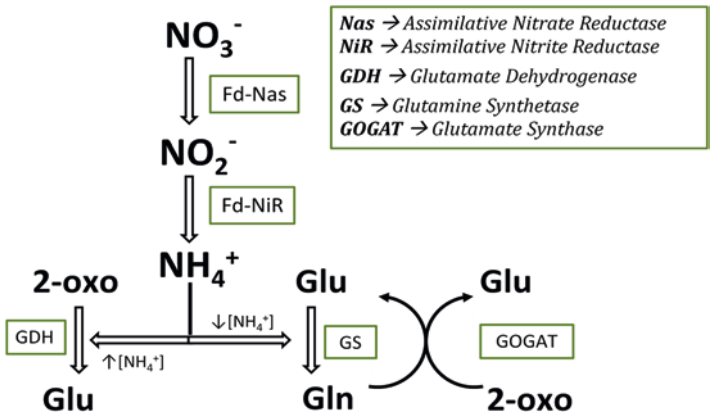

Fig. 9.1 Nitrate and ammonium assimilation pathways in *Haloferax mediterranei*. *2-oxo* 2-oxoglutarate, *Glu* L-glutamate, *Gln* L-glutamine

chapter (Fig. 9.1). All the known biochemical parameters already described from haloarchaeal protein transporters and enzymes involved in assimilatory nitrate reduction are summarized in the following sections.

2 Enzymes Involved in Nitrate/Nitrite and Ammonium Assimilation

2.1 NO_3^- and NO_2^- Transporters

The uptake of nitrogen compounds such as nitrate or nitrite is the first step to use these compounds as nitrogen sources. In prokarya, the genes encoding proteins required for NO_3^- uptake and its subsequent reduction are, in general, clustered (Moreno-Vivián and Flores 2007). Furthermore, it is common that NO_3^- is transported into the cells by an active system. At the time of writing, two kind of proteins involved in nitrate transport are described in prokarya: ATP-dependent ABC transporters (constitute by (i) an integral membrane subunit, (ii) a cytoplasmic ATP-binding component and (iii) a periplasmic substrate binding protein) and the monomeric NarK-type transporters belonging to the major facilitator superfamily (MFS-type permease). The last type of transporter depends on proton-motive force. ABC transporters can be found in Archaea, Bacteria and Eukarya (Wanner and Soppa 1999). Bacterial NarK-like transporters can be divided into two subgroups: NarK1 (proton: nitrate symporter that allows initiation of nitrate respiration) and NarK2 (nitrate: nitrite antiporter required for maintenance of a steady state rate) (Wood et al. 2002). Some of these proteins are involved in NO_3^-/ NO_2^- exchange rather than simply in the uptake of one of the mentioned ions. However, the transport mechanism by means of these MFS importers has not been elucidated yet. It has been stated that bacterial nitrate assimilation requires ATP-dependent ABC

nitrate transporters whereas nitrate respiration is mainly associated with proton-motive-force driven NarK transporters (Wood et al. 2002). Database comparisons of the genes involved in haloarchaea nitrate assimilation, in particular in *Hfx. mediterranei*, revealed that *nasB* gene (Q703N4) encodes a NO_3^- transporter, which is a membrane protein (Mr around 46.1 kDa) with 12 potential α helices and is most closely related with the NarK1 type of transporters. The best fits of *nasB* gene were with bacterial homologues belonging to the genera *Thermus*, *Paracoccus* and *Pseudomonas* (Lledó et al. 2005). *Hfx. mediterranei* NarK is the first Archaea NarK transporter reported to date, not being exclusive to Bacteria and Eukarya as it was initially suggested. This data suggest that NarK transporters could be involved in the nitrate transport not only in the assimilatory process but also in the denitrification pathway. This transporter could be in charge of the nitrate/nitrite exchange. However, more biochemical studies on that kind of transporters are required to properly understand how nitrate and nitrite are up taken in haloarchaea.

2.2 Ammonium Transporters

If ammonium is the nitrogen source for growth, the first step is the ammonium uptake facilitated by the ammonium transporter (Amt). Amt transporters are characterised by: high affinity and selectivity for ammonium, non-permeability to alkaline cations, permeability to methylammonium and quick saturation at low millimolar concentrations. This transporters are trimers containing 11-crossing membrane fragments. Each monomer contains a hydrophobic channel that conducts NH_3 but not any water or ions. The re-protonation of NH_3 on the receiving side raises the pH on that side in the absence of metabolism of NH_3, and there is no transfer of protons through the protein (Khademi and Stroud 2006). Crystallization of homologue proteins from the three domains pointed out that the functional entity corresponds to a trimer, with each monomer maintaining a conductive pore (Pantoja 2012). Although, Amt transporter is well characterised in Bacteria and Eukarya domains from a biochemical point of view, it still remains quite unknown in Archaea. In fact only the Amt from *Archaeoglobus fulgidus* (Andrade et al. 2005; Andrade and Einsle 2007) and *Hfx. mediterranei* (Pedro-Roig et al. 2013) have been studied to date from this domain.

2.3 Assimilatory Nitrate Reductase

Once nitrate enters into cells, it is reduced to nitrite by assimilatory nitrate reductase (Nas). Nas are cytoplasmic enzymes that catalyse the following reactions which implies the mobilization of two-electrons:

$$NO_3^- + NADH\ (or\ reduced\ \ ferredoxin) + H^+ \rightarrow$$
$$NO_2^- + NAD^+\ (or\ oxidized\ \ ferredoxin) + H_2O$$

Most of the characterised Nas use either NADH or ferredoxin as physiological electron donors, although some of them use flavodoxin instead of ferredoxin. Nas activity is only detected under aerobic conditions and it is induced by nitrate and repressed by ammonium, as described for bacterial Nas (Richardson et al. 2001; Luque-Almagro et al. 2011).

Ferredoxin-dependent Nas are usually monomeric enzymes while NADH-dependent Nas are heterodimers (Richardson et al. 2001). Fd-Nas and NADH-Nas are structurally and functionally different from the dissimilatory periplasmic nitrate reductases (Nap; EC 1.7.99.4) and the respiratory membrane-bound nitrate reductases (Nar; EC 1.7.99.4) present in many prokaryotes. Gene sequence analysis has revealed that *Hfx. mediterranei* ferredoxin-dependent Nas (Q703N5) is a molybdoenzyme and the synthesis of Mo-bis Pyranopterin guanine dinucleotide cofactor (Mo-bis-PGD) is mediated by MobA. This enzyme catalyses the binding of GTP to molybdopterin, resulting the active cofactor PGD. On the other hand, spectroscopic studies have revealed the presence of one [4Fe-4S] cluster. This monomeric enzyme (molecular mass around 75 kDa) is the only Nas isolated and characterised from haloarchaea up to know. In this case, the electrons could flux as follow: from the [2Fe-2S] cluster-containing ferredoxin (negative redox potential electron donor) to the [4Fe-4S] cluster and from this centre to the Mo-cofactor for the reduction of NO_3^- (Martínez-Espinosa et al. 2001a; Lledó et al. 2005; Bonete et al. 2008).

The best *Hfx. mediterranei* Nas protein fits were to Nas proteins of the genera *Pseudomonas*, *Xanthomonas* and *Synechococcus*. The comparison with the products of the putative archaeal genes coding for Nas showed that there was only a low overall similarity between these and assimilatory nitrate reductase from *Hfx. mediterranei*, with conserved residues predominantly those related to the cofactor binding sites. Nas kinetic parameters were determined by varying the concentration of one substrate (MV) at several concentrations of the other substrate (nitrate). The apparent K_m values for nitrate and MV were 0.95 ± 0.12 and 0.07 ± 0.12 mM, respectively. This halophilic Nas showed a strong dependence on the temperature and NaCl (maximum activity detected at 80 °C for NaCl concentrations between 3.1 and 2.2 M). Nas from *Hfx. mediterranei* can receive electrons from methylviologen and benzylviologen but not NAD(P)H. Nas was present during aerobic growth, and the activity decreases and even disappeared when the culture was near the stationary phase. In the presence of ammonium ion (or an alternative nitrogen source to nitrate) Nas activity is not detected (Martínez-Espinosa et al. 2001a).

2.4 Assimilatory Nitrite Reductases

Nitrite produced in the previous reactions is reduced to ammonium by ferredoxin-dependent assimilatory nitrite reductase (Q703N2) that catalyses the following reaction involving the mobilisation of six-electrons:

$$NO_2^- + 3\,NADH\,(or\,3\,reduced\ ferredoxin) + 5\,H^+ \rightarrow$$
$$NH_4^+ + 3\,NAD^+\,(or\,3\,oxidized\ ferredoxin) + 2\,H_2O$$

Assimilatory nitrite reductases are classified on the basis of the electron donor specificity: Fd-dependent Nir (described from eukaryotic and prokaryotic photosynthetic organisms) and NAD(P)H-dependent Nir (present in fungi and most heterotrophic bacteria). Fd-Nirs are cytoplasmic monomeric proteins containing a siroheme and a [4Fe-4S] cluster as redox centres. The electrons flow the [2Fe-2S] cluster-containing ferredoxin to the [4Fe-4S] cluster and from this centre to the siroheme for the reduction of nitrite. NADH-Nirs dimers and contain non covalently bound FAD, a [4Fe-4S] cluster, and siroheme as prosthetic groups (Fernández et al. 1998).

The Nir purified from *Hfx. mediterranei*, the first one purified from this kind of microorganisms, is a 66 kDa monomer that shows a strong sequence similarity with known ferredoxin-dependent nitrite reductases, such as those purified from cyanobacteria and bacteria. Some of its biochemical properties have been analysed in detail: K_m for nitrite around 8.6 mM and maximal activity at 60 °C in presence of 3.3 M NaCl. Like most of the bacterial assimilatory nitrite reductases described so far, the *Hfx. mediterranei* Nir contains siroheme and Fe-S centres as redox centres (Martínez-Espinosa et al. 2001b; Lledó et al. 2005).

2.5 Glutamine Synthetase-Glutamate Synthase (GS-GOGAT) Cycle and Glutamate Dehydrogenase (GDH)

In most bacteria and archaea, the formation of L-glutamate is the main way for ammonia and nitrogen assimilation. Three enzymes are involved in this process in *Hfx. mediterranei*. These are: glutamine synthetase (GS), which incorporates ammonium into glutamine, glutamate synthase (GOGAT), which converts the glutamine to glutamate, and glutamate dehydrogenase (GDH), which catalyzes the reversible reductive amination of 2-oxoglutarate to produce glutamate (Ferrer et al. 1996; Díaz et al. 2006; Martínez-Espinosa et al. 2006; Pire et al. 2014). The GS-GOGAT cycle requires ATP but has high affinity for ammonium, whereas GDH does not consume ATP but is less effective in cells growing in N-limited conditions. These enzymes are present in all three domains of life.

2.5.1 Glutamine Synthetase

Glutamine synthetase (GS) enzyme, which belongs to the GS- GOGAT route. It has been performed biochemical and molecular studies of GS protein from some hyperthermophilic and methanogenic archaea, prokaryotes and mammals (Adul Rahman et al. 1997; Reitzer 2003). This protein has a double function, producing glutamine and assimilating ammonium in collaboration with glutamate synthase. In brief, the GS can be classified into three types according to their molecular size and the number of subunits contained. GSI contains 12 subunits with a range of 44–60 kDa, and it is in archaea and bacteria groups (Brown et al. 1994; Robertson and Alberte 1996). GSII, is an octameric enzyme with subunit size of 35–50 kDa, and it is found in a

small number of soil-dwelling bacteria and eukaryotes (Kumada et al. 1993). At last, GSIII is a hexameric protein with 75 kDa subunits, which was found in some anaerobic bacteria and cyanobacteria (Reyes and Florencio 1994).

Few analyses have been carried out with GS from members of Archaea, and just two are from GS from haloarchaea; these enzymes, purified from *Hfx. mediterranei* and *Hbt. salinarium* are octamers belonging to the GS type II (Manitz and Holldorf 1993; Martínez-Espinosa et al. 2006). However, a few GSs described from methanogenic or hyperthermophilic archaea are dodecamers of about 600 kDa. So, GS from haloarchaea exhibits typical properties of GS from eukaryotes and soil bacteria species. The results obtained from *Hfx. mediterranei* suggest that GS from this haloarchaeon could allow the assimilation of the ammonium produced by assimilatory nitrite reductase (Martínez-Espinosa et al. 2006, 2007), while GDH would allow the assimilation of ammonium when this nitrogen source is present in the culture media at high concentrations. The biochemical characterization of *Hfx. mediterranei* GS shows an optimum pH value for activity around 8. Either in the presence of NaCl or KCl, the maximum stability was found at the highest salt concentration described by us (3.5 M and 2.5 M, respectively). The K_m value for ADP could not be calculated for the transferase activity, as all concentrations analysed produced similar results (K_m around 3.10 ± 0.5 mM). The apparent K_m for NH_2OH, Gln, ATP and Glu were 10.5 ± 3.5, 25 ± 1.8, 0.30 ± 0.08 and 4.9 ± 1.5 mM, respectively.

These values are similar to those described in *Hbt salinarium* GS and are in the range described for GS from different cyanobacteria and Archaea (Manitz and Holldorf 1993). Studies about the effectiveness of different metal ions on the activity assay revealed that Mn^{2+} was the most effective ion for transferase activity as it was expected due to its essential role as a cofactor.

2.5.2 Glutamate Synthase

Glutamate synthase (GOGAT or GltS), the second enzyme of the GS/GOGAT cycle catalyses the reductive transfer of the amide nitrogen from L-glutamine to 2-oxoglutarate to form two molecules of L-glutamate. There are different classes of GOGAT which differ for distribution among different organisms and tissues, subunit composition, cofactor content and physiological reductant (Vanoni and Curti 2005). The main GOGAT classification was (a) Bacterial NADPH-dependent GOGAT (NADPH-GOGAT), formed by two dissimilar enzyme subunits (α, the large subunit, and β, the small subunit, products of *gltB* and *gltD* genes, respectively). The α subunit consists of four domains, including the N-terminal amidotransferase domain, the FMN-binding domain and the C-terminal domain. The β subunit contains the NADPH binding site. (b) Ferredoxin-dependent GOGAT (Fd-GOGAT), from photosynthetic cells, consists of a single polypeptide chain similar to the α subunit of bacterial NADPH-GOGAT. (c) NADH-dependent GOGAT (NADH-GOGAT), from yeast, fungi and lower animals, consists of a single polypeptide chain with an N-terminal region similar to the bacterial alpha

subunit, linked to a C-terminal region similar to the bacterial beta subunit. Many different types of GOGAT are found in archaeal organisms.

In archaea there are different types of GOGAT that do not belong to any of the enzymes mentioned above. In *Archaeoglobus fulgidus, Methanococcus jannaschii,* and *Methanobacterium thermoautotrophicum* there are truncated *gltB* genes; in the archaeon *Pyrococcus sp*. KOD1, a homolog of the bacterial small subunit (*gltD* gene product) has been shown to function alone, and the genomes of other *Pyrococcus* strains contain only *gltD* genes (Nesbo et al. 2001). The enzyme from *Hfx. mediterranei* is ferredoxin-dependent as the enzyme from plant and cyanobacteria, and it is similar to the α-type subunit found in bacterial NADPH-GOGAT. The activity was only observed with one of the two different 2Fe-2S ferredoxins chromatographically isolated from *Hfx. mediterranei*. The enzyme also displayed typical halophilic behaviour, being fully stable, and producing maximal activity, at salt concentrations from 3 to 4 M NaCl, pH 7.5 and a temperature of 50 °C (Pire et al. 2014). Similar genes can be found in the available genomes of other halophilic archaea.

The only previous Fd-GOGAT characterised from a non phototrophic organism is that from *Hydrogenobacter thermophilus,* a hydrogen-oxidizing chemoautotrophic bacterium (Kameya et al. 2007). Among known Fd-GOGATs, there is a conserved insert region that has been designated the Fd loop, this insertion is not present in NADPH-GOGATs and is presumed to be involved in the interaction with ferredoxin, but Fd-GOGAT from *H. thermophilus* and from *Hfx. mediterranei* does not have such a conserved insertion (Kameya et al. 2007; Pire et al. 2014), suggesting that the insertion may be dispensable for the interaction with ferredoxin in these Fd-GOGATs. The enzyme from *H. thermophilus* is activated by some organic acids that are metabolized in the TCA cycle as succinate, oxaloacetate, malate, or citrate.

2.5.3 Glutamate Dehydrogenase (GDH)

Glutamate dehydrogenase, that converts 2-oxoglutarate into L-glutamate, and the inverse reaction, may be considered an important connection of both, carbon and nitrogen metabolism, of which these metabolites are key components. Regulating the concentration of glutamate and 2-oxoglutarate, cell may control the adequate function of the pathways responsible for nitrogen and carbon incorporation. The assimilation of nitrogen from ammonium by *Hfx. mediterranei* was reported to be mediated by glutamate dehydrogenase (GDH) (Ferrer et al. 1996). The demonstrated presence in this organism of the other via that determines the concentrations of glutamate and 2-oxoglutarate, the GS-GOGAT pathway, requires the study of the conditions that determine which via is preferred, or the contribution of each of them to assure the correct function of the metabolic pathways working at the same time at the particular conditions cell has to endure. The availability of nitrogen has been demonstrated to be determinant in bacteria. The via mediated by glutamate dehydrogenase, that directly converts 2-oxoglutarate and ammonium into L-glutamate, mainly functions when ammonium is available at enough high concentrations, meanwhile, under ammonium starvation is the GS/GOGAT the main responsible of the production of

L-glutamate. The higher affinity of GS for ammonium, accordingly to the low K_m values this enzyme displays for ammonium, makes this route the preferred when ammonium is not readily available in the required concentrations. However, exceptions may be found, and in some bacteria, GDH has been reported to be highly active under low ammonium concentration (Hochman et al. 1988).

GDHs of different sources display high coenzyme specificity. This feature determines its classification in three groups: NAD-dependent glutamate dehydrogenases, NADP-specific glutamate dehydrogenases, and those that are not NAD or NADP-specific, and so may function with both coenzymes. The halophilic archaeon *Hfx. mediterranei* has been reported to have at least two different GDHs: NADP-GDH and NAD-GDH, both of them isolated and fully characterized (Ferrer et al. 1996; Díaz et al. 2006). The NADP-dependent GDH from *Hfx. mediterranei* was probed to be a hexameric enzyme of 320 kDa, composed of six monomers of approximately 55 kDa. For the amination reaction at pH 8.5, the K_m values for its substrates were 0.18 mM for the coenzyme $NADP^+$, 0.34 mM for 2-oxoglutarate and 4.2 mM for ammonium.

The regulation of the activity of glutamate dehydrogenases from different sources by a variety of metabolites is another feature commonly reported. The effect of the metabolites on the activity depends on the particular glutamate dehydrogenase tested. For example, compounds such as GTP, ATP, ADP and AMP that were known to be allosteric modifiers of GDHs from mammals, displayed no effect on the activity of the NAD-GDH from *Hbt. halobium* (Bonete et al. 1996) and so indicated that they had no role in the regulation of this enzyme. However, this purified halophilic enzyme was highly affected by TCA intermediates, such as fumarate, oxalacetate, succinate and malate, that caused strong inhibition on both amination and deamination reactions, pointing out its close relation to this important metabolic pathway.

Initial rate studies carried out on this halophilic enzyme for the oxidative deamination reaction, also showed the regulatory effect of a variety of metabolites such as $NADP^+$, D-glutamate and glutarate, analogues of the reaction substrates, as well as dicarboxylic compounds such as adipate, besides the previously mentioned effect of TCA metabolites. On the other hand, amino acids were activators of the enzyme, except D-glutamate, that was competitive inhibitor, due to its similarity with the substrate, the L-isomer glutamate.

2.6 Ferredoxin as Physiological Electron Donors in Nitrate/Nitrite Assimilation and in GS/GOGAT Cycle

Ferredoxins are small proteins containing iron and sulphur atoms organized as iron-sulphur clusters. These metallic clusters can accept or release electrons, changing the oxidation states (+2 or +3) of the iron atoms. As a consequence of these redox reactions, ferredoxin acts as electron transfer agents in biological redox reactions (Beinert 2000). The *Hfx. mediterranei* ferredoxin involved in the electron transfer

during nitrate and nitrite assimilation is a small protein (Mr ≈ 21 kDa on SDS-PAGE) (Martínez-Espinosa et al. 2003; Zafrilla et al. 2011) that shows UV-visible, nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectra similar to those described from plant and bacteria [2Fe-2S] ferredoxins. UV-visible spectropotentiometric analysis determined a midpoint redox potential for the [2Fe-2S]2+/1+ transition of around −285 mV vs. SHE that was independent of salt concentration. This ferredoxin was a highly thermostable protein; the highest salt concentration present within the media (4 M) the highest thermal stability (80 °C). Ferredoxins from other halophilic archaea such as *Halobacterium halobium* (Geiger et al. 1978), *Haloarcula marismortui* (Frolow et al. 1996), and *Haloarcula japonica* (Sugimori et al. 2000) have been studied and in all cases they contain a cluster constituted by 2Fe and 2S.

3 Regulation of Enzymes Involved in Nitrate/Nitrite and Ammonium Assimilation

3.1 Regulation of Nitrate and Nitrite Reductases

Related to molecular biology analysis of the nitrate assimilation genes in the halophilic archaeon *Hfx. mediterranei*, Lledó et al. 2005 carried out the first studies about its identification and transcriptional analysis. This study presented the sequence determination and analysis of a segment of the *Hfx. mediterranei* genome. The bioinformatic analysis of the sequence revealed the presence of four open reading frames (ORF): *nasA* (2144 bp), *nasB* (1509 bp), *nasC* (633 bp) and *nasD* (1761 bp). These genes showed high similarity with their counterpart in bacteria and eukaryotes. The previous ORFs correspond to:

- *nasA*: protein with 707 amino acid residues identified as assimilatory nitrate reductase. This sequence also presents the typical residues involved in Fe-S clusters and Mo-MGD (Campbell and Kinghorn 1990). Based on the protein sequence analysis the halophilic nitrate reductase was classified into the monomeric ferredoxin Nas group.
- *nasB*: protein with 503 amino acid residues. It corresponds to nitrate transporter which belongs to the NarK family, a group of proteins not identified previously in Archaea. Specifically, the halophilic NarK matched with type I (Moir and Wood 2001), which are involved in the nitrate uptake in its assimilation pathway.
- *nasC*: protein with 211 amino acid residues wich corresponds to molybdopterin guanine dinucleotide biosynthesis protein. This data implied the presence of Mo cofactor in the halophilic nitrate reductase.
- *nasD*: protein with 587 residues identified as monomeric ferredoxin-dependent nitrite reductase. It has also identified residues involved in the Fe-S cluster formation.

The sequence of this 6720 bp segment of the *Hfx. mediterranei* genome represents a novel organization of nitrate assimilation genes. Moreover, unlike what goes on in bacteria (Lin and Stewart 1998), the genes are transcribed as two independent messengers, the first one as a polycistronic (*nasABC*) and the second one as monocistronic (*nasD*) (Lledó et al. 2005). This atypical arrangement raises different questions in relation to regulation of these two operons, because each one is under the control of different promoters. An interesting characteristic of both promoters was the presence of palindromic sequences, which could be a suitable candidates for the binding of transcriptional regulators as in other microorganisms belonging to the Archaea *Domain*.

The preliminary analysis related to the nitrate assimilation regulation was carried out by RT-PCR with the object to determine the effect of nitrogen source on the expression of *nasABC* and *nasD* (Lledó et al. 2005; Martínez-Espinosa et al. 2007). When *Hfx. mediterranei* was grown in the presence of ammonium as nitrogen source, no transcription of *nasABC* or *nasD* was detected. However, the expression of these operons was detected when *Hfx. mediterranei* was grown with nitrate (Fig. 9.2), indicating that these genes were needed for nitrate assimilation. The expression of *nasA* was higher when the optical density (OD) of the culture rose above 0.4, supporting previous studies related to Nas activity assays where the maximum Nas activity was detected at OD = 0.9. Otherwise, the expression of *nasD* was higher when *Hfx. mediterranei* was grown in the presence of nitrite than in the presence of nitrate, suggesting that nitrite could have a positive effect on *nasD*.

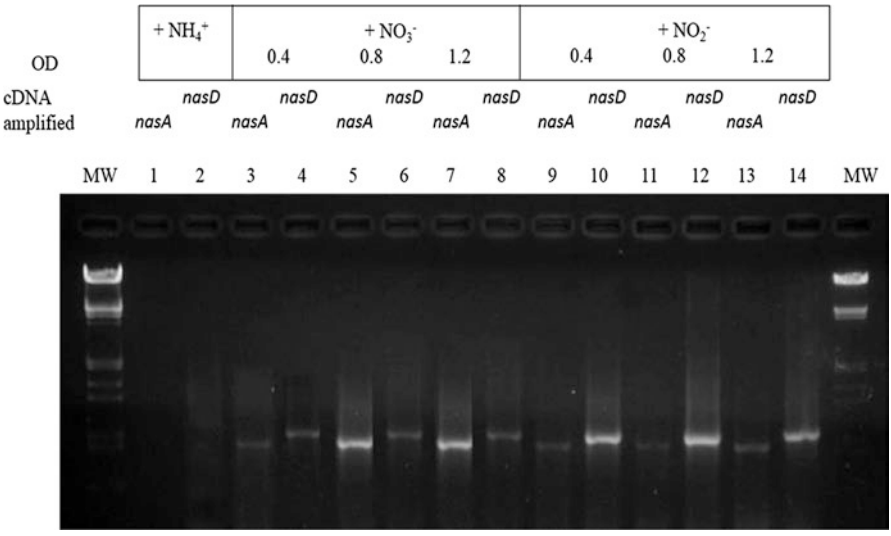

Fig. 9.2 Analysis of the expression of *nasA* and *nasD* genes by agarose gel electrophoresis of double-stranded DNA fragments generated in RT-PCR reactions. Reactions had been performed with total RNA isolated from ammonium medium (Lanes 1 and 2), nitrate (Lanes 3, 4, 5, 6, 7 and 8) or nitrite (9, 10, 11, 12, 13, and 14) supplemented medium from different OD and a pair of primers that amplify *nasA* or *nasD* cDNA. Lane MW: molecular size markers (Lledó et al. 2005)

Therefore, these results concluded that both operons, *nasABC* and *nasD,* were regulated at transcriptional level, being the ammonium the key element which repressed the transcription of nitrate and nitrite reductases genes. Moreover, nitrate and nitrite was involved in a specific control of the assimilation pathway because their presence increased the expression level of *nasABC* and *nasD.*

Martínez-Espinosa et al. (2009) carried out physiological and biochemical studies with *Hfx. mediterranei* in the presence of different concentrations of nitrate (0.5 mM–100 mM) and nitrite (0.5–2 mM). These studies revealed that in the presence of nitrate concentrations higher than 5 mM, the activity of nitrate reductase appeared in first place followed by the activity of nitrite reductase. Under these conditions, the intracellular nitrite concentrations increased shortly before nitrite reductase activity was detected, suggesting that the nitrite accumulation could act as a signal to increase the activity of this enzyme. The expression of *nasA* and *nasD* under the described conditions was analyzed by real-time quantitative PCR experiments (Fig. 9.3a and b), showing that their mRNA concentrations increased during exponential phase whereas they decreased at the beginning of the stationary phase. The maximum *nasA* and *nasD* mRNA concentrations were detected before the maximum enzyme activity in all conditions. Specifically, the espression level of *nasA* mRNA not showed significant differences in cultures with a range of 5–25 mM nitrate, whereas from 25 to 100 mM nitrate the mRNA content increased reaching the maximum expression at 100 mM nitrate (Fig. 9.3a). Moreover, the *nasA* mRNA expression decreased faster after the maximum expression in those media containing 100 mM NO_3^-. This fact allowed to propose that the product of nitrate assimilation could repress the expression of *nasA* mRNA. On the other hand, the *nasD* mRNA level showed a clear dependence on nitrate concentration (Fig. 9.3b) and, always, the maxima *nasD* expressions were detected after the maximum Nas activity. Therefore, the accumulation of nitrite inside the cells could act as inductor of *nasD* expression.

In relation to the experiments carried out when *Hfx. mediterranei* was grown in the presence of nitrite as sole nitrogen source, it was observed that nitrite was consumed by cells under all concentrations assayed and stationary phase was reached at a relatively low optical density (Martínez-Espinosa et al. 2009). Surprisingly, *nasA* mRNA was detected in the cultures with nitrite as nitrogen source independently of its concentration. The concentration of *nasA* mRNA under this conditions was lower than in presence of nitrate, therefore the nitrate has a positive effect on the expression of this gene. This basal *nasA* expression, in media with a different nitrogen source of nitrate, is not frequent as it has described by Chai and Stewart (1998). Consequently, this data suggests that other kind of regulation could be working in this halophilic microorganism. Regarding *nasD,* its mRNA expression was logically detected in the cultures with nitrite as nitrogen source. The Fig. 9.4 shows like its expression depends on nitrite concentration as well as on the optical density of the culture.

More recently, a transcriptome analysis of the operon *nasABC* and *nasD* has been carried out in the presence of inorganic nitrogen salt (nitrate and ammonium) as well as in the presence of four amino acids (glutamate, glutamine, aspartate, asparagine) as the sole nitrogen source (Esclapez et al. 2014). Dot-blot analysis of

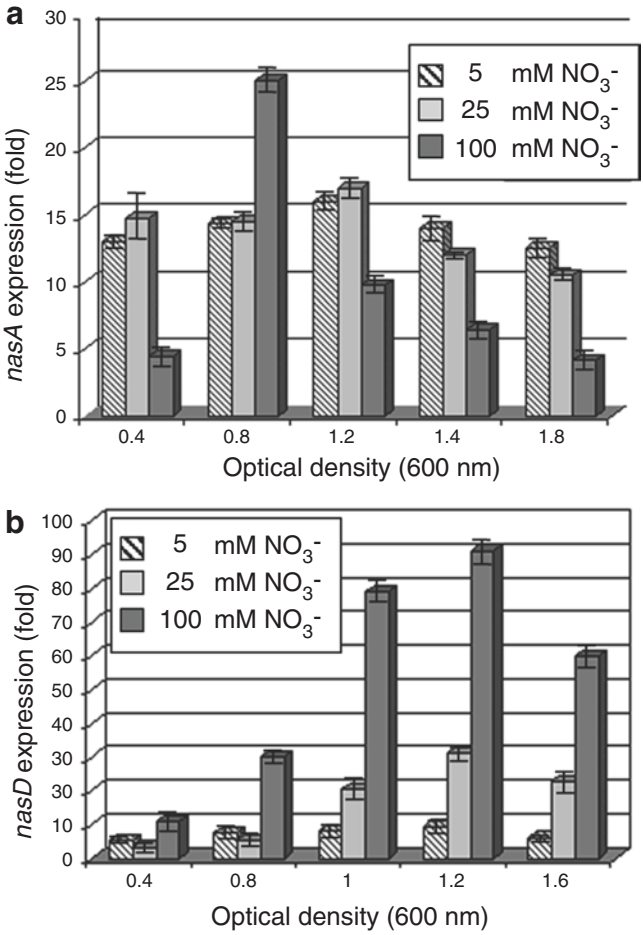

Fig. 9.3 *nasA* and *nasD* mRNA expression levels under different growth conditions during different optical density OD. Comparisons between results obtained from cultures grown in 5 mM NO_3^- (*hatched bar*), 25 mM NO_3^- (*light shaded bar*) and 100 mM NO_3^- (*dark shaded bar*) as sole nitrogen source. Data plotted are the average of the results obtained from quadruplicate experiments. Bars represent the standard deviations (Martínez-Espinosa et al. 2009)

mRNA isolated from different cultures showed hybridization with specific probes in the samples corresponding to the exponential growth phase, obtaining the stronger signals when the higher nitrate concentration was (Fig. 9.5a). Northern analysis showed hybridization with *nasA* and *nasD* probes in media with nitrate as nitrogen source, but any hybridization was observed when the samples came from complex medium (Fig. 9.5b). Therefore, nitrate and nitrite reductases are expressed in the presence of nitrate in the exponential growth phase. These data were confirmed by RT-PCR experiments, which determined that the *nasA* and *nasD* expression was 10-fold and 15-fold higher in the nitrate culture compared to the ammonium culture. These data are in agreement with other previous studies (McCarty and Bremner 1992;

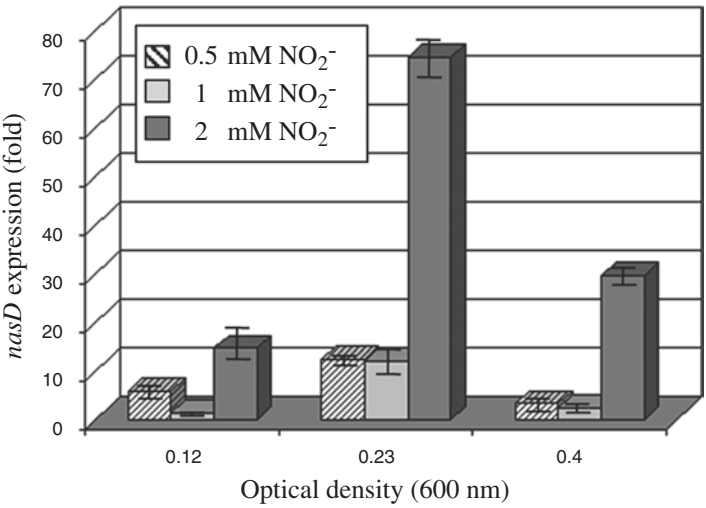

Fig. 9.4 *nasD* mRNA expression level under different growth conditions during different optical density OD. Comparisons between results obtained from cultures grown in 0.5 mM NO_2^- (*hatched bar*), 1 mM NO_2^- (*light shaded bar*) and 2 mM NO_2^- (*dark shaded bar*) as sole nitrogen source. Data plotted are the average of the results obtained from quadruplicate experiments. Bars represent the standard deviations (Martínez-Espinosa et al. 2009)

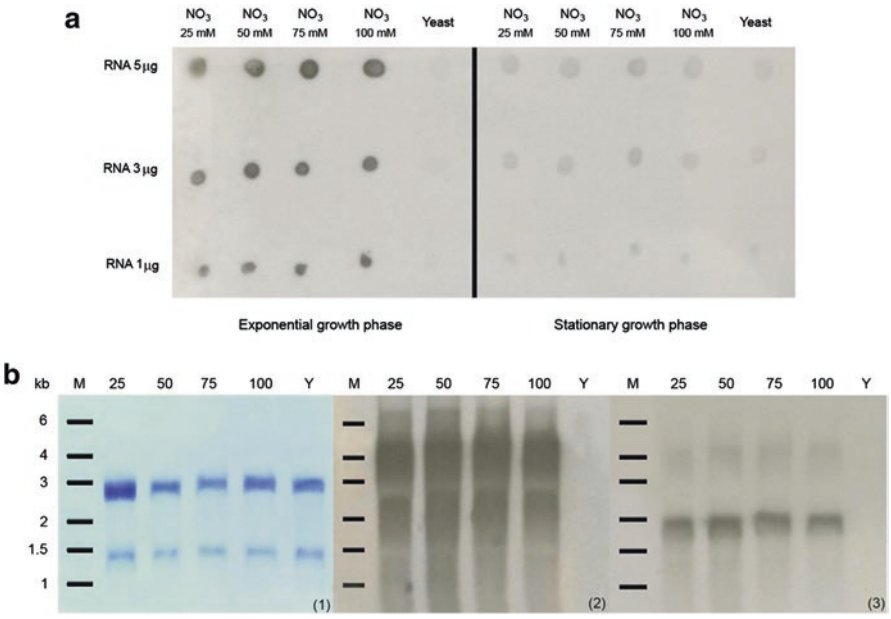

Fig. 9.5 (**a**) Dot-blot using *nasA*-specific probe. The samples of mRNA were obtained in exponential and stationary phase from cultures with different nitrate concentration. (**b**) Northern blot of mRNA obtained in exponential growth phase from cultures with nitrate in a range of 25–100 mM: (1) Methylene blue-stained membrane indicates the 23S and 16S rRNA genes and was used as control for the quality and quantity of total RNA, (2) Northern analysis using *nasA* probe, (3) Northern analysis using *nasD* probe (Esclapez et al. 2014)

Lledó et al. 2005; Bonete et al. 2008; Martínez-Espinosa et al. 2009), but the effect of organic nitrogen source have not been studied in halophilic microorganisms.

To analyse the effect of organic nitrogen sources on *nasABC* and *nasD* transcription, dot-blot and Northern blot analysis were carried out in cultures with glutamate, glutamine, aspartate or asparagine as sole nitrogen source. The mRNA from these cultures was isolated in the exponential phase, because of that is the phase in which nitrate and nitrite reductases are expressed. Dot-blot as well as Northern-blot analysis revealed hybridization with specific probes of *nasA* and *nasD* in the samples from cultures with nitrate, glutamate or aspartate as nitrogen source. However, any signal was obtained in the samples from cultures with ammonium, glutamine, asparagine or yeast extract. Hence, the presence of glutamate, aspartate or nitrate produces the expression of nitrate and nitrite reductases, obtaining the strongest signals in the presence of nitrate. Otherwise, the ammonium, glutamine or asparagine acts as repressor of the transcription of genes related to nitrate assimilation pathway. Previous studies carried out with plants postulated that glycine, glutamine and asparagine inhibit the stimulation performed by nitrate over nitrate reductase enzyme, whereas the amino acids with only one amine group, as glutamate and aspartate, induce its expression (Radin 1977). Later, it has also described the inhibitor effect of glutamine, ammonium or analog compounds over assimilatory nitrate reductase activity in soil (McCarty and Bremner 1992). The last study related with the use of amino acids as nitrogen source suggested that is the internal amino acids pools which acts as intracellular signal of nitrogen level (Miller et al. 2008). Taking into account the data obtained with the halophilic microorganism, *Hfx.mediterranei*, it could postulated that the nitrogen sources which inhibit the expression of nitrate and nitrite reductases are those involved in the synthesis of amino acids coming from ammonium, as glutamine and asparagine.

The last studies related to the expression of *nasABC* and *nasD* genes were performed by means of microarray technology in three culture media with different nitrogen sources: (a) cells grown stationary (AmSt) and exponentially (AmEx) in ammonium, (b) cells grown exponentially in nitrate (NiEx), and (c) cells shifted to nitrogen starvation (NSta) conditions (Esclapez et al. 2015). The transcriptional profiles showed significant differences between the cultures with ammonium as nitrogen source and those with nitrate or nitrogen starvation. These results are in agreement with previous analysis carried out with different methodologies (Lledó et al. 2005; Martínez-Espinosa et al. 2009; Esclapez et al. 2014). Therefore, it could be concluded that the absence of ammonium is the real cause for the expression of assimilative nitrate and nitrite reductases. Furthermore, the absence of ammonium not only modify the expression of genes involved in nitrate assimilation pathway but also change the transcription level of proteins involved in nitrogen metabolism, genes encoding transport proteins, enzymes involved in protein biosynthesis, transcriptional regulators and other proteins. Related to the genes involved in nitrogen assimilation, *nasA, nasB, nasC* and *nasD* genes are over-expressed under nitrogen starvation and in cultures with nitrate as sole nitrogen source in relation to ammonium in exponential growth phase (Table 9.1). Therefore, it may be concluded that nitrate is not a real inductor since *nas* gene expression with nitrate (like under nitrogen starvation) is a consequence of the absence of ammonium rather than an induction by nitrate (Martínez-Espinosa et al. 2007; Esclapez et al. 2014, 2015).

Table 9.1 Expression level of *nasABC* and *nasD* genes in NiEx-AmEx and NSta-AmEx contrasts

Annotation	Gene name	NSta-AmEx		NiEx-AmEx		NCBI accession number
		Log_2FC	SD	Log_2FC	SD	
Ferredoxin-nitrite reductase	*nasD*	5.54	0.03	4.12	0.02	AHZ23086.1
Ferredoxin-nitrate reductase	*nasA*	5.46	0.50	4.70	0.50	AHZ23083.1
Nitrate/nitrite transporter	*nasB*	4.22	0.07	3.60	0.11	AHZ23084.1
Molybdopterin guanine biosynthesis protein A	*nasC*	4.12	0.23	2.50	0.30	AHZ23085.1

3.2 *Regulation of Ammonium Assimilation: Glutamate Dehydrogenases, Glutamine Synthetase and Glutamate Synthase*

There are two alternative routes to assimilate ammonium which allow the great variety of living organisms cope with changing environments where they have to survive. So, when ammonium is readily available, is glutamate dehydrogenase (GDH), and the route it represents, the preferred to ensure the appropriate uptake of ammonium, meanwhile, when ammonium is not available, or its concentration is not enough high, the GS-GOGAT pathway is the working way to incorporate nitrogen into the cell.

Meanwhile the amination reaction catalyzed by GDH provides the nitrogen, as amino-group, that will be used in many biosynthetic pathways, the oxidative deamination reaction of GDH, on the other hand, converting L-glutamate in 2-oxoglutarate, will provide carbon to the tricarboxylic acid cycle (TCA), producing the adequate concentrations of L-glutamate to balance the glutamine to glutamate ratio and also regulating the necessary levels or concentrations of 2-oxoglutarate, pivotal and essential metabolite, since its belonging to the TCA, as well as its participation in the transamination reactions. These two different roles, let us name them catabolic and anabolic role, respectively, are commonly carried out by GDHs activities depending of NADH or NADPH as cofactors, that also differ in their properties and regulation (Bonete et al. 1986, 1987; Ferrer et al. 1996; Ingoldsby et al. 2005; Díaz et al. 2006; Tomita et al. 2010). As stated before, amino acids display an activator effect on the NAD-GDH from *Hbt salinarum*, meanwhile TCA intermediates and other metabolites inhibit its activity (Bonete et al. 1996; Pérez-Pomares et al. 1999)

Although it is reasonable that the presence of these two activities may be related to the essential roles previously discussed, in the metabolism of organisms (Smith et al. 1975; Santero et al. 2012), including extremophiles such as halophilic Archaea, the exact role of the different GDH proteins still need to be further investigated, studies that becomes even more interesting when, not only two, but, at least four putative *gdh* genes have been found in *Hbt salinarum* genome (Ingoldsby et al. 2005).

Recently Tomita et al. (2010) has shown that glutamate dehydrogenase from *Thermus thermophilus* displays interesting features regarding enzyme regulation. Studies on the genome of this extreme thermophile bacteria shows two genes,

putatively forming an operon, *gdhA* and *gdhB*, which correspond to two different glutamate dehydrogenases. The products of these two genes were purified and characterized by Tomita et al. (2010) in order to assign them its real function. One of the products, GdhA, showed no GDH activity and the other, GdhB, displayed GDH activity, being 1.3 fold higher the rate for the reductive amination than that found for the oxidative deamination. The co- expression and purification of GdhA with His-tag-fused GdhB also displayed glutamate dehydrogenase activity, let us name it GdhA–GdhB. The GdhA–GdhB activity for the reductive amination was lower than that for GdhB alone, meanwhile for the oxidative deamination was higher. GdhA–GdhB had decreased reductive amination activity and increased oxidative deamination activity, in a ratio of 3.1-fold oxidative deamination versus reductive amination.

Just as reported for the halophilic archaeon *Hbt salinarum* NAD-glutamate dehydrogenase (Bonete et al. 1996), and previously discussed, amino acids had an activating effect on the hetero-complex GdhA–GdhB, and also it was higher for hydrophobic amino acids. The most effective activating amino acid for GdhA–GdhB was leucine, which, at 1 mM leaded to an activity 9.74 fold higher than that without leucine for the reductive amination, and 2.45 fold for the oxidative deamination. The activating effect of leucine was much lower for GdhB alone. The kinetic analysis of this activating effect showed that leucine enhanced the turnover number of glutamate dehydrogenase. This product acted as a hetero-oligomeric GDH system, where the role for GdhA subunit was regulatory, and GdhB subunit was catalytic. In this hetero-complex, GdhA acts modulating the activity of GdhB through the formation of the hetero-complex that depends on the concentration of the activator hydrophobic amino acid. This study of Tomita et al. illustrated the allosteric regulation of a hetero-oligomeric glutamate dehydrogenase (Tomita et al. 2011).

The regulation of glutamate synthase is not well known and there are different approaches depending on the organism. In plants there are two forms of GOGAT enzyme: Fd-GOGAT, which is usually present in high activities in the chloroplasts of photosynthetic tissues, and NADH-dependent enzyme, which is also present in plastids of non-photosynthesizing cells (Bowsher et al. 2007). Both enzymes supply glutamate during the nitrogen assimilation, and glutamate dehydrogenase does not represent a significant alternate route for glutamate formation. GDH may be expected to function mainly in the deaminating direction providing 2-oxoglutarate and ammonium that can be used in respiration and amide formation respectively (Miflin 2002; Forde and Lea 2007) and also would act with NADH-GOGAT controlling the homeostasis of Glu in the plant (Labboun et al. 2009).

In *Escherichia coli*, the GS-GOGAT pathway is used when the cell is not under energy limitation. At low ammonium concentration is the main way for glutamate synthesis and it is essential for regulation of the glutamine pool. In ammonium and phosphate abundance and when the cell is limited for energy and carbon, the GDH pathway is used in glutamate synthesis (Helling 1994).

There are a number of transcriptional regulators that control the GOGAT synthesis in bacteria. The *gltB* and *gltD* genes code for the large and small subunits, respectively, of the GOGAT. In *E. coli* expression of *gltBD* is influenced by regulators including the leucine-responsive regulatory protein, LRP and CRP (Reitzer 2003).

Synechocystis sp. strain PCC 6803 utilizes the GS-GOGAT pathway as the primary pathway of ammonia assimilation, but the presence of GDH appears to offer a selective advantage for the cyanobacterium under no exponential growth conditions (Chávez et al. 1999). In *Corynebacterium glutamicum*, transcription of the operon formed by the genes *gltB* and *gltD* is under control of AmtR, a repressor of nitrogen-regulated genes in this organism (Beckers et al. 2001), which regulates the transcription of other nitrogen genes including *amtB* and *glnK*. Schulz et al. (2001) found that disruption of the *hkm* gene, encoding a putative histidine kinase upstream of *gltBD* in *Corynebacterium glutamicum*, reduced the levels of GOGAT activity two-fold under nitrogen-rich and nitrogen-limiting conditions. Transcription of *hkm* was moderately induced by nitrogen starvation, indicating that the Hkm protein may play a role in signal transduction of the nutritional status of the growth medium (Schulz et al. 2001).

In *B. subtilis,* the two enzymes responsible for the unique pathway of ammonium assimilation are glutamine synthetase and glutamate synthase since GDH is absent in this organism. The *gltAB* operon requires a specific positive regulator, GltC, for its expression and is repressed by TnrA, a regulator of several other genes of nitrogen metabolism (Belitsky et al. 2000).

In haloarchaea, studies at transcriptional level have been done to elucidate the role of the different enzymes implied in the different pathways. Figure 9.6a shows the comparison of the expression profiles of *gdh-1*, *glnA* and *gltS*, tested at exponential phase, from different media (each containing ammonium, nitrate, glutamate or glutamine as nitrogen source). The results revealed that *gltS* expression was 70-fold higher in the nitrate culture compared with the ammonium culture. The presence of nitrate also enhanced the expression of *glnA*, but repressed the expression of *gdh-1*. This is in agreement with the hypothesis that the GS/GOGAT pathway operates under conditions of ammonium restriction, whereas ammonium assimilation by GDH would occur preferentially at high ammonium concentrations, although under these conditions the levels of transcription of *gdh-1, gltS* and *glnA* were very similar (Fig. 9.6b). In medium containing glutamate as the only nitrogen source, *gdh-1, glnA* and *gltS* expression was lower than that seen in the ammonium medium. However, in the medium with glutamine, *glnA* expression was repressed, whereas the expression of *gltS* and *gdh-1* was enhanced, when compared with levels in media containing ammonium. This profile suggests that high levels of glutamate in the cell can be a signal to repress ammonium assimilation, but high levels of glutamine are a signal of ammonium abundance; indeed, expression of both *gltS* and *gdh-1* was higher than in media containing ammonium. The RT-qPCR carried out by Pire et al. (2014) strongly suggests that the GS/ GOGAT pathway could be the preferred route for ammonium assimilation under conditions of ammonium starvation or deficiency. In the medium with ammonium as nitrogen source, *gdhA-1* and *gdh1* were transcribed at similar level, with a relative change of 1.1 ± 0.3 for the expression of *gdhA-1* compared with the expression of *gdh1*. Both enzymes work in the amination reaction (Ferrer et al. 1996; Díaz et al. 2006), but whereas the gene expression of *gdhA-1* did not change when the nitrogen source was nitrate instead of ammonium, the expression of gdh1 was highly repressed. Although the expression

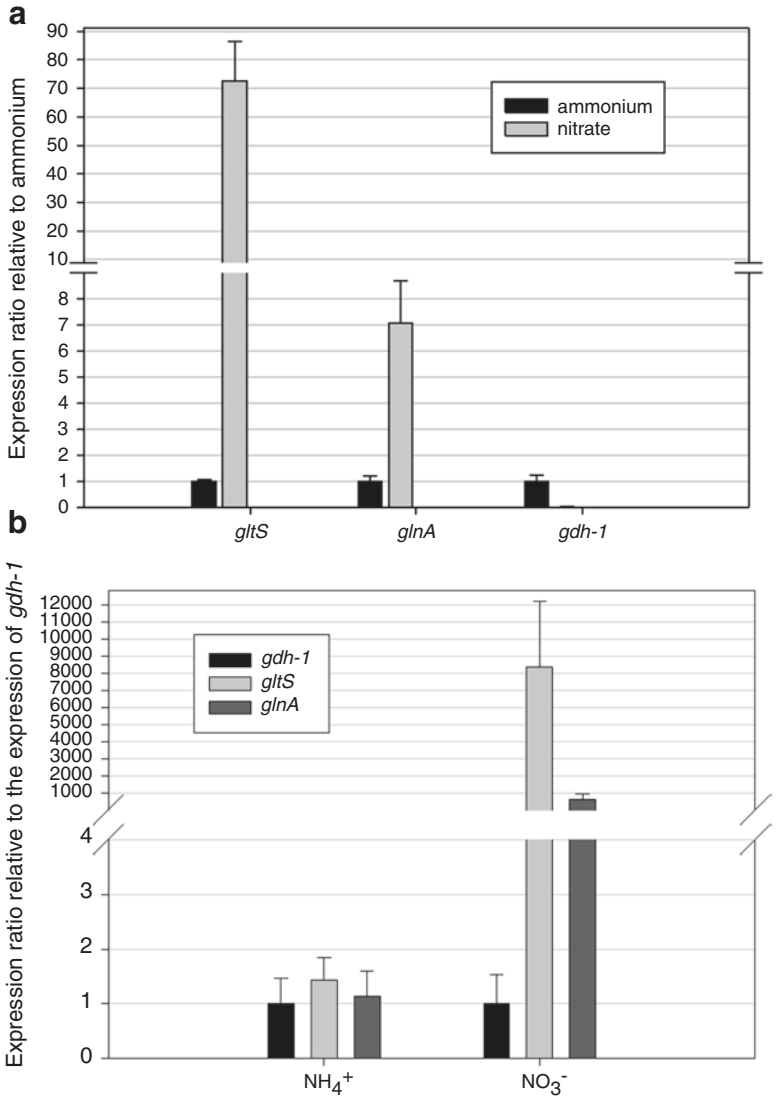

Fig. 9.6 Transcription level of *gltS*, *glnA*, and *gdh-1* genes from cultures at exponential phase with ammonium and nitrate as a nitrogen source. (**a**) The results from ammonium culture were used for calibrating. (**b**) The results for *gdh-1* expression were used for calibrating (Pire et al. 2014)

of *gdhA-1* seems to be constitutive, previous studies of the enzyme showed that the specific activity of NADPH-GDH was higher in ammonium than in nitrate, suggesting an inhibition of the enzyme activity (Ferrer et al. 1996).

Not only the transcription of *gdh-1* was highly repressed in a media with nitrate, but also the transcription of *gltS* and *glnA* was enhanced in the same media.

The GDH reaction would assume ammonium uptake when this is available in the medium, since ammonium is the preferred nitrogen source over nitrate or other nitrogen- containing compounds (Bonete et al. 2008). However, the Northern blot analysis, as well as the RT-qPCR results, showed a basal level of Fd-GOGAT transcription even when ammonium is available.

As shown in Fig. 9.6b, the transcription level of *gltS* in media containing ammonium is similar to that of *gdh-1*. Under these conditions, GDH activity predominates due to its higher affinity for 2-oxoglutarate and ammonium. These results would indicate that the level of 2-oxoglutarate is essential. It has been proposed that the "in vivo" rate of GOGAT reaction is controlled by the production of glutamine by GS, but whereas GS activity is determined by the cellular levels of glutamine and 2-oxoglutarate, GS would also be modulated by GOGAT through its influence on the 2-oxoglutarate concentration (Vanoni and Curti 1999). In our case, according to the K_m values, 2-oxoglutarate would be a key metabolite in determining whether assimilation takes place via the GS/GOGAT route (Pire et al. 2014).

All these results indicated that in *Hfx. mediterranei* the ammonia produced by the nitrate and nitrite reductase reactions is incorporated into the carbon skeletons mainly by the GS/GOGAT cycle. The global gene expression in *Hfx. mediterranei* in three culture media with different nitrogen sources (ammonium, nitrate, and in cells shifted to nitrogen starvation), has been analyzed (Esclapez et al. 2015). The main differences in the transcriptional profiles have been identified between the cultures with ammonium as nitrogen source and the cultures with nitrate or nitrogen starvation. Some transcriptional regulators have been identified, two ArsR type and other putative transcriptional regulators. Analysis of these transcriptional regulators, would allow identifying nitrogen regulators involved in the ammonium deficiency adaptation in haloarchaea.

4 Nitrogen Regulatory Proteins (PII) in Haloarchaea

In 1969, the PII signal transduction proteins were discovered when posttranslational modification studies were carried out with *Escherichia coli* glutamine synthetase (Shapiro 1969). Currently it is known that PII proteins are involved in regulatory processes involved in the assimilation of nitrogen (Ninfa and Jiang 2005; Leigh and Dodsworth 2007; Forchhammer 2008). Apparently, they can act as devices that collect the level of 2-oxoglutarate and adenylylate energy charge (Forchhammer 2010). Proteins regulated by PII are: AmtB ammonium transporter, which is able to retain GlnK to complex with it when the cellular nitrogen level is sufficiently high (Javelle et al. 2004); glutamine synthetase adenylyltransferase (GlnE), which modificates covalently the activity of glutamine synthetase (Jiang et al. 2007); DraG/DraT, which regulates the nitrogenase enzyme in nitrogen fixing bacteria (Dixon and Kahn 2004) and NAGK, important enzyme involved in arginine biosynthesis in cyanobacteria (Heinrich et al. 2004), among others.

Previous articles have been shown with the GlnB-K protein family in Archaea have been carried out, specifically with *Methanosarcina mazei* (Ehlers et al. 2002, 2005)

and *Archaeoglobus fulgidus* (Helfmann et al. 2010; Litz et al. 2011). With halophilic Archaea, the study of these proteins began in 2011, when the existence of two homologues PII gene copies in the genome of *Hfx. mediterranei* (Pedro-Roig et al. 2011) was observed, with 84 % of identical amino acids, classified as GlnKs ($GlnK_1$ and $GlnK_2$), and both linked to genes encoding for ammonium transporters (*amt*). The two pairs of genes are located consecutively in the genome, but separated by a 300 nt gap of non-coding sequence. The C-terminal region is highly conserved in both GlnKs (PS00638, PROSITE), but only a fragment of the uridylylation site of PII proteins is conserved (PS00496, PROSITE). The amino acid sequence WRGEEY is also present in both GlnK proteins of *Hfx. mediterranei*, with two changes with regard to other proteins non halophilic, the first residue, W, is changed to Y, and the fourth residue, E, is changed to A or S. In the latter case, the substitution may be related to the halophilic adaptation. There is a tyrosine residue which can participate in the uridylylation, like in other PII proteins, at position 60 for $GlnK_1$ and 61 for $GlnK_2$. Studies carried out by the technique of cross-linking using glutaraldehyde concluded that GlnKs proteins have a trimeric quaternary structure (45 kDa) (Pedro-Roig et al. 2013b). The crystal structure has also been determined to $GlnK_2$ protein, being the first structural study of haloarchaea PII protein, and confirming the high degree of structural conservation in all PII proteins of all organisms in which they are located (Palanca et al. 2014).

4.1 GlnK Proteins in Different Nitrogen Sources and Their Role in the Regulation of Nitrogen Metabolism in Hfx. mediterranei

Hfx. mediterranei was grown in several different conditions: with yeast extract as a source of organic nitrogen, or either ammonium or nitrate as inorganic nitrogen sources (glucose was added in excess as a carbon source for these synthetic media). In the presence of nitrate both GlnKs were observed, while that in the presence of ammonium or yeast no signal could be detected (Pedro-Roig et al. 2011). With GS, the same thing happens (Martínez-Espinosa et al. 2006), so it can deduce that there is a relationship between PII and GS, activating the last one. Growth of *Hfx. mediterranei* in 75 mM nitrate, in exponential phase neither GS activity nor GlnK intense signal were observed. While at the same concentration of nitrate in stationary phase, high activity of GS and GlnK intense signal (Fig. 9.7b, lane 9) were observed. Therefore, it has been suggested an activating function for GlnK in ammonium assimilation in *Hfx. mediterranei*, through the GS/GOGAT via (Pedro-Roig et al. 2011). This fact was proved *in vitro.* Biosynthetic activity measurements were realized in the presence and absence of GlnK, and an increase of GS enzymatic activity up to 50 % as a result of purified recombinant GlnK presence in the assays was obtained. Moreover, in SDS-PAGE analysis from cells grown in nitrate, a sole band was seen, corresponding to $GlnK_1$, present under these conditions, so you could think they have different electrophoretic mobility (Pedro-Roig et al. 2013b).

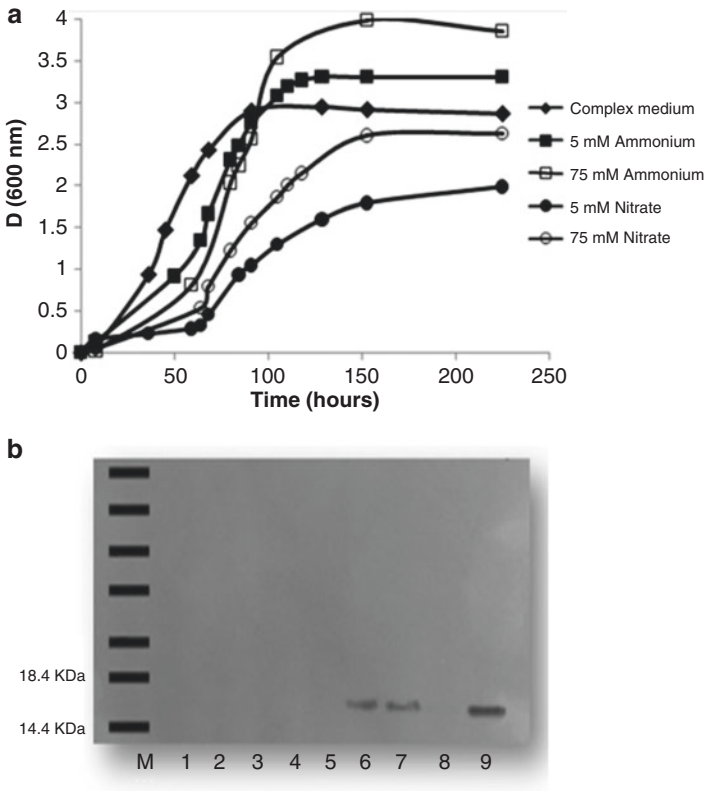

Fig. 9.7 *Hfx. mediterranei* growth curves with different nitrogen sources (**a**) and analysis of GlnK expression by SDS-PAGE and Western blot (**b**): Lane M, molecular-mass markers; lane 1, complex medium, exponential phase; lane 2, complex medium, stationary phase; lane 3, 5 mM ammonium, exponential phase; lane 4, 5 mM ammonium, stationary phase; lane 5, 75 mM ammonium, exponential phase; lane 6, 5 mM nitrate, exponential phase; lane 7, 5 mM nitrate, stationary phase; lane 8, 75 mM nitrate, exponential phase; lane 9, 75 mM nitrate, stationary phase (Pedro-Roig et al. 2011)

Biosynthetic activity of GS was increased in presence of 10 mM 2-oxoglutarate and in presence and absence of GlnK, showing an increase of 12-fold with the metabolite alone, and 18-fold in the presence of PII protein. 2-oxoglutarate is a metabolite that promotes assimilation of ammonium by GS when there is nitrogen deficiency. GS activity increases in the presence of GlnK because a complex between both two proteins is formed, as it has been demonstrated by gel filtration chromatography followed by SDS-PAGE and immunoblotting with specific antibodies for the regulatory protein (data not shown) (Pedro-Roig et al. 2013b). The presence of 2-oxoglutarate is important to form GS-GlnK while the GS activity must be increased, and ammonium assimilation should be produced by the route GS/GOGAT when the cell has low nitrogen. In the GlnK–GS complex from *Hfx. mediterranei*, the stoichiometry remains unclear, but one dodecamer of GS to four trimers of PII is calculated.

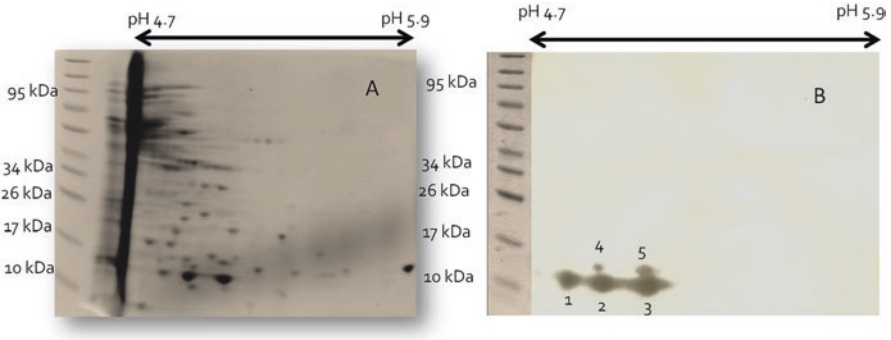

Fig. 9.8 (**a**) 2DE of 500 g of total cell protein isolated from 20-h nitrogen starved cells using an IPG strip pH 4.7–5.9 and an *Any kD TGX* gel, both from Bio-Rad. The gel was stained with Coomassie Blue. (**b**) Anti-GlnK immunoblot of a 50 g replicate of the 2DE in (**a**). Specific *Hfx. mediterranei* anti-GlnK antibodies were used, and ECL detection was performed using HRP-conjugated secondary antibodies and luminol as substrate (Pedro-Roig et al. 2013a)

4.2 Post-Translational Modification of PII Homologues from Haloferax mediterranei

To know if PII proteins of haloarchaeal species were post-translationally modified it has been used two-dimensional electrophoresis (2DE) and with specific detection using antibodies for PII, applying the immunoblot technique, and subsequent analysis of MALDI-TOF-MS. With this type of study should determine PTM for these proteins as they undergo a small displacement in the isoelectric point. The theoretical pI for $GlnK_1$ protein from *Hfx. mediterranei* is 5.15 and for $GlnK_2$ is 5.11. Specific antibodies were used for GlnK from *Hfx. mediterranei*, using as key the T-loop area of both proteins, and applying immunoblotting technique, five different signals which coincided with the pI values and expected size were obtained (Fig. 9.8b) (Pedro-Roig et al. 2013a). Spots 1, 2 and 3 (Fig. 9.8b) belong to $GlnK_1$, and spots 4 and 5 belong to $GlnK_2$. The high primary structure identity (84 %) between the two GlnK from *Hfx. mediterranei* could mask differentiation between both proteins with this technique; however, through the mass spectra, which analyzed peptides with different masses for each protein, allowed specific identification.

Previously, it has been proposed that there was no presence of $GlnK_2$ in *Hfx. mediterranei* grown with nitrate (Pedro-Roig et al. 2013b). When the cell is nitrogen starved, with the immunoblotting technique the second GlnK (Pedro-Roig et al. 2013a) also appears. This is important physiologically, since when the cell is in the presence of a nitrogen source, only is necessary one of the two GlnKs to metabolic regulation, but in total absence of nitrogen, both GlnKs are present. This indicates that GlnKs are activators of nitrogen assimilation in shortage of this component, which coincides with that both of them activate the GS *in vitro* (Pedro-Roig et al. 2013b).

In the spectra from spots 2 and 4 in Fig. 9.8b an increase in mass of 306 Da was observed, with respect to the theoretical mass of the peptide, which did not occur in spots 3 and 5. This mass matches with an UMP group (monoisotopic mass of 306.03), so it could be concluded that in the spots 2 and 4, the $GlnK_1$ and $GlnK_2$ proteins are uridylylated, respectively, while in the spots 3 and 5, the proteins are not modified. It could be said that the modification occurs at the conserved tyrosine residue (Y60 for $GlnK_1$, Y61 for $GlnK_2$), since, as previously mentioned, only a fragment of the uridylylation site of the PII protein is conserved (PS00496, PROSITE). This could be an important conclusion: PII proteins from the Archaea Domain undergo post-translational modifications. However, in the *Hfx. mediterranei* genome, a halophilic homologue of the uridylyltransferase/uridylyl-removing enzyme (encoded by *glnD*) has not found (Pedro-Roig et al. 2013a). Therefore, there should be some other similar halophilic Archaea protein that can perform this function.

4.3 Cotranscription of glnK and amtB Genes From Haloferax mediterranei Under Conditions of Ammonium Limitation

A transcriptional level, the two *amtB-glnK* genes pairs from *Hfx. mediterranei* have been studied (Pedro-Roig et al. 2013). When the medium was rich in ammonia or yeast extract, there was no evidence of *amtB-glnK* transcript, so that the pairs of genes are not transcribed or transcripts suffered degradation due to ammonia excess. Conversely, when the medium was rich in nitrate, the *amtB-glnK* transcripts of the two pairs of genes in RNA samples (data not shown) were obtained. With the Northern blot was found that *amtB-glnK* genes are cotranscribed in pairs, and the pair *amtB1-glnK1* is expressed primarily in conditions of nitrate in the medium, rather than *amtB2-glnK2*. Furthermore, transcription levels suffer regulation by the availability of nitrogen in the medium, with no expression in ammonium source in the medium and maximum when the medium lacks ammonium.

5 Biotechnological Applications

Human activity has led to an increase in the concentration of salts, such as nitrates and nitrites, in soils and groundwater. Companies engaged in the manufacture of pesticides, herbicides, explosives and dyes, contribute to this problem. Nitrate and nitrite have a significant involvement in agriculture, environment and public health. Most organisms are usually affected by even low nitrate and nitrite concentrations. *Hfx. mediterranei* is the first reported haloarchaea having the ability to assimilate nitrate and nitrite, due to the presence of nitrate (Nas) and nitrite (NiR) reductases in a high salt concentration environment. Physiological characterization carried out in *Hfx. mediterranei*, has shown that this microorganism is able to grow aerobically on minimal culture with nitrate (up to 2 M concentration)

or nitrite (up to 50 mM concentration) as unique nitrogen sources (Martínez-Espinosa et al. 2009). The high nitrite concentration tested in the assay cited above is one of the highest yet described for a microorganism belonging to Prokarya. The growth of *Hfx. mediterranei* in these toxic conditions for most microorganisms studied up to now, involves removing nitrate and nitrite in the culture media. The concentrations of nitrate and nitrite in wastewater are usually lower than the concentrations studied in *Hfx. mediterranei*. So it is possible to think that this microorganism could be employed in bioremediation processes for salted wastewater or brines. In the same way, Nas and NiR enzymes could be immobilized to be used in bioremediation techniques or like sensors to detect the presence of nitrate and nitrite.

On the other hand, carotenoids have been studied because of their potential functions on nutrition and human health in the last years. Numerous studies have shown that these pigments have some physiological roles in the retardation of cancer and heart affections by quenching of singlet oxygen or free radicals, increasing the production of antibodies *in vitro*, etc. (Edge et al. 1997; Carpenter et al. 1997; Palozza et al. 1998; Chew et al. 1999). The carotenoids use in the field of nutrition as processed food coloring agents, is also studied (Bauernfeind 1981). Traditionally there have been studies with carotenoids producing organisms such as *Haematococcus pluvialis, Blaskeslea trispora* and *Dunaliella salina* (Olaizola 2000; Mehta et al. 2003; Raja et al. 2007). However there are very few studies in extreme halophilic microorganisms that are excellent candidates because of their characteristics for the production of such pigments. In *Hfx. alexandrinus* has conducted a study on the production of canthaxanthin (Asker and Ohta 2002) and another in *Hfx. mediterranei* in which the influence of the composition of the culture media for the production of C_{50} carotenoids is studied (Fang et al. 2010). Based on these studies, there have been realized various assays in *Hfx. mediterranei* in our research group in which it has been found that according to the carbon/nitrogen rate in the culture media different carotenes are produced (unpublished data).

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