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Characterisation of chlorate reduction in the haloarchaeon *Haloferax mediterranei*

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ABSTRACT

Background: Haloferax mediterranei is a denitrifying haloarchaeon using nitrate as a respiratory electron acceptor under anaerobic conditions in a reaction catalysed by pNarGH. Other ions such as bromate, perchlorate and chlorate can also be reduced.
Methods: Hfx. mediterranei cells were grown anaerobically with nitrate as electron acceptor and chlorate reductase activity measured in whole cells and purified nitrate reductase.
Results: No genes encoding (per)chlorate reductases have been detected either in the *Hfx. mediterranei* genome or in other haloarchaea. However, a gene encoding a chlorite dismutase that is predicted to be exported across the cytoplasmic membrane has been identified in *Hfx. mediterranei* genome. Cells did not grow anaerobically in presence of chlorate as the unique electron acceptor. However, cells anaerobically grown with nitrate and then transferred to chlorate-containing growth medium can grow a few generations. Chlorate reduction by the whole cells, as well as by pure pNarGH, has been characterised. No clear chlorite dismutase activity could be detected.
Conclusions: Hfx. mediterranei pNarGH has its active site on the outer-face of the cytoplasmic membrane and reacts with chlorate and perchlorate. Biochemical characterisation of this enzymatic activity suggests that *Hfx. mediterranei* or its pure pNarGH could be of great interest for waste water treatments or to better understand biological chlorate reduction in early Earth or Martian environments.
General significance: Some archaea species reduce (per)chlorate. However, results here presented as well as those recently reported by Liebensteiner and co-workers [1] suggest that complete perchlorate reduction in archaea follows different rules in terms of biological reactions.

1. Introduction

During the last 10 years, perchlorate (ClO_4^-) and chlorate (ClO_3^-) have been detected in several water supplies, ground waters, agricultural crops and even in soils as a result of human activities [2]. Perchlorate is used in the manufacture of propellants, explosives and pyrotechnic devices [3]. Perchlorate salts are extremely soluble, non-volatile, non-reactive and chemically very stable. The high water solubility and poor adsorption of perchlorate to soil and organic matter make its high mobility in the environment possible [4]. The concerns about perchlorate toxicity are its interference with iodide uptake by the thyroid gland, and the related potential carcinogenic effects [5]. Chlorate is present in several herbicides and defoliants, and it is released when chlorine dioxide (ClO_2) is used as a bleaching agent in the paper and pulp industry [6]. In humans, ClO_3^- may cause thyroid lesions and anaemia. Because of these health concerns, several organizations such as the World Health Organization or the Environmental Protection Agency has advised that (per)chlorate in water intended for human consumption should be minimized [7].

Perchlorate and chlorate are ideal electron acceptors for microorganisms due to their high redox potentials (ClO_4^-/Cl^- $E_o = 1.287$ V; ClO_3^-/Cl^- $E_o = 1.03$ V) [8]. It has been proposed that in perchlorate-respiring bacteria (PCRB) the (per)chlorate-reduction pathway consists of the (per)chlorate reductase, which sequentially reduces perchlorate to chlorate and in turn chlorate to chlorite (ClO_2^-), via sequential two-electron transfers [6,7]. Finally, chlorite dismutase transforms chlorite into chloride and oxygen [9–13]. Perchlorate-respiring bacteria (PCRB) are ubiquitous in the environment, and are mainly facultative anaerobes and denitrifiers [14,15]. Perchlorate reductases isolated from PCRB react with both perchlorate and chlorate [6], while chlorate reductases expressed by chlorate-respiring bacteria (CRB) do not reduce perchlorate [16]. It has also been demonstrated that perchlorate and chlorate reductases isolated from some PCRB recognize nitrate as substrate [17].

Nitrate is also often present in environments where perchlorate or chlorate is faced as contaminants [18]. In the denitrification pathway,

Abbreviations: DT, dithionite; MV, methylviologen; NarGH, respiratory nitrate reductase; PCRB, perchlorate-respiring bacteria; CRB, chlorate-respiring bacteria

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nitrate is sequentially reduced to dinitrogen gas: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ [19]. Several nitrate reductases involved in anaerobic nitrate reduction also reduce chlorate. However, these nitrate reductases have active sites facing the membrane potential negative side (nNars) and the nitrate transporters that deliver nitrate into the cytoplasm do not recognize chlorate, thus preventing the potentially damaging intracellular reduction of chlorate to cytotoxic chlorite. In previous studies on the respiratory nitrate reductase (NarGH) from *Haloferax mediterranei*, a denitrifying halophilic archaeon able to use nitrate as nitrogen source for growth or as electron acceptor under anaerobic conditions [20–22], it was demonstrated that this enzyme has an active site facing the membrane potential positive face (pNars) and is able to reduce chlorate [18]. The extra-cytoplasmic active site could be accessible to chlorate and so, this reaction might take place in the environment leading to the question of whether it could support growth and also whether it could reduce perchlorate, a substrate for which the role as electron acceptor in the reactions catalysed by nitrate reductases has been poorly described in haloarchaea.

Recent results reveal that (per)chlorate reductases establish a distinct group with the archaeal p-type NarG nitrate reductases as the closest relatives into dimethyl sulfoxide (DMSO) reductase family [23]. It has also been proposed that chlorate reduction was built multiple times from type II dimethyl sulfoxide (DMSO) reductases and chlorite dismutases [24,25]. This work summarises the biochemical characterisation of the NarGH chlorate reductase activity and discusses different strategies that might be used by haloarchaea to deal with the chlorite produced during chlorate reduction.

2. Materials and methods

2.1. Strains, media and growth conditions

Hfx. mediterranei (ATCC 33500T) was grown anaerobically with nitrate (100 mM) as electron acceptor as previously described [18], in a 25% (wt/vol) mixture of salts (25% SW) [26] and 0.5% yeast extract (complex media). Cultures with chlorate (100 mM) as electron acceptor were prepared in the same way. Growth was monitored by measuring the optical density at 600 nm. In some experiments, *Hfx. mediterranei* cells were grown as already described to induce the denitrification pathway (nitrate as electron acceptor). After that, cells were harvested by centrifugation at 15,000 ×*g* for 20 min at 4 °C in a Beckman J2-21 centrifuge, washed with 25% SW, centrifuged again at 15,000 ×*g* for 20 min at 4 °C and transferred to fresh anaerobic complex medium (25% SW and 0.5% yeast extract) containing chlorate at different concentrations ranging from 5 to 100 mM.

2.2. Purification of respiratory nitrate reductase and characterisation of the NarGH chlorate reductase activity

All the purification steps were carried out at 25 °C following the protocol previously described [20]. The chlorate reductase activity of the NarGH was measured using two different methods: i) colorimetric o-toluidine assay, which allows chlorate quantification [27] and ii) methylviologen method (substrate-dependent oxidation of reduced methylviologen) [28]. For the o-toluidine assay, the reaction mixture contained, in a final volume of 1 ml: 50 mM Tris–HCl pH (7–9), 0–2 M NaCl or KCl, 5 mM MV (electron donor), 50 mM $KClO_3$ (substrate), 10 mM $Na_2S_2O_4$ (freshly prepared in 0.1 M $NaHCO_3$) and 40 μl of pure enzyme (final protein concentration around 0.03 mg protein per ml was constantly present in the reaction mixture). The enzymatic activity was tested at temperatures between 25 and 70 °C, but most of the assays were developed at 35 °C. After 5 min of incubation to allow enzymatic reaction, 0.25 ml of o-toluidine (0.4 g/l) and 1.25 ml of concentrated HCl were added to the reaction mixture. O-Toluidine and HCl additions destroy the protein and as a consequence, the enzymatic reaction is stopped. The absorbance related to the yellow holoquinone finally produced in the colorimetric reaction was checked at 490 nm. NarGH chlorate reductase activity is expressed as micromoles of $KClO_3$ reduced per minute and chlorate reductase specific activity is expressed as micromoles $KClO_3$ reduced per minute per milligram of protein. All the assays were carried out in triplicate and against a control assay without enzyme. The control without enzyme was used for two different purposes: i) to check that there is no chlorate reduction when removing the enzyme and ii) to quantify chlorate concentration within the reaction mixture at zero time. This ensures that the kinetics of chlorate reduction take into account the chlorate concentration within the reaction mixture at zero time. To determine the optimal pH for chlorate reductase activity, 50 mM MES (pH 5.5.–6.7), 50 mM MOPS (pH 6.5–7.9) and 50 mM carbonate (pH 9–11) buffers were also prepared containing the aforementioned reaction mixtures. For the methylviologen assay, chlorate reductase activity was measured spectrophotometrically in quartz cuvettes equipped with rubber septa by monitoring the oxidation of reduced MV ($\varepsilon_{600\ nm} = 13{,}700\ M^{-1}\ cm^{-1}$) in the presence of chlorate at different temperatures [28]. The reaction mixture contained, in a final volume of 800 μl, 50 mM Tris buffer pH 8, 0–2 M NaCl or KCl, 5 mM MV and an appropriate amount of pure enzyme (final protein concentration around 0.03 mg protein per ml was constantly present in the reaction mixture). The assay mixture was flushed with nitrogen for 10 min, and 5 mM dithionite solution (degassed and freshly prepared in 0.1 M $NaHCO_3$) was added until an absorbance of 3.0 at 600 nm was obtained. The reactions, incubated for 1 min at 40 °C, were initiated by the addition of $KClO_3$ (nitrogen flushed) to a final concentration between 0 and 50 mM. Alternative electron acceptors were tested in the same assay system, except that chlorate was replaced by ClO_4^-, NO_3^-, IO_3^-, BrO_3^- and SeO_4^{2-} (potassium salts). All the assays were carried out in triplicate and against controls without enzyme or without the electron acceptors.

The MV assay method was also used to follow the chlorate reduction using whole cells previously grown with nitrate or chlorate as electron acceptors. In this case, harvested cells were resuspended in 50 mM Tris buffer containing 0.5 M NaCl up to a final O.D. around 0.2. The reaction mixture (1.2 ml final volume) contained 1 ml resuspended cells, 5 mM MV, 5 mM dithionite solution and 0–25 mM substrate. The assay was developed at room temperature. All the assays were carried out in triplicate and against controls without cells or without the electron acceptors. Data obtained by MV method were processed using the Michaelis–Menten equation. The values of V_{max} and K_m were determined by nonlinear regression analysis of the corresponding Michaelis–Menten curves (rate vs. $[ClO_3^-]$) using the algorithm of Marquartd–Levenberg with the SigmaPlot program (Jandel Scientific, version 1.02). The protein content was determined by the Bradford method, with bovine serum albumin (fraction V) as a standard.

In order to check the effect of other anions (e.g. bromate and (per) chlorate) on nitrate reduction catalysed by *Hfx. mediterranei* pNarGH, the nitrate reduction was also measured as previously described [20]. In that instances, the standard reaction mixture contained 4 mM MV (artificial electron donor), 18 mM KNO_3, and different chlorate, perchlorate and bromate concentrations (from 0 up to 18 mM). We followed the nitrite production by pNar using Griess method [29]. Nitrate reductase specific activity is expressed as micromoles of NO_2^- appearing per minute per milligram of protein [20].

3. Results

3.1. Chlorate reduction by Hfx. mediterranei cells

In order to analyze the capacity of *Hfx. mediterranei* cells to reduce chlorate, the cell growth was checked in anaerobic media using nitrate or chlorate as electron acceptors. When nitrate is present within the anaerobic media, denitrification is induced and as a consequence, *Hfx. mediterranei* is able to use nitrate as electron acceptor to support growth, as previously described [19–22,30]. However, no growth was

observed if chlorate was added as the unique electron acceptor to the anaerobic media. Specific growth rate (μ) and cell doubling time (t_d) for cultures under each of the assayed conditions are summarised in Table 1. These results suggest that this haloarchaeon is unable to express chlorate inducible genes coding for a (per)chlorate reductase system. Recently, the *Hfx. mediterranei* genome draft has been obtained and no genes encoding (per)chlorate reductases have been detected (www.ncbi.nlm.nih.gov/genome/?term=haloferax+mediterranei).

However, when nitrate-respiring cells (anoxic conditions) were harvested at the beginning of the stationary phase of growth, washed and transferred to a fresh anaerobic chlorate media, the cells were able to use chlorate as electron acceptor. In this case, the optical density reached at stationary phase of growth is 0.8 (after 150 h of batch culture), t_d is 27 h and a μ of 0.019 h^{-1} is achieved. When the cells pre-grown with nitrate were transferred into chlorate-containing medium more than twice, cells were not able to grow anymore. This suggests that pre-induced respiratory nitrate reductase could be involved physiologically in chlorate reduction supporting limited cell culture growth. In earlier studies, it was stated that (per)chlorate and nitrate reduction were catalysed by the same enzyme (a nitrate reductase) in bacteria [31]. This implies that the inability of several denitrifiers to grow using (per)chlorate is due to: (1) the failure to induced nitrate reductase in the presence of chlorate alone and (2) the toxicity chlorite produced by nitrate reductase when chlorite dismutase is absent.

This explanation is supported by experiments where chlorate, perchlorate and bromate reduction were measured using whole *Hfx. mediterranei* cells previously grown with nitrate as electron acceptor (Fig. 1A), where the order of the reaction velocities was $ClO_3^- > NO_3^- > BrO_3^- > ClO_4^-$, while no reduction activity was detected in presence of IO_3^- or SeO_4^-, similarly to the purified enzyme (Fig. 1B). This characteristic could be the explanation for the *Hfx. mediterranei* NarGH chlorate reductase activity.

3.2. Characterisation of the NarGH chlorate reductase activity

A preliminary report that pNarGH could reduce chlorate has previously been made [32]. A more detailed study was undertaken of this reaction in the present study to explore the use of this enzyme (and/or the whole cells) in waste water treatments. The capacity of pure NarGH to reduce substrates such as iodate, selenate, bromate and perchlorate has been checked. The results obtained (Fig. 1B) have been compared with the nitrate reduction. Consistent with the results from the whole cell experiments, no activity was detected when iodate or selenate were used as substrate. The absence of NarGH reactivity towards selenate was previously pointed out and it has been proposed that this fact could be taken into account to distinguish pNars from nNars (the latter type of Nars are highly reactive towards selenate) [22]. Nevertheless, pure *Hfx. mediterranei* NarGH reduced chlorate, bromate and perchlorate with reaction velocities in the order of 0.25 μmol min^{-1} mg^{-1}, 0.15 μmol min^{-1} mg^{-1} and 0.11 μmol min^{-1} mg^{-1}, respectively. The order of effectiveness was $ClO_3^- > NO_3^- > BrO_3^- > ClO_4^-$, which is the same pattern observed from the activity measurements using whole cells (Fig. 1A). This pattern correlates with that observed in whole cells suggesting it is due to the NarGH present in the membranes of these cells.

Table 1
Specific growth rate (μ) and cell doubling time (t_d) calculated from *Hfx. mediterranei* cultures anaerobically grown in complex media [20] in presence or absence of electron acceptor.

Electron acceptor	Optical density (600 nm) at stationary phase of growth	Batch culture time (hours)	μ (h^{-1})	t_d(h)
None	0.15	150	0.0058	79
Nitrate	2	150	0.11	6.5
Chlorate	0.15	150	0.0060	75
Nitrate growing cells transferred to chlorate media	0.8	150	0.019	27

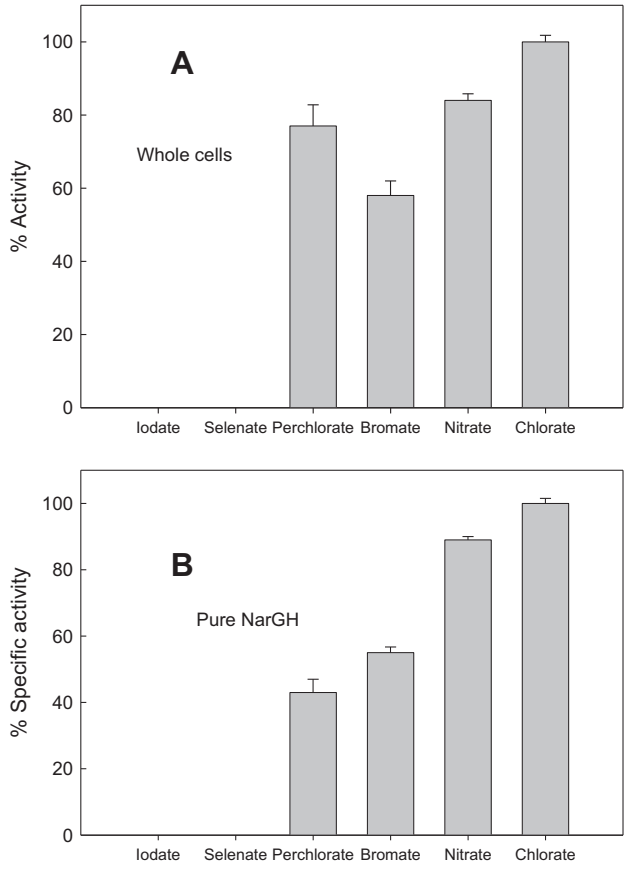

Fig. 1. Reduction of different substrates by whole *Hfx. mediterranei* cells (A). Cells were grown under anaerobic conditions with nitrate as electron acceptor as cited in Section 2. Harvested cells were resuspended in fresh complex culture medium containing 5 mM MV, 5 mM DT. Substrates final concentration was 20 mM. The reaction mixture was incubated at 40 °C for 15 min. 100% Activity = 0.76 U. Reduction of different substrates by pure NarGH (B). Assays were carried out in 50 mM Tris buffer pH 8 at 40 °C for 15 min. Substrates' final concentration was 30 mM. 100% Activity = 0.28 μmoles MV oxidized min^{-1} mg $prot.^{-1}$ MV assay was used in this experiment.

The pH dependence of enzyme activity in the range of 5 to 11 revealed that the optimal pH for pNarGH chlorate reductase activity was around pH 8 at 40 °C (which is the average temperature detected in the *Hfx. mediterranei* natural environment) (Fig. 2A). pNarGH nitrate reductase activity also showed maximum activity at pH values around 8 [20]. Bacterial chlorate reductases recently described had a temperature optimum between 40 and 70 °C, which is perhaps unexpected because most bacteria from which they were purified are mesophilic [16].

NarGH chlorate reductase activity was also measured at different NaCl or KCl concentrations at 40 °C (Fig. 2B). In those reaction mixtures containing NaCl the enzymatic activity increased when the NaCl concentrations were increased up to 1.4 M and at higher NaCl concentrations the activity remained stable. However, in those assays carried out in the presence of KCl, the enzymatic activity increased when the KCl concentrations were increased up to 1 M, but at higher KCl concentrations, a decrease in the specific enzymatic activity could be detected. This result could be related to the fact that in pNarGH the catalytic subunit is oriented to the positive side of the membrane [22] and in natural salt-marsh environments, NaCl is the predominant salt (instead of KCl which is predominant in the cytoplasm).

In another set of experiments, NarGH chlorate reductase activity was measured using an assay mixture with different NaCl concentrations at temperatures from 25 °C to 70 °C (Fig. 3). The maximum specific

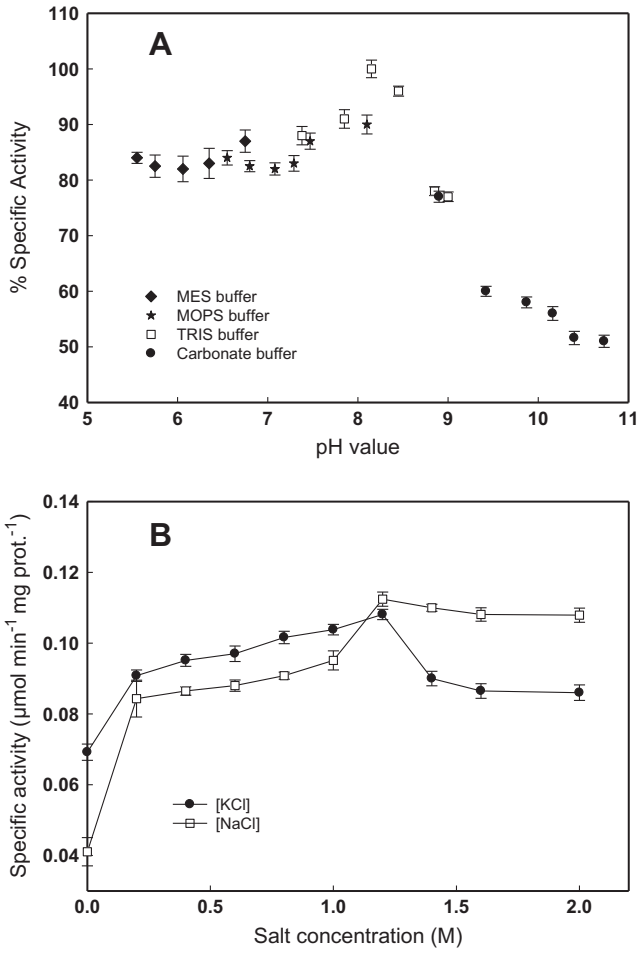

Fig. 2. Optimum pH (A) and effect of salts concentration on chlorate reductase activity (B) Reactions mixtures were incubated at 40 °C for 10 min. In panel A, 100% Activity correspond to 0.13 μmoles $KClO_3$ reduced min^{-1} mg $prot.^{-1}$. O-Toluidine assay was used in this experiment.

chlorate reductase activity was detected in the presence of low NaCl concentrations at 35 °C. At salt concentrations higher than 0.4 M, the maximum specific activity could be detected at higher temperatures (between 40 and 45 °C). The data was subject to an Arrhenius analysis that revealed that the lowest activation energy (around 2.2 ± 0.2 J mol^{-1} in the presence of 0.8–1.6 M NaCl) was observed at the highest NaCl concentrations (versus 5 ± 0.5 J mol^{-1} in the presence of 0–0.4 M NaCl. These results strongly support the fact that activation energy of a halophilic enzyme is lower at high salt concentrations. However, the higher the salt concentrations, the lower NarGH chlorate reductase activity was observed. This is not the pattern expected from halophilic enzymes, which are characterised by high enzymatic activity values at high salt concentrations. The pattern here described for NarGH chlorate reductase activity differs from the behaviour previously described for NarGH nitrate reductase activity. In the latter, nitrate reduction was not strongly dependent on temperature at different NaCl concentrations [20].

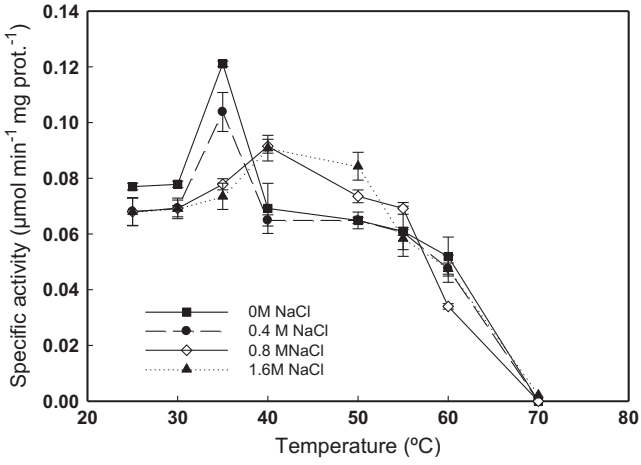

Fig. 3. Effect of temperature and salt concentration on chlorate reductase activity. Assays carried out in 50 mM Tris buffer pH 8, at 40 °C, 10 min. O-Toluidine assay was used in this experiment.

Like other halophilic nitrate reductases from the genus *Haloferax*, NarGH from *Hfx. mediterranei* presented a remarkable thermophilicity and worked well up to 70 °C with nitrate as a substrate and this activity did not show a direct dependence on salt concentration [20]. However, working with chlorate as substrate, NarGH exhibited higher specific activity at low temperatures in the presence of low salt concentrations (Fig. 3). This may reflect the different molecular geometry of the substrates (planar triangular for nitrate, triangular pyramidal for chlorate or tetragonal pyramidal for perchlorate).

Kinetic parameters of NarGH were determined using different concentrations of chlorate (as substrate), in the presence of 50 mM Tris buffer (pH 8.0) containing 0.2 M NaCl. The halophilic enzyme followed Michaelis–Menten kinetics. Apparent V_{max} and K_m values for chlorate were 0.280 ± 0.003 μmol min^{-1} mg $prot.^{-1}$ and 2.41 ± 0.16 mM, respectively. These values are higher than those described in several chlorate reductases isolated from bacteria such as *Pseudomonas chloritidismutans* [16], which is in agreement with the fact that although NarGH is able to reduce chlorate, it is not its natural substrate. The turnover numbers as well as the specificity constant have been obtained taking into account the molecular mass of the NarGH isolated from *Hfx. mediterranei* [20] and the V_{max} obtained from reactions where chlorate or nitrate act as substrates; results are summarised in Table 2.

Finally, nitrate reduction activity has been measured in the presence of different anions such as perchlorate, chlorate and bromate using pNarGH from *Hfx. mediterranei* or the whole cells. As previously mentioned, it is quite common to find environmental samples or waste water samples containing nitrate and (per)chlorate. We expected to see competitive inhibition of nitrate reduction due to the presence of (per)chlorate ions in the reaction mixture. That's why, we hypothesised that when the highest oxyanion concentration was added, the lowest nitrite concentration would be produced. However, the opposite effect was observed: the nitrite production was even more extensive when bromate, chlorate and perchlorate were present within the reaction mixture containing nitrate as substrate (Fig. 4). From these results, we conclude that in the presence of nitrate, these oxyanions are neither inhibitors nor alternative substrates. We suspect that the high redox potential defining each of those chemical compounds has an important role in this enzymatic mechanism. Thus, they could act as electron carriers in the reduction of nitrate to produce nitrite. Another interesting feature to highlight from these results is that the reaction in presence of nitrate + other oxyanion requires more time to take effect (nitrite is not detected prior to 2–3 min of incubation), but when nitrite production starts, the reaction is quicker than that taking place in absence of bromate, chlorate and perchlorate. These results are relevant when trying to explore the feasibility of bioremediation of waste water samples containing more than one of those compounds.

4. Discussion

The results reported here indicate that *Hfx. mediterranei* is able to use (per)chlorate as final electron acceptor in the absence of oxygen using the nitrate reductase (pNarGH) involved in denitrification process. Some recent reports support the existence of separate pathways for (per)chlorate and nitrate reduction under anaerobic conditions,

Table 2
Turnover numbers (Kcat) and kinetic constants for NarGH. Kinetic parameters related to nitrate where obtained at pH 8 in presence of 3.6 M NaCl and MV as electron donor. Regarding to chlorate, kinetic parameters where obtained in presence of 0.5 M NaCl and MV as electron donor. Activity is expressed as μmoles MV oxidized min^{-1} mg $prot.^{-1}$.

Substrate	K_m (mM)	V_{max} (μmoles MV oxidized min^{-1} mg $prot.^{-1}$)	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})	Reference
Nitrate	0.8	0.25	0.042	0.052	[20,22]
Chlorate	2.4	0.28	46	19	This paper

although they have not completely eliminated the potential of shared enzymes, being used for (per)chlorate and nitrate reduction in some bacteria [33]. The physiological study previously discussed suggests that chlorate respiratory enzymes are not inducible in *Hfx. mediterranei* by chlorate under anaerobic conditions, which make sense taking into account that genes coding for (per)chlorate reductases have not been identified in *Hfx. mediterranei* genome.

One interesting aspect to be pointed out is what happens to the chlorite produced by NarGH during chlorate reduction by *Hfx. mediterranei*. The analysis of the *Hfx. mediterranei* genome draft shows that the gene encoding a putative chlorite dismutase is present (Fig. 5), while no genes coding for (per)chlorate reductases have been detected either in the *Hfx. mediterranei* genome nor in other haloarchaea up to now. Database searches (HALOLEX: www.halolex.mpg.de/public/) have pointed out that chlorite dismutase gene from *Hfx. mediterranei* is a homolog to *pitA* from *Haloferax volcanii*, which is a fusion between chlorite dismutase-like and antibiotic biosynthesis monooxygenase-like domains within a single open reading frame. This fusion has been also described from other haloarchaea and may represent a modification to limited oxygen availability [34]. Preliminary studies to detect chlorite dismutase activity in *Hfx. mediterranei* whole cells and extracts have been carried out in our laboratory following oxygen production in the presence of chlorite using Durham tubes and oxygen electrodes. An activity toward chlorite could not clearly be identified either in whole cells or in cell extracts. Some recent work from some bacteria suggests that some chlorate reduction genes might constitute transposons flanked by insertion sequences which show the potential to move horizontally [24]. The phylogenetic analysis carried out using bacterial genomes reveals that chlorate reduction was evolved multiple times from type II DMSO reductases and chlorite dismutases [24]. It has also been suggested that chlorite dismutase has been mobilised at least once from (per)chlorate reducers to build chlorate respiration [24]. More studies focused on that subject should be done in archaea to understand how chlorate respiration was built in this domain.

Other studies from hyperthermophilic archaea highlight that although (per)chlorate can be used as electron acceptor using enzymes belonging to the type II subgroup of DMSO reductase family, no chlorite dismutase activity has been detected. In that case, the authors demonstrate that chlorite is eliminated by interplay of abiotic and biotic redox reactions involving sulphur compounds instead of being enzymatically split into chloride and oxygen [1]. This work has been carried out using *Archaeoglobus fulgidus*, which is a hyperthermophilic archaeon that thrive in environments resembling those of early Earth.

In the study presented here, we have not added specific sulphur compounds to check the mentioned interplay of abiotic and biotic redox reactions and no evidence on that subject has been reported so far from haloarchaea. So, it would be worth exploring these abiotic and biotic redox reactions involving sulphur in the future within in member of the *Halobacteriaceae* family. Regarding this, it is interesting to draw attention to: i) the natural salted water where these microorganisms live contains sulphur salts, ii) when growing the haloarchaea in the lab, salted water also contains sulphur salts (ammonium sulphate, magnesium sulphate, etc.) and iii) some of the protocols used to purify proteins from haloarchaeal involve buffers containing sulphur compounds (i.e. ammonium sulphate). Therefore, we cannot dismiss the possibility that abiotic and biotic redox reactions involving sulphur compounds take place in haloarchaea. Some works related to bacterial communities in marine sediments have also stated recently that there are (per)chlorate effects on metabolic pathways related to sulphur [35].

This research as well as other recent published reports suggest that haloarchaea can reduce perchlorate and chlorate anaerobically. This fact has recently been connected to studies of the anaerobic Martian environment where perchlorate amongst the salts as detected by the Phoenix Lander on Mars, may support halophilic life in a similar way to the halophilic environments on Earth [36].

The results presented show that (per)chlorate anions can be efficiently removed from the media where the *Hfx. mediterranei* cells are growing. Perchlorate and chlorate reduction take place efficiently thanks to *Hfx. mediterranei* cells, even in the presence of low salt concentrations. This fact could suggest that changes in the structure of the enzyme, and therefore the chlorate reductase activity, could be caused by salt.

If this is the case for other denitrifying halophilic archaea, then those types of microorganisms could be excellent models for the bioremediation of brines [37]. Most of the ground waters or waste waters contaminated with chlorate also contain nitrate [16] and several

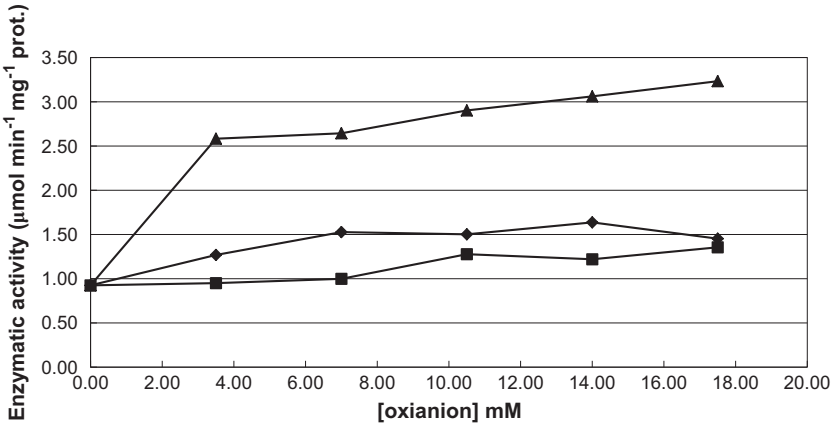

Fig. 4. Nitrate reductase activity in presence of different oxyanions. (Triangle: 18 mM nitrate + bromate; rhombus: 18 mM nitrate + perchlorate; square: 18 mM nitrate + chlorate). MV was used in this assay as electron donor. Nitrite production by pNarGH was follow using Griess method.

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H mediterranei          --MVEAPQTDEGWFALHDFRTVDWDAWRDAPEHERRRAIEEGVAYLNSHE 48
H volcanii              --MVEAPQTDEGWFALHDFRTVDWDAWRDAPDRERRRAIEEGVAYLDAHE 48
H lacusprofundi         --MVEAPQTEEGWFALHDFRSIDWDAWRDAPERERKRAIEEGKAFLKHRE 48
H turkmenica            MERRQPPQTEEGWYVLHDFRSIDWDAWRDAPERRRSRAIEEGIEYLSAAE 50
N magadii               MERRQPPQTDEGWYVLHDFRSIDWDAWRDAPEHRRSRALEEGIDYLTAAN 50

H mediterranei          AVEDAAEGTSAIFSVLGHKADFMVVHFRPTLDDISRAERQFEQTALAEFT 98
H volcanii              AVEDAEAGASAVFSVLGHKADFVVVHFRPTLDDISRAERQFERTALAAFT 98
H lacusprofundi         LVADADEGDSGLFSVLGHKADLLFVHFRPTLDDLSSIERRFEDTALANFT 98
H turkmenica            SVADAEEGDSATFAVLGHKADLLVLHLRPTLADLDALERRFEGTALAEFT 100
N magadii               DVADAEEGDSAVFSVLGHKADLLLLHLRPTLAALDTLERQFEQTALAEFT 100

H mediterranei          EQPTSYVSVTEVSGYVSDDYFEGNKEDIDTGLLRYIEGKLQPDIPDDTYM 148
H volcanii              EQPTSYVSVTEVSGYVSDDYFEGNEEDIDAGLLRYIEGKLKPDIPEDTYM 148
H lacusprofundi         ERTTSYVSVTEVSGYVSDEFFE-DPESVDTGLKRYIEGKMTPEIPDDEYV 147
H turkmenica            ERADSYLSVTEVSGYMSQDYFDEDAEVEDTGMARYIETRLKPEIPDSEFL 150
N magadii               ERADSYLSVTEVSGYMSQEYFEEDGEIEDTGTKRYIESRLKPTIPDSEFV 150

H mediterranei          SFYPMSKRRGEKHNWYDLPFDERRELMSVHGDTGRKYAGKIKQVIASSVG 198
H volcanii              SFYPMSKRRGEEHNWYDLSFDERRDLMSTHGDTGRQYAGKIKQVIASSVG 198
H lacusprofundi         CFYPMSKRRGEEYNWYDLSFEDRADLMADHGEVGKEYAGKIKQVIASSVG 197
H turkmenica            SFYPMDKRRGPEDNWYDLPFDERAEHLSSHGDIGKDYAGRVTQIISGSIG 200
N magadii               SFYPMDKRRGPEHNWYDLPFDERADHLSSHGELGRNYAGRVTQIISGSVG 200

H mediterranei          FEEFEWGVTLFGDDPTDIKDIVYEMRFDEVSAKYGEFGEFYVGRRFPPSD 248
H volcanii              FDDYEWGVTLFGDDPTDIKDIVYEMRFDEVSSKYGEFGQFYVGRRFPPSD 248
H lacusprofundi         FDSHEWGVTLFGSDPTDIKDIVYEMRFDPASSRYGEFGEFYIGRRFPPED 247
H turkmenica            LDDFEWGVTLFGDDPTDVKELLYEMRFDPSSSRFAEFGRFLSARRFPPED 250
N magadii               LDDFEWGVTLFADDPTDVKELLTEMRFDPSSSAFAEFGRFLSARRFPPAN 250

H mediterranei          LGAFLAGDAVPT----SEFGDESHHHAHAHG-EGGHHHGEGGHAHGEDGH 293
H volcanii              LGAFLAGDGVPT----SEFGDESHHGAHAHG-EGG-HHGEGG-----DGH 287
H lacusprofundi         LGAYFAGETVPTPAGDGDTGDTEDGHGHAHG-EGHDHAGSGGGS--AHGD 294
H turkmenica            LGAFLAGERIPR-----EGEESHGEHPHAGGESGGHHHGESGGHHEGSGD 295
N magadii               LGAFLAGEEVP--------QEQTSGHQHAGG--HGHGNGNGHGHGSDSSG 290

H mediterranei          HHGESGHGHGEGGHH-------GDSDDEA----DETDIRGQLDDLNIY 331
H volcanii              HH----HDDGDGDHPH-------GDDGDEA----ADEDIRGQLEDLNIY 321
H lacusprofundi         HPHGEEETSGEGDHPHSGEEGGHGGEDGDDP----SDADIRGELADLNIY 340
H turkmenica            HHHGDSSSSGRGDHGGS-------GGPHGD-----DDEDLRSELEDMGVY 333
N magadii               HHHGDSG-HGHGHGHGS-------GDPHDDAGADEDDESVRSELEELGVY 332

H mediterranei          AGKPHGEDVYATVLYSEADADEVFEEVEGLRGNFDHYPTHVKTAVYEAND 381
H volcanii              AGKPHGEDVYATVLYSEADADELFEEVEGLRGNFDHYPTHVKTAVYEANE 371
H lacusprofundi         AGKPSGEDVYATVLYSEADVDELFDEVEGLRGNFDHYGTHVKTAVYEGRV 390
H turkmenica            AGQPHGEDVHAVVLYSAADAEELFEEVDGLRGNFDHYDTHVKTAVYEPQD 383
N magadii               AGQPHGEDVHAVVLYSAADAGELFEEVEGLRTNFDHYDTHVKTAVYEPQN 382

H mediterranei          RD---RNAVVSIWETASAAETAAGFLSELPGIVERAGEESG--FGTMGMF 426
H volcanii              RG---RVAVVSIWETASAAETAAGFLSELPGIVERAGEGSG--FGTMGMF 416
H lacusprofundi         TD---RAAVVSIWDTASAAETAAGFLSELPEVVARAGEESG--FGTMGMF 435
H turkmenica            GGDDSETAVVSLWETERAASTAAGFLADLPDIVRQAGDDEGDSWGTMGMF 433
N magadii               DDSDAETAVVSLWETDRAANTAAGFLADLPDIVRQAGDDEDDSWGTMGMF 432

H mediterranei          YTVKPEHREDFVEKFGVVGGLLDDMDGHFDTDLMVNLEDENDMFIASQWR 476
H volcanii              YTVKSEHRGDFVEKFGTVGGLLEEMDGHFDTDLMVNVEDEDDMFIASQWR 466
H lacusprofundi         YTVKPEHQEDFTDTFDDVGEILAEMDGHVETDLMMNVEDENDMFIASQWH 485
H turkmenica            YSVKPEHRGDFLGTFEEAGELLAEMDGHRKTDLLINREDENDMFIASRWD 483
N magadii               YTVKPEHRGDFIGVFDDAASILAEMDGHRKSDLLVNREDENDMFIASRWD 482

H mediterranei          SQEDAMGFFRSDEFRDTVQWGRDVLADRPRHVFLA 511
H volcanii              SQDDAMEFFRSDAFRDTVQWGRDVLADRPRHVFLA 501
H lacusprofundi         AKEDAMAFFGSDEFRETVQWGREVLADRPRHVFLA 520
H turkmenica            SREDAMQFFRSDAFSEAVEFGRDVLTDRPRHVFLA 518
N magadii               SREDAMQFFRSDEFAETVEFGRDVLADRPRHVFLA 517
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Fig. 5. Protein sequence alignment *Hfx. mediterranei* chlorite dismutase-like protein with *Hfx. volcanii* PitA and *Halorubrum lacusprofundi* B9LRB6, *Haloterrigena turkmenica* D2RQG0 and *Natrialba magadii* D3STR5 using Clustal 2.0.12. Histidine residues lying in the region linking chlorite dismutase-like and monooxygenase-like domains are in bold and underlined.

previous studies have shown that (per)chlorate and nitrate are simultaneously degraded by several bacteria [38]. A better understanding of the factors that regulate the expression of the enzymes involved in (per) chlorate, chlorate, and nitrate reduction are important as several microorganisms could play a key role in water bioremediation. The processes based on bioremediation could replace or even improve those protocols where perchlorate and nitrate are removed from brines by ion exchange techniques [5]. Several studies have highlighted that (per)chlorate-reducing bacteria removed (per)chlorate at such slow rates to make them impractical for application in treatment systems. Moreover, the enzymes involved in this pathway are only active at low salt concentrations [5]. Other previous studies reported rapid

perchlorate and nitrate removal using mixed cultures in presence of 10% NaCl [39]. Other systems use permeable barriers containing vegetable oil that would promote the degradation of (per)chlorate [7]. However, each process has a limitation, namely salt tolerance.

5. Conclusions

Respiratory nitrate reductases, mainly those belonging to the pNarGH group [20] may have a relevant role in (per)chlorate reduction in those halophilic archaea lacking genes coding for (per)chlorate reductases. Biological per(chlorate) reduction by ancient archaea might have taken place during pre-anthropogenic times thanks to (per)chlorate reductases or even nitrate reductases. As a consequence of these enzymatic activities members of the archaea domain may have prevented perchlorate accumulation in early Earth giving rise to the environmental conditions characterising the Earth [1]. Taking into account the biochemical parameters defining NarGH chlorate reductase (reported here) and NarGH nitrate reductase activities in *Hfx. mediterranei* [20,22], waste water treatment approaches could consider the relevance of halophilic denitrifiers to explore a role in bioremediation in the near future. Preliminary results obtained in our lab reveal that chlorate removal by *Hfx. mediterranei* cells is more efficient than per(chlorate) removal. In those media containing 5 mM chlorate, the final chlorate concentration quantified after 150 h of incubation was 0.2 mM. On the other hand, nitrate reduction is not inhibited in the presence of either (per)chlorate or bromate, it can even be concluded that bromate is able to slightly stimulate nitrate reduction (see results summarised in Fig. 4). So, the same microorganism could reduce nitrate and chlorate in presence of other ions thanks to the nitrate reductase under microaerobic or anaerobic conditions. These results are quite interesting in terms of waste water bioremediation purposes because most of the waste water samples containing nitrate also contain chlorate and other oxyanions. The removal ratio for chlorate estimated in our study is around 4.8 mM chlorate in approximately 6 days. Although the removal process is not really fast, the removed concentration is one of the highest described at the time of writing when using microorganism for waste water bioremediation [40,41]. Furthermore, one of the advantages of using *Hfx. mediterranei* cells or its pNarGH is that nitrate reduction is not inhibited in the presence of (per)chlorate (and vice versa) at high salt concentrations. These results open a new way to explore bioremediation processes making haloarchaeaon-based remediation of brines and waste waters feasible.

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