Nitrate reduction in *Haloferax alexandrinus*: the case of assimilatory nitrate reductase.

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Abbreviations: Nas, assimilatory nitrate reductase; Fd-Nas, ferredoxin assimilatory nitrate reductase dependent; Nar, respiratory nitrate reductase; DT, dithionite; DTT, dithiothreitol, MV, methylviologen.

Abstract

Haloferax alexandrinus strain TM JCM 10717^T = IFO 16590^T is an extreme halophilic archaeon able to produce significant amounts of canthaxanthin. Its genome sequence has been analysed in this work using bioinformatics tools available at Expasy in order to look for genes encoding nitrate reductase-like proteins: respiratory nitrate reductase (Nar) and/or assimilatory nitrate reductase (Nas). The ability of the cells to reduce nitrate under aerobic conditions was tested. The enzyme in charge of nitrate reduction under aerobic conditions (Nas) has been purified and characterised. It is a monomeric enzyme (72±1.8 kDa) that requires high salt concentration for stability and activity. The optimum pH value for activity was 9.5. Effectiveness of different substrates, electron donors, cofactors and inhibitors were also reported. High nitrite concentrations were detected within the culture media during aerobic/microaerobic cells growth.

The main conclusion from the results is that this haloarchaeon reduces nitrate aerobically thanks to Nas and may induce denitrification under anaerobic/microaerobic conditions using nitrate as electron acceptor. The study sheds light on the role played by haloarchaea in the biogeochemical cycle of nitrogen, paying special attention to nitrate reduction processes. Besides, it provides useful information for future attempts on micro-ecological and biotechnological implications of haloarchaeal nitrate reductases.

Key words: N-cycle, halophiles; Archaea; nitrate reductase; assimilatory nitrate pathway; denitrification.

Introduction

Haloferax alexandrinus was described in 2002 as an extreme halophilic archaea able to produce significant amounts of canthaxanthin (Asker and Ohta, 2002a; Asker and Ohta, 2002b), a carotenoid of high interest for several biotechnological uses (Rodrigo-Baños et al. 2015). Shortly after that, lipidic characterisation of the *Hfx. alexandrinus* strain TM JCM 10717^T = IFO 16590^T was also reported (Asker et al. 2002). Even taking into account the potential use of this haloarchaea as carotenoid producer, studies about this strain are scarce.

During the last decade, several haloarchaeal genomes have been fully sequenced and annotated. The sequence of the *Hfx. alexandrinus* strain TM JCM 10717^T = IFO 16590^T was reported first in 2013 and later modified in 2015 (http://www.ncbi.nlm.nih.gov/genome/16378?genome_assembly_id=176792). More recently, the genome sequence of *Hfx. alexandrinus* strain Arc-Hr has been published (http://www.ncbi.nlm.nih.gov/genome/16378?genome_assembly_id=204114). Although genomic "era" for archaea started late compare to other organisms, currently it is possible to carry out genomics in parallel to biochemical studies for many of the most representative species of the class Halobacteria, commonly named haloarchaea (Gupta et al. 2015; Gupta et al. 2016).

Haloarchaea constitute the main microbial populations in salty environments, and consequently, the play an important role in the main biogeochemical cycles. Nitrogen is a basic element for life and it accounts for approximately 6% of the dry mass on average. The biogeochemical cycle of nitrogen (N-cycle) makes possible nitrogen interconversions from the most strongly reduced state, as [NH_3], in the −3 oxidation state, to the most highly oxidized state, nitrate ion, [NO_3]$^-$, in the +5 oxidation state (Richardson et al. 1999; Thomson et al. 2012). This cycle is constituted by several pathways with bacteria and archaea playing an important role. Nitrate can be used as nitrogen source for growth under aerobic conditions (assimilatory nitrate reduction) or as final electron acceptor under anaerobic conditions (denitrification) (Bothe and Ferguson, 2006).

In nitrate assimilation, first NO_3^- is incorporated into the cells by high/low-affinity transporters and further reduced to NH_4^+, via NO_2^-, by two sequential reduction reactions catalysed by assimilatory nitrate reductase (Nas; EC 1.6.6.2) and assimilatory nitrite reductase (Nir; EC 1.7.7.1). These two enzymes are located within the cytoplasm. The NH_4^+ produced is further incorporated into 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) (Martínez-Espinosa et al. 2006; Pire et al. 2014).

Two classes of assimilatory nitrate reductases (Nas) have been described from microorganisms: the ferredoxin- or flavodoxin-dependent Nas and the NADH-dependent enzyme (Moreno-Vivián et al. 1999). The Fd-Nas are usually monomers with a molecular mass between 75 to 85 kDa (Mikami and Ida, 1984; Rubio et al. 1996), while NADH-Nas proteins are heterodimers of 45 kDa FAD-containing diaphorase and 95 kDa catalytic subunit with molybdenum cofactor and a putative N-terminal [4Fe-4S] centre (Richardson et al. 2001).

Apart from assimilatory nitrate reductases, there are other two other types of nitrate reductases-like proteins (Richardson et al. 2001; Sparacino-Watkins et al. 2014): respiratory nitrate reductases (Nar) and dissimilatory nitrate reductases (usually termed Nap). These reductases differ in their cellular location and function: respiratory membrane-bound enzyme (Nar) plays a key role in the generation of metabolic energy by using nitrate as a terminal electron acceptor (nitrate respiration/denitrification) (Richardson et al. 2001; Torregrosa-Crespo et al. 2016). This enzyme is an heterotrimer as well as the periplasmic nitrate reductase (Nap), which participates in the dissipation of excess of reducing power for redox balancing (nitrate dissimilation) (Richardson et al. 2001).

In silico studies revealed that genes encoding the main proteins involved in nitrogen cycle have been found in archaeal genomes (Cabello et al. 2004). However, physiological and biochemical characterisation of such as kind of proteins is still poor in Archaea domain. Particularly, proteins involved in NO_3^- reduction to NO_2^- related to both, assimilation or denitrification, have only been studied in members of the *Haloferax* and *Haloarcula* genera (Yoshimatsu et al. 2000; Yoshimatsu et al. 2002; Yoshimatsu et al. 2007; Torregrosa-Crespo et al. 2016; Hattori et al. 2016). Besides, assimilatory nitrate reduction pathway has only been explored in the haloarchaea *Hfx. mediterranei* at the time of writing this work (Martínez-Espinosa et al. 2001a; Martínez-Espinosa et al. 2001b; Martínez-Espinosa et al. 2006; Pire et al. 2014, Esclapez et al. 2015).

This work summarises the *in silico* analysis of the *Hfx. alexandrinus* strain TM JCM 10717^T = IFO 16590^T genome looking for the sequences encoding nitrate reductases-like proteins. Biochemical

characterisation of the enzyme catalysing nitrate reduction to nitrite (Nas) under aerobic conditions is also reported. This is the second study about Nas (and consequently about assimilatory nitrate reduction) in haloarchaea. The results show that *Hfx. alexandrinus* is able to use nitrate as sole nitrogen source for growth under aerobic conditions. Potential capability to use nitrate as final electron acceptor (under anaerobic/microaerobic conditions) is also expected.

Materials and methods

Genome analysis

Haloferax alexandrius strain TM JCM 10717^T = IFO 16590^T genome available at NCBI (http://www.ncbi.nlm.nih.gov/genome/16378?genome_assembly_id=176792) was used to perform *in silico* analysis with the aim to identify genes coding for nitrate reductase like proteins. Standard bioinformatics tools available at Expasy portal were used (http://www.expasy.org/) (Gasteiger et al. 2003). Genomics were carried out using: ClustalW software for multiple sequence alignment (http://embnet.vital-it.ch/software/ClustalW.html) (Thompson et al 1997) and BLAST software for biological sequence similarity search and search on protein sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). Protparam (http://web.expasy.org/protparam/) was used to get physical-chemical parameters of the nitrate reductases predicted like proteins.

Growth conditions

Hfx. alexandrinus strain TM JCM 10717^T = IFO 16590^T strain from Japan collection of microorganisms was used (RIKEN BioResource Center). The cells were grown in culture media containing the following mixture of salts: (g l^{-1}) 250 NaCl, 20 $MgSO_4$ x $7H_2O$, 2 KCl, 3 $Na_3C_6H_5O_7$, 0.05 $FeSO_4$ x $7H_2O$, 0.0002 $MnSO_4$ x H_2O and 0.5 KH_2PO_4 (Asker and Ohta, 2002a). This medium also contained: glucose and KNO_3, 5 and 10 g l^{-1}, respectively. The pH value of the culture medium was adjusted to pH 7.4 using 1 M KOH. *Hfx. alexandrinus* was grown aerobically at 37 ºC in 500-ml batch cultures in 1 L x 20 erlenmeyer flasks using a rotary shaker (New Brunswick innova44) at 180 rpm. Growth was monitored during 10 days measuring the optical density at 600 nm. Nitrite excreted within the media by the cells was quantify using diazo coupling method (Snell and Snell, 1949).

Assimilatory nitrate reductase purification

In order to purify Nas, cells were harvested at mid exponential phase of growth (100 hours of incubation) by centrifugation at 30,000 g for 20 min in a Beckman Avanti J 30 centrifuge. All the

purification steps were carried out at room temperature following the protocol previously described by Martínez-Espinosa and co-workers (Martínez-Espinosa et al. 2001b) with some minor changes.

Step 1: Preparation of crude extract. The freshly harvested cells were washed using the mixture of salts previously described and centrifuged at 30,000 g for 20 min at room temperature. After that, the cells were resuspended in 50 mM phosphate buffer pH 7.4, containing 2.5 M $(NH_4)_2SO_4$ (buffer A). The cells were disrupted by sonication (3' x 8 pulses in ice) and the suspension was centrifuged at 105,000 g for 1.5 hours at 4 ºC. The supernatant was collected and used as the source of enzyme.

Step 2: Sepharose-4B chromatography. The supernatant from the previous step was chromatographed on a Sepharose-4B column (2.5 x 30 cm) equilibrated with buffer A. After introducing the sample, the column was washed with two volumes of buffer A. Elution was carried out with a decreasing linear gradient of 2.5-0.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 7.4 at a flow rate of 48 ml h^{-1}. The total volume of the gradient was 1.5 l. Fractions containing Nas activity were pooled and applied to a DEAE-cellulose column.

Step 3: DEAE-cellulose chromatography. A DEAE-cellulose column (1 x 6 cm) was equilibrated with two column volumes of buffer A. The column was washed using the same buffer at a flow rate of 30 ml/h. The enzyme was eluted with 50 mM phosphate buffer pH 7.4 (buffer B), containing 4.3 M NaCl at a flow rate of 30 ml h^{-1}. Fractions containing Nas activity were pooled and applied to a gel filtration column.

Step 4: Sephacryl S-300 chromatography. Fractions containing Nas activity were loaded on a Sephacryl S-300 column (Pharmacia HiPrep 16/60), previously equilibrated with buffer B containing 2 M NaCl. Buffer B was also used for protein elution (flow rate of 30 ml h^{-1}). After elution, the fractions containing Nas activity (15 ml in total) were immediately dialysed against 100 volumes of 50 mM phosphate buffer pH 7.4, containing 4.3 M NaCl to stabilise the Nas protein (Martínez-Espinosa et al. 2001b).

Protein determination, nitrate reductase assay and enzymatic activity characterisation.

The protein content was determined by the Bradford method, with bovine serum albumin (fraction V) as a standard.

Nitrate reductase activity was measured by colorimetric determination of nitrite as previously described. The appearance of nitrite was followed using the diazo coupling method (Snell and Snell, 1949; Martínez-Espinosa et al. 2001b).

Nas specific activity is expressed as nmol of NO_2^- appearing per min per mg of protein. Enzymatic activities were explored at different pHs (using phosphate, TRIS-HCl or carbonate/bicarbonate buffers), temperatures ranging from 20 ºC to 90 ºC and in presence of different salt concentrations (0-2 M NaCl or KCl). All the assays were carried out in triplicate and against a control assay without enzyme.

The kinetic results were processed using the Michaelis-Menten equation. The values of V_{max} and K_m were determined from the analysis of the corresponding Michaelis-Menten curves using Excel software.

To analyse the effect of several electron donors and inhibitors on the Nas activity, NADH, NADPH, azide, cyanide, EDTA and sulphite were added to the reaction mixture at 1 mM final concentration.

UV-visible spectra from pure protein sample was obtained to identify signals from metal cofactors. The oxidised spectrum was obtained first and the reduced by re-running the same sample after addition of a few crystals of sodium DT (which was used as reductant reactive).

Gel electrophoresis and estimation of Nas Mr value

The Mr of Nas was estimated by SDS-PAGE taking into account that molecular masses of halophilic proteins are over estimated in SDS-PAGE (around 13-17%) (Johnsen et al. 2004). Molecular mass markers were proved by Sigma (marker M4038).

Results

Haloferax alexandrius strain TM JCM 10717^T = IFO 16590^T genome is available at NCBI (http://www.ncbi.nlm.nih.gov/genome/16378?genome_assembly_id=176792). This genome is fully sequenced and annotated. Recently, it has been stated that annotation errors are quite common in haloarchaeal genomes (Pfeiffer et al. 2015) and nomenclature used is usually confusing. In order to explore potential capability of *Hfx. alexandrinus* to reduce nitrate, the genome previously mentioned was

analysed. Two different sequences encoding nitrate reductases like proteins were located (table 1). Both of them are annotated as "nitrate reductases". Similarities search using Blast and sequences alignments using ClustalW from Expasy stated that one of the sequences (Accession number: ELZ94752.1) corresponds to the respiratory nitrate reductase beta subunit (in charge of the electron transfer during nitrate reduction under anoxic conditions), whilst the other sequence (Accession number: ELZ88427.1) shows the highest similarity to the assimilatory nitrate reductases (in charge of the nitrate reduction to nitrite under aerobic conditions). Sequences coding for the respiratory nitrate reductase alpha subunit (catalytic subunit) were not identify.

Figure 1 displays sequence alignments of the *Hfx. alexandrinus* ELZ88427.1 sequence and other halophilic assimilatory nitrate reductase like proteins. It has the best scores with Nas from *Hfx. volcanii* (99% identity) and with Nas from *Hfx. mediterranei* (83 % identity). *Hfx. mediterranei* is the only haloarchaea from where assimilatory and respiratory nitrate reductases have been isolated and biochemically characterised up to now (Martínez-Espinosa et al. 2001b; Torregrosa-Crespo et al. 2016).

The N terminal of the *Hfx. alexandrinus* protein ELZ88427.1 contains a twin arginine "-RR-". The twin arginine ('RR') motif (also termed Tat signal peptide) is involved in proteins translocation to the outside of the cytoplasmic membrane (Maillard et al. 2007). The conserved consensus sequence for this motif (S/T-RR-X-FLK) has been identified in few archaeal respiratory nitrate reductases (Torregrosa-Crespo et al. 2016). However, the N terminal of the protein ELZ88427.1 is not similar to the consensus Tat signal peptide. Consequently, this protein may be is the assimilatory nitrate reductase, a cytoplasmic enzyme reducing nitrate to nitrite aerobically to allow cells growth. ~~This protein has not the signal peptide (Tat signal) to be exported to the membrane as it is the case of respiratory nitrate reductases.~~

Comentario [RM1]: It must be removed to avoid repetition as the reviewer 2 suggested

Protparam was use to get physical-chemical parameters of the *Hfx. alexandrinus'* ELZ88427.1 sequence, finding that it has 713 amino acidic residues (predicted Mr = 76049.8 Da) from which the total number of negatively charged residues (Asp + Glu) reach 110 against a total number of positively charged residues (Arg + Lys) of 57. Other predicted parameters were: pI: 4.52; instability index (II): 35.15; and aliphatic index: 72.95.

Once it was verified that the genome contains a gene encoding a putative assimilatory nitrate reductase (Nas), cells were grown aerobically in minimal culture media containing 100 mM KNO_3 as

sole nitrogen source for growth, in order to explore *Hfx. alexandrinus* capacity to reduce nitrate aerobically.

As it is displayed in Figure 2, cells were able to grow aerobically using nitrate. Nas activity was detected between 72 and 168 hours of incubation and it reached the maximum value when the absorbance of the culture was around 0.47 (at 600 nm). This maximum activity value was observed shortly after the beginning of the exponential phase of growth and in that moment high nitrite concentration within the media was quantify (up to 18.8 mM). This growth phase is characterised by oxygen depletion (culture media is initially aerobic but it becomes microaerobic as soon as the biomass increases shortly before the stationary phase of growth) (Hochstein and Lang, 1991; Torregrosa-Crespo et al. 2016). Consequently, under these circumstances, the respiratory pathway could also be induced as it has been previously described in *Hfx. mediterranei*, which is known as a denitrifier (Mancinelli and Hochstein, 1986; Torregrosa-Crespo et al. 2016). The nitrite excretion here detected as well as the presence of genes coding for at least three of the four enzymes involved in denitrification indirectly suggest that *Hfx. alexandrinus* could induce denitrification under microaerobic conditions (see discussion section). The growth rate calculated under these growth conditions was 0.010 ± 0.002 (h^{-1}).

To purify Nas, cells were harvested at the beginning of the exponential phase of growth (where maximum Nas activity was detected under aerobic conditions). The purification scheme is summarised in Table 2. It involves Sepharose-4B, DEAE-cellulose, Sephacryl S-300 chromatographies. These protocols was previously tested to purify Nas from *Hfx. mediterranei* and it allows successful purifications of halophilic proteins (pure concentrate protein samples in a short period of time with low cost). Nas from *Hfx. alexandrinus* was purified 70-fold, and the specific activity of purified enzyme was 0.23 U/mg protein. These values are lower than those obtained from *Hfx. mediterranei* Nas purification (the enzyme was purified 177-fold and specific activity was 0.55 U/mg protein (Martínez-Espinosa et al. 2001b). *Hfx. alexandrinus* Nas activity decreased about 40% in one week when the crude extract was stored at temperatures around 4 ºC. At temperatures higher than 4ºC, the activity depletion in the crude extract was even more dramatic (60-80 %). However, the activity of the pure sample was more stable (2-3 weeks stored at 4 ºC). Consequently, it was necessary to start the purification process immediately after getting the crude extract. This pattern was also observed during the *Hfx. mediterranei* Nas purification and it

could be due to: i) the action of different proteases, ii) interactions between Nas and other enzymes in the crude extract or iii) the instability of the iron-sulphur clusters and other metallocofactors (MoCo for instance) in presence of oxygen.

SDS-PAGE of the purified enzyme showed one band of Mr 72 ± 1.8 kDa (figure 3). It is important to highlight that molecular masses of halophilic proteins are usually overestimated by SDS-PAGE. This effect is due to the presence of large amounts of negatively charged amino acids (Johnsen et al. 2004). Taking into account the magnitude of the Mr overestimation (13-17%), a molecular mass of around 70 kDa for *Hfx. alexandrinus* Nas would be expected. This value correlates with the molecular mass predicted from the protein sequence (Table 1).

Fractions containing Nas activity from DEAE cellulose chromatography were combined and used for the characterisation assays. After DEAE-cellulose column, Nas sample was bright brown colour which agrees with those results obtained from other assimilatory nitrate reductases. This colour is mainly due to the presence of Fe-S clusters in the Nas. To confirm the presence of Fe-S clusters in the protein, protein samples from DEAE cellulose as well as pure protein fractions from Sephacryl S-300 were used to get UV-Vis spectra in the fully oxidised and fully reduced forms. In addition to the expected absorbance maximum at 280 nm (due to protein), there was a broad band showing a maximun peak at 404 nm in teh fully oxidised protein sample, which is consistent with the presence of Fe-S clusters. These clusters usually exhibit a maximum between 400 and 460 nm. This peak shifted up to 450 nm in the fully reduced protein. These results are similar to those obtained from *Hfx. mediterranei* Nas (Martínez-Espinosa et al. 2001b).

The effect of several electron donors such as NADH, NADPH or MV on Nas activity was tested. Reduced methylviologen (MV) was the best electron donor (*in vitro*) for *Hfx. alexandrinus* Nas, as it was previously described for its homolog from *Hfx. mediterranei* (Martínez-Espinosa et al. 2001b). Nas from *Hfx. alexandrinus* did not use electrons from either NADH (1 mM) or NADPH (1 mM mM) (in presence or absence of DT within the reaction mixture). Dithionite (DT) was not able to reduce nitrate in absence of MV. These results suggest that Nas from *Hfx. alexandrinus* could be a ferredoxin dependent enzyme (Martínez-Espinosa et al. 2001b). Conserved Cys residues that may serve as ligands to Fe atoms (Fe-S clusters) are highlighted in figure 1.

Several inhibitors of nitrate reductases were also tested. Dithiothreitol (DTT: 1 mM) was not effective as Nas inhibitor (only 2% inhibition was determined compared to the control). Sulphite and EDTA caused 30 % and 52 % inhibition respectively, at 1 mM final concentration. Azide (1 mM) and cyanide (1 mM) strongly inhibited the enzyme (90% and 98 % inhibition, respectively).

pH-dependence of enzymatic activity (figure 4) as well as the effect of salt concentration (table 3) on enzymatic activity were also analysed. Optimum pH for activity was slightly alkaline (9.5). The effect of NaCl and KCl at different concentrations (up to 2 M) was studied finding that the highest salt concentration the highest activity value. However, Nas activity was significantly higher in presence of KCl than in presence of NaCl (table 3). Like other halophilic nitrate reductases from genus *Haloferax* Martínez-Espinosa et al. 2001b), Nas from *Hfx. alexandrinus* showed a remarkable thermophilicity and worked well up to 50 ºC in presence of high salt concentrations.

Kinetic parameters of halophilic Nas were determined varying the concentration of one substrate (MV) at several fixed concentrations of the other substrate (nitrate), in the presence of 120 mM bicarbonate/carbonate buffer (pH 9.0) containing 1 M NaCl. The halophilic enzyme followed a Michaelis-Menten kinetic. K_m values for nitrate and MV were 45 ± 5.2 and 6.46 ± 0.74 μM, respectively. V_{max} values for nitrate and MV were 61.1 ± 3.4 and 19.01 ± 1.7 U/mg prot., respectively. The value of K_m for nitrate is under the range of the values obtained from other nitrate reductases (reported K_m values are between 0.1 and 1.6 mM (Alvarez Ossorio e al. 1992; Martínez-Espinosa et al. 2001b).

Discussion

Nitrate cycle in archaea, and in particular in haloarchaea, has been poorly described up to now. Taking into account that haloarchaea constitute the major microbial populations in salty environments, it is worthy to explore how relevant is their contribution in the main biogeochemical cycles. Nevertheless, the nature of the archaeal cells in terms of cell membranes composition, molecular biology machineries, etc, makes difficult (but at the same time interesting) to study haloarchaeal metabolic pathways from biochemical and molecular biology points of view.

New efforts have been done to sequence haloarchaeal genomes and to improve genome annotations, thus improving current knowledge about this group of extremophiles. The *in silico* analysis

of the *Hfx. alexandrinus* strain TM JCM 10717^T = IFO 16590^T genome (which annotation is not completely detailed at the time of writing this work), revealed that there are two genes coding for nitrate reductases-like proteins: assimilatory nitrate reductase (which catalyses the nitrate reduction to nitrite under aerobic conditions) and the beta subunit (also termed NarH) of the respiratory nitrate reductases (which catalyses the reduction of nitrate to nitrite under anaerobic conditions). It was impossible to identify genes coding for the large subunit of the respiratory nitrate reductases (NarG, also called alpha subunit) in *Hfx. alexandrinus* genome. Potential capacities of *Hfx. alexandrinuns* to carry out nitrate assimilation and nitrate respiration were checked first by *in silico* searches looking for genes encoding the structural enzymes catalysing both pathways. Genes coding for all the enzymes required to assimilate nitrate (ferredoxin dependent nitrite reductase: ELZ88359.1; glutamine synthetase: ELZ90622.1; glutamate synthase: ELZ92264.1; glutamate dehydrogenase: ELZ95726.1), as well as most of the enzymes involved in denitrification (copper containing nitrite reductase: ELZ87995.1; nitric oxide reductase: ELZ88003.1; nitrous oxide reductase accessory protein: WP_006600978.1) have been identify. The presence of genes encoding structural enzymes of denitrification as well as nitrite excretion within the media at the end of the exponential phase of growth under the culture conditions used in this study suggest that *Hfx. alexandrinus* could be denitrifier. Consequently, this haloarchaea could potentially use nitrate in both senses, as nitrogen source for growth or as final electron acceptor to respire. Regarding to denitrification, it remains unclear whether or not there are genes coding for the catalytic subunit of the respiratory nitrate reductase as well as the nitrous oxide reductase (the last enzyme in the denitrification pathway). Genome annotation errors are quite common in haloarchaeal genomes (Pfeiffer et al. 2015). Several aspects such as start codon misassignments, disrupted genes as well as poor knowledge based on experimental characterisation of the genes/proteins functions contribute to this persistent problem hampering research in the biosciences related to extreme microbes.

This *in silico* analysis was the starting point to study assimilatory nitrate reduction in *Hfx. alexandrinus*. The cells were able to grow aerobically in minimal culture media in presence of 100 mM nitrate as sole nitrogen source (Figure 2). These culture conditions were used to purify and characterise the assimilatory nitrate reductase (Nas) from *Hfx alexandrinus*, which is the first enzyme of the pathway. Nas has been purified as a monomer showing similar biochemical characteristics than those reported from

Hfx. mediterranei Nas (Martínez-Espinosa et al. 2001b), in terms of molecular mass, optimal pH for activity and effect of high salt concentration on activity at stability (even in presence of high temperature). Like other non-halophilic and halophilic nitrate and nitrite reductases, cyanide and azide were strong inhibitors for *Hfx. alexandrinus* Nas (Alvarez Ossorio et al. 1992; Hochstein et al. 1991; Ken-Ichi et al. 1996; Moreno-Vivián et al. 1999; Martínez Espinosa et al. 2001a; Martínez Espinosa et al. 2001b). These compounds are thought to inhibit by metal chelation and the primary site of action is probably the molybdenum (McDonald et al. 1974). Nas activity from *Hfx. alexandrinus* showed a remarkable thermophilicity working well up to 50 ºC in the presence of high salt concentrations (2 M NaCl or KCl) as it was expected taking into account the environmental conditions of the ecosystems inhabited by this haloarchaea. Nas activity was higher in presence of KCl than in presence of NaCl under all the conditions assayed, which makes sense taking into account that KCl is the salt that haloarchaea accumulated intracellularly to be isotonic with their environment (Oren, 2013). One important feature to be highlighted is that *Hfx. alexandrinus* Nas has greater affinity for its substrate than *Hfx. mediterranei* Nas (Martínez-Espinosa et al. 2001b). K_m value for nitrate in the case of *Hfx. alexandrinus* Nas was 0.045 mM, which is approximately 1/21 of the value reported for nitrate from *Hfx. mediterranei* Nas (Martínez-Espinosa et al. 2001b). However, *Hfx. mediterranei* grows much better aerobically in presence of nitrate than *Hfx. alexandrinus*, as it can be concluded comparing the growth rates of *Hfx. alexandrinus* (μ = 0.010 ± 0.002 h^{-1}) and *Hfx. mediterranei* grown aerobically in presence of nitrate as sole nitrogen source (Martínez-Espinosa et al. 2001a; Martínez-Espinosa et al. 2001b).

In conclusion, *Hfx. alexandrinus* strain TM JCM 10717^T = IFO 16590^T is able to use nitrate as sole nitrogen source for growth under aerobic conditions thanks to Nas. In 2002, Asker and Ohta (Asker and Ohta, 2002a) described this specie as a strict aerobe unable to grow anaerobically by using alternative electron acceptors such as nitrate or DMSO, or by fermenting l-arginine. Results from genomics here presented as well as nitrite excretion during the cells growth in the presence of nitrate confirm that *Hfx. alexandrinus* strain TM JCM 10717^T = IFO 16590^T may induce denitrification under anaerobic/microaerobic conditions using nitrate as electron acceptor. On the other hand, the same authors detected aerobic reduction of nitrate and nitrite without gas production (Asker and Ohta, 2002a), which is consistent with the induction of the assimilatory nitrate pathway. These results about *Hfx. alexandrinus*

strain TM JCM 10717^T = IFO 16590^T Nas constitute the second study about assimilatory nitrate reduction in haloarchaea providing useful information about haloarchaeal Nas.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Legends to figures

Figure 1. ClustalW alignment of the predicted *Hfx. alexandrinus* Nas with Nas sequences from *Hfx. mediterranei*, *Haloferax* sp. Q22, *Haloferax volcanii* DS2 and *Halalkalicoccus paucihalophilus*. Conserved Cys residues that may serve as ligands to Fe atoms (Fe-S clusters) are highlighted.

Figure 2. *Haloferax alexandrinus* growth in minimal culture media with 100 mM KNO_3 as a nitrogen source (▲) and evolution of Nas activity during the growth of the cells (♦). The plotted results correspond to the average of the values obtained from three different experiments.

Figure 3. SDS-PAGE of the Nas purification process. Lane 1: Marker; lane 2: Sephacryl; lane3: DEAE cellulose; lane 4: Sepharose 4 B; lane 5: Crude extract**.** The Mr values of standard protein markers are indicated in kDa

Figure 4. Optimum pH for *Hfx. alexandrinus* Nas activity. The results represented correspond to the average value obtained from three different activity assays.