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ORIGINAL PAPER

The effect of ammonium on assimilatory nitrate reduction in the haloarchaeon *Haloferax mediterranei*

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Abstract Physiology, regulation and biochemical aspects of the nitrogen assimilation are well known in *Prokarya* or *Eukarya* but they are poorly described in *Archaea* domain. The haloarchaeon *Haloferax mediterranei* can use different nitrogen inorganic sources (NO_3^-, NO_2^- or NH_4^+) for growth. Different approaches were considered to study the effect of NH_4^+ on nitrogen assimilation in *Hfx. mediterranei* cells grown in KNO_3 medium. The NH_4^+ addition to KNO_3 medium caused a decrease of assimilatory nitrate (Nas) and nitrite reductases (NiR) activities. Similar effects were observed when nitrate-growing cells were transferred to NH_4^+ media. Both activities increased when NH_4^+ was removed from culture, showing that the negative effect of NH_4^+ on this pathway is reversible. These results suggest that ammonium causes the inhibition of the assimilatory nitrate pathway, while nitrate exerts a positive effect. This pattern has been confirmed by RT-PCR. In the presence of both NO_3^- and NH_4^+, NH_4^+ was preferentially consumed, but NO_3^- uptake was not completely inhibited by NH_4^+ at prolonged time scale. The addition of MSX to NH_4^+ or NO_3^- cultures results in an increase of Nas and NiR activities, suggesting that NH_4^+ assimilation, rather than NH_4^+ per se, has a negative effect on assimilatory nitrate reduction in *Hfx. mediterranei*.

Keywords Halophile · Archaeon · Nitrate assimilation · Ammonium assimilation · Nitrogen regulation · Nitrate reductase · Nitrite reductase

Abbreviations

Nas	Assimilatory nitrate reductase
NiR	Assimilatory nitrite reductase
NR	Nitrate reductase
MSX	L-Methionine-D,L-sulfoximine
GDH	Glutamate dehydrogenase
GS	Glutamine synthetase
GOGAT	Glutamate synthase
MV	Methylviologen
DT	Dithionite

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Introduction

Nitrate is a major source of inorganic nitrogen used by most plant, algae, yeast and bacteria. It is transported into the cells by an active transport system and reduced to ammonium by the sequential action of assimilatory nitrate (Nas) and nitrite reductases (NiR) (Moreno-Vivián et al. 1999; Richardson 2001; Martínez-Espinosa et al. 2001a, b).

Ammonium produced by NiR can be further incorporated into carbon skeletons by glutamate dehydrogenase (GDH) (Ferrer et al. 1996) or glutamine synthetase–glutamate

synthase (GS–GOGAT) pathway (Flores et al. 1983). Some microorganisms use only the GS–GOGAT system (Fisher 1999), while others employ only GDH, but most microorganisms can use both pathways for ammonia assimilation (Burkovski 2003). In this case, when ammonium is available in excess, it is usually assimilated by GDH (Cohen-Kupiec et al. 1999), while GS and GOGAT are operative when the ammonium concentration is relatively low (Hochman et al. 1987). In methanogenic archaea, many members use GDH in ammonium assimilation and GS solely in glutamine production. However, for a few methanogenic archaea, such as *Methanococcus maripaludis*, a dual function of GS has been described: ammonium assimilation for the cell and glutamine synthesis for protein synthesis (Cohen-Kupiec et al. 1999). Most hyperthermophilic archaea also show high GDH activity, and this enzyme plays a major role in ammonium assimilation (Diruggiero and Robb 1996). In fact, *Thermococcus profundus* has no detectable GS activity (Kobayashi et al. 1995) and the GS of *Pyrococcus* sp. has very low biosynthetic activity, suggesting that the GS reaction is biased towards glutamate production. To date, GS-GOGAT pathway is not described in haloarchaea. Only, GSs from *Halobacterium salinarum* and *Hfx. mediterranei* have been purified and its properties characterised (Manitz and Wilhelm-Holldorf 1993; Martínez-Espinosa et al. 2006).

When microorganisms able to assimilate nitrate are exposed to ammonium, the ability of the cells to use nitrate is drastically hampered (Flores et al. 1983). Ammonium exerts this effect by acting at two different levels: long or short term. In the first case, a prolonged incubation of the cells in ammonia media results in the repression of the genes encoding the nitrate/nitrite transporter and Nas and NiR enzymes (Flores et al. 1983). In the short term, ammonium inhibits nitrate and nitrite uptake without affecting activity levels of the enzymes of nitrate-reducing system. This pattern has been reported in both eukaryotic (Kleinhofs and Warner 1990) and prokaryotic (Raju et al. 1996) organisms. Two assumptions have been proposed to explain the regulation of nitrate uptake system. On one hand, some studies have led to the conclusion that the inhibitory effect of ammonium on nitrate uptake was due to ammonium per se (Franco et al. 1984) or ammonium effect on different periplasmic proteins (Dobao et al. 1994). More recent studies indicate that the regulatory mechanism of the *nir* operon (Tapia et al. 1995), *nasAB* operon (Gutierrez et al. 1997) or *nasF* operon (Chai and Stewart 1998) is closely linked to the repression caused by ammonium assimilation. On the other hand, it has been described some transcriptional regulatory proteins activating genes involved in nitrate and nitrite assimilation in bacteria (Magasanik 1996; Wray et al. 1996, 2000; Fisher 1999).

Albeit assimilatory nitrate/nitrite reduction and its regulation has been characterised in some bacteria and eukaryotic organisms; knowledge of this pathway in members of the Archaea domain is scarce. In this paper, we investigate the ammonium effect on the regulation of nitrate and nitrite assimilation from a member of halophilic archaea.

Materials and methods

Growth conditions

Haloferax mediterranei (ATCC 33500/R4) was used. Cultures were grown in a 25% (w/v) mixture of inorganic salts (25% SW) as described by Rodríguez-Valera et al. (1980). This minimal mineral medium was supplemented with 0.005 g/l $FeCl_3$; 0.5 g/l KH_2PO_4; 5 g/l glucose and 100 mM KNO_3, 5 mM KNO_3, 100 mM $(NH_4)_2SO_4$ or a combination of two of these nitrogen sources. All experiments were carried out at least three times.

In all cases, the pH value of the culture media was adjusted to 7.3 using KOH or HCl. The media were inoculated with a seed culture grown for 5 days. 0.5 l batch cultures of *Hfx. mediterranei* were grown aerobically at 37°C in 1 l erlenmeyer flasks on a rotary shaker at 200 rpm. Growth was monitored by measuring the optical density at 600 nm.

Cell-free extracts

During the growth, aliquots (12 ml) of cell suspensions were harvested and washed twice by centrifugation (30,000×*g* for 20 min at 4°C) in 25% SW and resuspended in 50 mM phosphate buffer, pH 7.3 containing 2.5 M $(NH_4)_2SO_4$. The cells were disrupted by sonication at 150 W for the eight periods of 3 min each at 4°C. The suspension was centrifuged at 30,000×*g* for 20 min.

Determination of nitrate, nitrite and ammonium

The nitrate disappearance of the medium was determined after a 50-fold dilution of 1 ml of the medium using the UV method (Ministerio de Agricultura, Pesca y Alimentación 1986). The nitrite concentration of media (consumed or excreted) was determined after a 40-fold dilution of 25 µl of media, using the diazo-coupling method (Martínez-Espinosa et al. 2001a, b). Ammonium was assayed by the phenol-hypochlorite method according to Weatherburn (Weatherburn 1967).

Nitrate reductase, nitrite reductase and glutamine synthetase assays

Nitrate and nitrite reductase activities were measured by the colorimetric determination of nitrite produced or consumed as previously described by using the diazo-coupling method (Martínez-Espinosa et al. 2001a, b). All assays were carried out in triplicate and against a control assay without enzyme.

GS transferase activity was determined as previously described (García-Fernández et al. 1994) over 30 min at 45°C. The composition of the reaction mixture was 100 mM L-glutamine, 10 mM sodium hydroxylamine, 50 μM manganese chloride, 10 μM ADP and 50 mM sodium arsenate in 0.2 M morpholinepropanesulfonic acid buffer (MOPS, pH 7.3).

MSX treatment of cells

MSX was added to the cultures (at zero time) at a final concentration of 2 μM, and subsequently every 12 h. MSX was sterilised by filtration (0.22 μm).

Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted (using the high pure RNA isolation kit (Roche), from *Hfx. mediterranei* cells grown in complex and minimal medium and harvested at different OD of growth. cDNA synthesis was performed in a total volume of 20 μl with 1.5 μg RNA, 30 pmol reverse oligonucleotide, 20 mM of the four deoxynucleotide triphosphates, and 20 U AMV reverse transcriptase (Sigma) in the commercial buffer and in the presence of 20 U RNase inhibitor. The reaction was carried out for 1 h at 50°C. Controls were performed in the absence of AMV retrotranscriptase. About 5 μl cDNA aliquots were used as a template in the PCR amplification step with different combinations of oligonucleotide pairs (0.5 μM each) in a total volume of 50 μl with 0.2 mM dNTPs and 1.5 U *Pfu* DNA polymerase (Fermentas). The initial denaturation step was 5 min at 95°C, followed by 35 cycles of synthesis comprising 1 min of denaturation at 95°C, 1 min of annealing at 50°C, and elongation at 72°C for 2 min. The amplification was ended with 10 min elongation at 72°C. About 10 μl aliquots of the various PCRs were analysed by agarose gel electrophoresis. The sequence of the primers used to target *nasA* (which encodes the assimilative nitrate reductase, Q703N5) and *nasD* (codes for the assimilative ferredoxin-dependent nitrite reductase, Q703N2) genes is shown in Table 1.

Results

The effect of ammonium on nitrate growing cells

To characterise the effect of ammonium on assimilatory nitrate reduction, different assays were carried out. Figure 1 shows the effect of ammonium on nitrate cultures. When *Hfx. mediterranei* cells were grown with 100 mM KNO_3 (N excess) as the sole nitrogen source under aerobic conditions (Fig. 1a), Nas and NiR activities were induced, and nitrite was excreted into the media up to a final concentration around 20 mM. This excretion indicates that the production of nitrite is faster than its reduction to ammonium under these conditions. As it is shown in Fig. 1a, maximum Nas activity was detected at OD = 0.9, while maximum NiR activity appeared shortly after this OD (=1.2). So the development of Nas activity before NiR activity could explain the nitrite accumulation into the culture media.

To analyse if low NH_4^+ concentrations are able to inhibit assimilatory NO_3^- reduction, when nitrate is in excess, 5 mM $(NH_4)_2SO_4$ was added to nitrate-growing cells at the mid-exponential phase of growth (OD = 1.2), since in this phase the highest Nas and NiR activities were detected. It was observed that the nitrite concentration previously excreted by cells decreased (Fig. 1b). The depletion of the nitrite concentration, in the presence of ammonium, could be related with two different aspects. One aspect is the chemical characteristics of this nitrogen specie, since nitrite can diffuse freely as nitrous acid to the cytoplasm in the presence of ammonium. The culture media here described are not buffered; under these conditions, the growth of *Hfx. mediterranei* caused a sudden drop in pH, due to the production of organic acids when glucose is used as carbon source (Oren and Gurevich 1994). By this reason, pH becomes 5.5 at the mid-exponential phase of growth or near to 4 at the stationary phase of growth. The other aspect could be related with the inhibition of Nas activity caused

Table 1 Primers used in RT-PCR

Gene	Primer	Sequence	T_m (°C)	bp
nasA	NASfor	GAACACGACACTCTGCATGGC	61.8	21
	NASrev	CGTCCGCTCCATGTTCATCG	55.7	20
nasD	NIRfor	GGACTCGATAAACGACATCGGA	60.3	22
	NIRrev	GGCGACGGCGTCGGGTCCTT	62.6	20

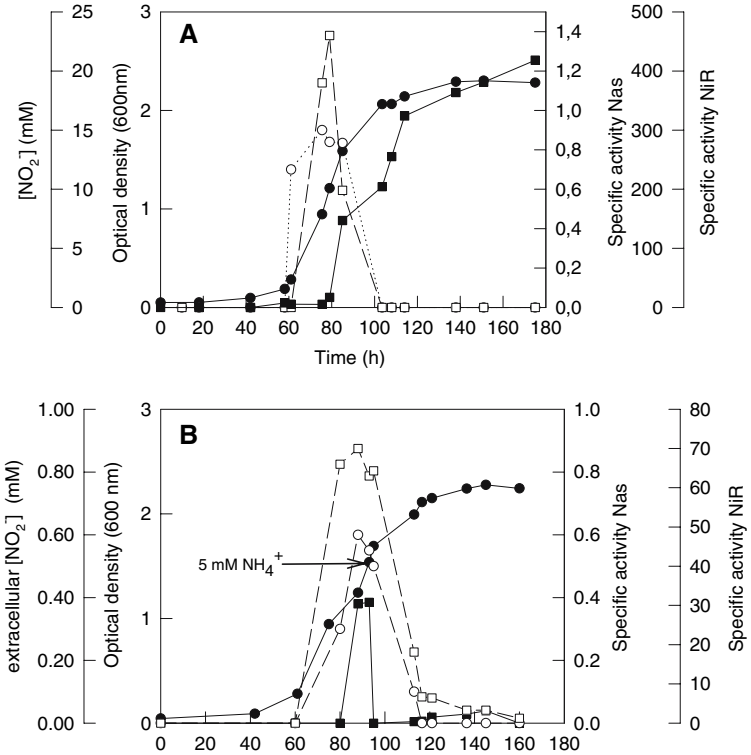

Fig. 1 Effect of 5 mM $(NH_4^+)_2SO_4$ addition on nitrate assimilation. **a** Control: evolution of *Hfx. mediterranei* growth, Nas and NiR activities in minimal medium containing 100 mM KNO_3. **b** *Hfx. mediterranei* cultures growing in minimal medium containing 100 mM KNO_3 were supplied with $(NH_4^+)_2SO_4$ when excretion of nitrite started (0.38 mM of nitrite into the medium). Optical density at 600 nm (*filled circle*), specific activity Nas (mU/mg prot) (*open circle*), specific activity NiR (mU/mg prot) (*open square*) and nitrite excreted into the medium (*filled square*)

by the presence of ammonium in the culture media. After the addition of ammonium, Nas activity was 65% of the maximum activity detected from cells growing in nitrate medium.

In relation to the nitrite reductase activity, we observed that it was less than 15% of the maximum NiR activity, obtained from cells growing in nitrate medium without ammonium. However, NiR activity can be detected even 60 h after the addition of ammonium probably due to the assimilation of nitrite previously excreted into the medium.

In the second kind of experiments, *Hfx. mediterranei* cells growing in 100 mM KNO_3 were harvested at mid-exponential phase of growth, washed with the mineral medium (25% SW) and transferred to minimal medium with 100 mM $(NH_4)_2SO_4$. The aim of this set of experiments was to analyse the changes of Nas and NiR activities as well as cells growth when NO_3^- is replaced by the same concentration of NH_4^+. The transference of these cells to an ammonium medium (100 mM $(NH_4)_2SO_4$) caused a decrease of Nas and NiR activities (Fig. 2). NiR activity was undetected 45 h after the transference, but Nas was not completely inhibited (Fig. 2).

In order to study if the inhibition of nitrate assimilation by ammonium was reversible, cells growing in ammonium medium (100 mM $(NH_4)_2SO_4$) were transferred to 5 mM nitrate medium (Fig. 3). Nas and NiR activities increased progressively, indicating that the inhibition by ammonium is reversible in *Hfx. mediterranei* as well as in cyanobacteria (Flores et al. 1983).

The effect of ammonium on nitrate-growing cells has been analysed not only from a physiological and biochemical point of view, but also from the regulation of gene expression. In connection with the last aspect, RT-PCR was used to determine the effect of nitrogen source on the expression of *nasA* and *nasD*. The expression of both genes, *nasA* and *nasD*, was analysed by isolating total RNA from *Hfx. mediterranei* cultures grown to different optical densities in minimal medium containing various nitrogen sources (nitrate, nitrite or ammonium). As can be seen in Fig. 4, in ammonium medium, the transcription of *nasA* and *nasD* was not detected, but in nitrate minimal medium both were present, indicating that these genes were needed for nitrate assimilation. As can be seen in the figure, *nasA* expression increased when the OD of the nitrate cultures was higher than 0.4. This result agrees with the Nas activity assays where the maximum Nas activity was detected at OD = 0.9. Besides, *nasA* mRNA was observed in minimal medium supplemented with nitrite, indicating that although the *nasA* expression is higher in nitrate medium (nitrate has positive effect on *nasA*), basal *nasA* expression takes place with nitrite as nitrogen source. Related to *nasD*, the expression of this gene was higher in nitrite cultures than in nitrate cultures, suggesting that nitrite has positive effect on *nasD*.

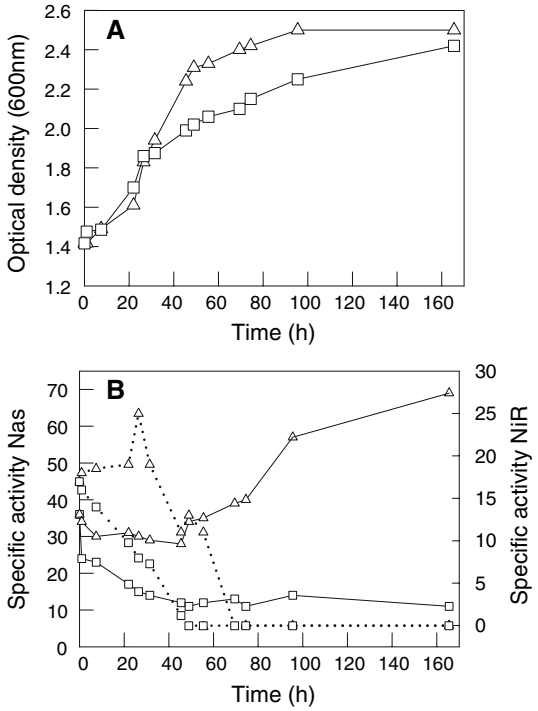

Fig. 2 Effect of the cells transference to nitrate or ammonia media. Cells growing in 100 mM KNO_3 were harvested at mid-exponential phase and transferred to medium containing 100 mM KNO_3 (control: *open triangle*) or 100 mM NH_4^+ (*open square*). **a** optical density; **b** specific activity Nas (mU/mg prot.) (*solid line*) and specific activity NiR (mU/mg prot.) (*dotted line*)

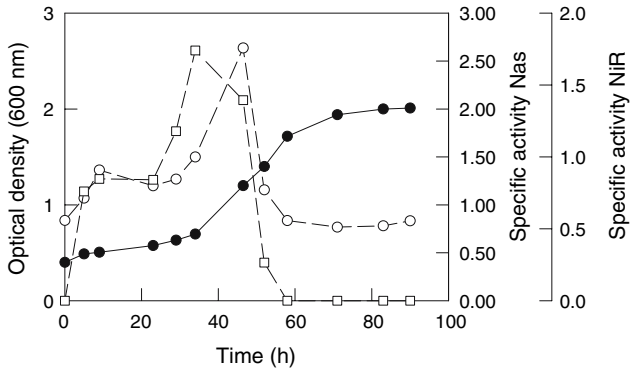

Fig. 3 Induction of Nas and NiR by nitrate. At zero time, cells growing in minimal medium containing 100 mM $(NH_4)_2SO_4$ were transferred to a medium with 5 mM KNO_3. Optical density (*filled circle*), specific activity Nas (mU/mg prot.) (*open circle*) and specific activity NiR (mU/mg prot.) (*open square*)

Changes in enzyme activities after the transfer of nitrate-growing cells to ammonium nitrate or ammonium nitrite medium

In order to determine which nitrogen source is preferentially consumed when two nitrogen sources are present in the culture media at low concentrations *Hfx. mediterranei* cells growing in 100 mM KNO_3 media were harvested at OD 1.2, washed and transferred to minimal culture media containing 5 mM $(NH_4)_2SO_4$ plus 5 mM KNO_3 or 2 mM $(NH_4)_2SO_4$ plus 2 mM KNO_2. In ammonium nitrate medium, Nas activity increased over 28 h after the transfer, but it began to decay quickly after that (Fig. 5). Either no effect or an inhibitory effect of ammonium on Nas activity could be expected, but an increase in this activity was found in the initial 30 h. It is possible to think that this ammonium concentration is insufficient to provoke an inhibition of Nas activity in the presence of nitrate in short term. This result also suggests that nitrate has a positive effect on Nas activity. With regard to NiR activity, we observed an increase of activity in the initial 5 h, but after that NiR activity decreased quickly (Fig. 5). Ammonium was preferentially consumed (1.3 mM $(NH_4)_2SO_4$ vs. 0.5 mM KNO_3 consumed at the stationary phase of growth).

In cells growing in ammonium nitrite medium, Nas activity was constant during incubation period. Nas activity values were lower than those observed from cells growing in ammonium nitrate medium, due to a positive effect of NO_3^- on Nas activity. In relation to NiR activity, we observed a similar pattern to those obtained from cells growing in ammonium nitrate medium (Fig. 5). NiR activity levels were slightly higher in ammonium nitrite medium than those in ammonium nitrate cultures, probably due to a positive effect of nitrite on NiR activity. Ammonium and nitrite concentrations have been quantified in these cultures at the stationary phase of growth, detecting that in ammonium nitrite medium, the two nitrogen sources were consumed (0.5 mM $(NH_4)_2SO_4$ vs. 0.35 mM KNO_2 consumed), although cells could have an ammonium preference.

The effect of MSX on growth and enzyme activities

To determine whether GS activity is detected in crude extracts when MSX is present in the culture medium, this compound was added at different concentrations (0.5–10 μM) every 12 h, to *Hfx. mediterranei* cultures growing with ammonium or nitrate. In these experiments, GS activity was not detected in crude extracts when MSX concentrations were around 1.5–2.5 μM. So, we supplied *Hfx. mediterranei* cultures (ammonium or nitrate) with 2 μM MSX (every 12 h) to estimate the effect of MSX on growth and enzyme activities (Nas and NiR) (Fig. 6). The growth of *Hfx. mediterranei* was less than that observed in minimal medium without MSX (Figs. 1, 6).

In MSX-ammonium medium as well as MSX-nitrate medium, the specific activities of Nas and NiR increased

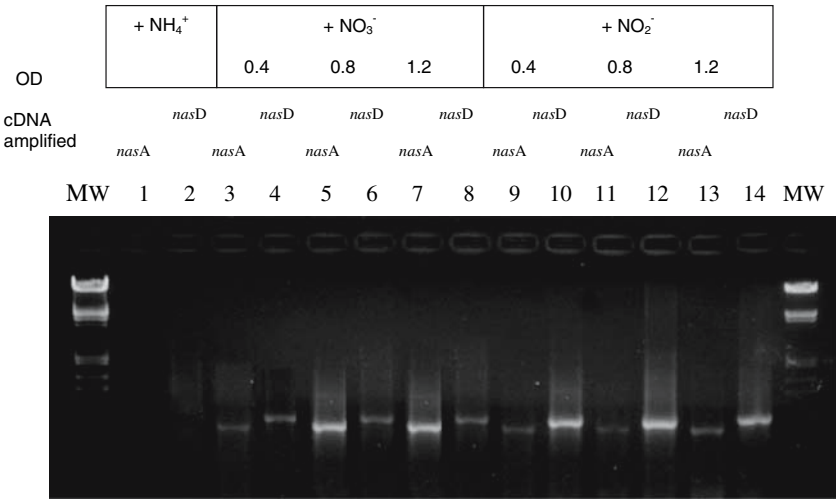

Fig. 4 Analysis of the expression of *nasA* and *nasD* genes by agarose gel electrophoresis of double-stranded DNA fragments generated in RT-PCR reactions. Reactions had performed with total RNA isolated from ammonium medium (*lanes 1* and *2*), nitrate (*lanes 3*, *4*, *5*, *6*, *7* and *8*) or nitrite (*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

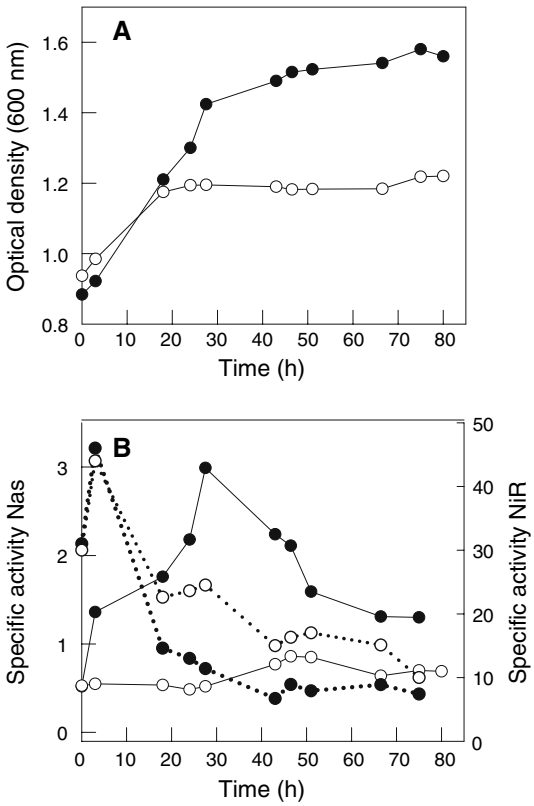

Fig. 5 Effect of the cells transference to media containing two nitrogen sources. Cells growing in 100 mM NO_3^- were harvested at mid-exponential phase and transferred to medium containing 5 mM NH_4^+ + 5 mM NO_3^- (*filled circle*) or 2 mM NH_4^+ + 2 mM NO_2^- (*open circle*). **a** Optical density; **b** specific activity Nas (mU/mg prot.) (*dashed line*) and specific activity NiR (mU/mg prot.) (*dotted line*)

(Fig. 6), reaching similar levels in both media. The addition of MSX to *Hfx. mediterranei* cultures reverses the negative effect of ammonium.

Discussion

In this work, we present the effect of ammonium on the assimilatory nitrate reduction in *Hfx. mediterranei* as a haloarchaea model, and constitute the first study of this mechanism in a member of the *Halobacteriaceae* family. *Hfx. mediterranei* is able to use nitrate as nitrogen source for growth under oxygenic conditions thanks to the assimilatory nitrate pathway. Under these conditions nitrite was excreted into the media shortly before the stationary phase of growth and it was concomitant with the loss of both activities, Nas and NiR. This accumulation of nitrite into the medium has also been described in bacteria (Dobao et al. 1994).

It has been extensively reported that nitrite strongly inhibits bacterial metabolism by inactivating FeS proteins (Moreno-Vivián et al. 1986) and some aerobic enzymes such as cytochrome oxidase, aconitase and fumarase (Martínez-Luque and Castillo 1991), although some *Rhodococcus* strains are able to tolerate nitrite concentration as high as 60 mM (Blasco et al. 2001). To explain the nitrite accumulation into culture media two different assumptions could be considered: (i) Nas and NiR activities are maximal at different optical density values during the culture growth, so that the production of nitrite by Nas is faster than its consumption by NiR. Due to its toxicity, nitrite is not accumulated into the cell and this fact explains the excretion of this nitrogen specie. However, this pattern is not enough to explain the increase of NO_2^- concentration in the culture media up to concentrations as high as 20 mM; (ii) nitrite excretion is probably the result of two different nitrate reductase activities (assimilatory and respiratory) higher than assimilatory nitrite reductase activity. In the presence of 100 mM KNO_3, we have detected two different nitrate reductases: assimilatory cytoplasmic nitrate

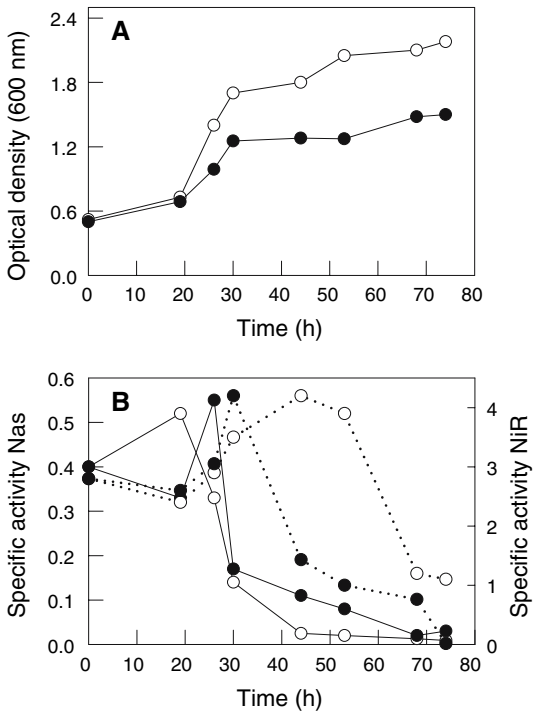

Fig. 6 Effect of MSX on growth and activities. At zero time, cells growing in minimal medium with 100 mM KNO_3 (optical density = 1.2), were transferred to a medium containing 5 mM NH_4^+ + MSX (*filled circle*) or 5 mM NO_3^- + MSX (*open circle*). **a** Optical density; **b** specific activity Nas (mU/mg prot.) (*dashed line*) and specific activity NiR (mU/mg prot.) (*dotted line*)

reductase, which was detected at the early exponential phase of growth (Martínez-Espinosa et al. 2001a), when oxygen concentration into culture media was high; and a membrane-associated nitrate reductase enzyme, which activity appeared shortly before the stationary phase of growth when oxygen availability decreased. These two nitrate reductase activities were well defined because they were detected at different times during the growth. The first appeared when the optical density of the cultures was between 0.28 and 1.6 (maximal Nas activity was quantified when optical density was 0.9). Nas activity was affected by NH_4^+ addition as it has been discussed before. However, the high nitrate concentration present in the medium (100 mM) as well as oxygen depletion shortly before the stationary phase of growth, caused a progressive increase of the respiratory nitrate reductase activity, which maximal value was observed at the stationary phase of growth (OD around 2.4). The last peak of activity corresponds to the membrane-associated enzyme, which has also been described in our research group (results not shown) (Lledó et al. 2004).

However, when low NH_4^+ concentrations were added to NO_3^- media, the NO_2^- concentration appeared as a product of Nas activity was lower than in those culture media, which only contained NO_3^- as nitrogen source. The decrease of NO_2^- availability could induce the assimilation of NO_2^-, previously excreted into the culture media. These facts explain the nitrite reabsorption in the presence of nitrate and ammonium (Fig. 1b). Although ammonium has a strong negative effect on active nitrite transport (Tapia et al. 1996), nitrite can be consumed in the presence of ammonium as it has been shown is this work or in bacteria (Caballero et al. 1986). Besides, nitrite could behave as an effective competitive inhibitor of nitrate transport (Rodríguez et al. 1992). It is also possible to think that this nitrite assimilation was promoted by carbon–nitrogen balance effect (100 mM KNO_3 plus 5 mM $(NH_4)_2SO_4$ vs. 30 mM glucose) on the assimilatory nitrate/nitrite pathway. In *Rhodobacter capsulatus* E1F1, there are evidences that suggest a role of the C/N balance in the regulation of nitrate uptake and nitrate reductase activity (Dobao et al. 1994).

When cells were transferred from 100 mM NO_3^- to 100 mM NH_4^+, both assimilatory activities (Nas and NiR) decreased as it has also been described for many other organisms such as *Phormidium laminosum* (Tapia et al. 1996). Nevertheless, both activities increased progressively when NH_4^+ growing cells were transferred to fresh NO_3^- media. So, the regulatory pattern of assimilatory pathway in *Hfx. mediterranei* is similar to the pattern described for halotolerant bacteria (Hochman et al. 1987), bacteria (Moreno-Vivián et al. 1999) and cyanobacteria (Flores et al. 1983), i.e., ammonium causes an assimilatory nitrate pathway inhibition and nitrate is an inductor. Moreover, Nas activity was also detected in minimal medium supplemented with nitrite, which suggests that although nitrate is an inductor of the assimilatory pathway, nitrite also has positive effect on Nas induction. The results obtained from physiological experiments were in agreement with those obtained from the analysis of the expression of *nasA* and *nasD* genes, i.e, ammonium repressed the expression of the assimilatory genes. However, in the presence of nitrate or nitrite as nitrogen sources, *nasA* and *nasD* expression levels increased under different cultures conditions. This mechanism is a general control, which responds to the availability of the preferred nitrogen source: ammonium.

In addition to the short-term inhibitory effect of ammonium on nitrate uptake, ammonium can exert a negative effect on nitrate assimilation by affecting the activity levels of the enzymes involved in the assimilatory pathway. This ammonium effect, which was described many years before in bacteria and cyanobacteria (Flores et al. 1983), requires a more prolonged time scale to be expressed. Results presented in this work suggest that ammonium also causes the same long-term effect on both assimilatory activities, Nas and NiR. On the other hand, it has been observed that in the presence of ammonium nitrate or ammonium nitrite, ammonium is preferentially consumed by *Hfx. mediterranei* cells. In cyanobacteria, the

simultaneous presence in the medium of nitrate and ammonium is equivalent to that of ammonium alone (Flores et al. 1983).

Finally, the effect of MSX on *Hfx. mediterranei* growth and enzymes activities was also analysed. It has been described that MSX is an irreversible inhibitor of GS activity (Shapiro and Stadtman 1970). In preliminary experiments, we detected transferase GS activity in *Hfx. mediterranei* cells growing in minimal media with 25–100 mM KNO_3, and this activity was inhibited by MSX as it has been previously described for other microorganisms (Shapiro and Stadtman 1970; Tapia et al. 1995). The presence of MSX in the culture media caused a modification of the rate growth, since the ammonium assimilation was inhibited preventing glutamine synthesis as it has been reported so far (Franco et al. 1984).

It is known that the addition of MSX to cultures causes an increase of C/N ratio because the last is not assimilated. Under these conditions, there is N limitation, and consequently, the amino acids pool inside the cells decreases. The N deficit results in a selective increase of nitrogen-assimilating enzymes (Nas and NiR), which could be a physiological adaptation to facilitate the quick utilization of nitrogen much more effectively than in a normal situation. These results are similar to that obtained from *P. laminosum* (Tapia et al. 1995; Tapia et al. 1996) and *Plectonema boryanum* (Kikuchi et al. 1996). Furthermore, it has been reported that transcription of the *nirA* operon of *Synechococcus* sp. strain PCC 7942 is induced by the removal of ammonium from the medium or by the inhibition of ammonium fixation with MSX (Shapiro and Stadtman 1970). This induction could also explain the increase of halophilic Nas and NiR activities (Fig. 6), so it is possible to propose that glutamine or a related metabolite, rather than ammonium itself, could act as a repressor of nitrate utilisation. The reversion of negative ammonium effect on assimilatory nitrate pathway has also been described in the cyanobacterium *P. laminosum* (Franco et al. 1984).

Concluding remarks

The results reported show the effect of ammonium on assimilatory nitrate reduction in *Hfx. mediterranei*. In addition, it is also revealed that *Hfx. mediterranei* is able to assimilate ammonium via GS as well as GDH (Ferrer et al. 1996; Martínez-Espinosa et al. 2006), so GS-GOGAT pathway could also be operative in this halophilic archaeon.

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