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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.

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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

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