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Perchlorate and chlorate reduction by the Crenarchaeon *Aeropyrum pernix* and two thermophilic Firmicutes

Martin G. Liebensteiner,[1] Martijn W.H. Pinkse,[2,3] Bart Nijsse,[4] Peter D.E.M. Verhaert,[2,3] Nicolas Tsesmetzis,[5] Alfons J.M. Stams[1,6] and Bart P. Lomans[7]*

[1]*Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB, Wageningen, The Netherlands.*

[2]*Analytical Biotechnology Section, Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands.*

[3]*Netherlands Proteomics Centre, Julianalaan 67, 2628 BC Delft, The Netherlands.*

[4]*Laboratory of Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.*

[5]*Shell International Exploration and Production Inc., 3333 Highway 6 South, Houston, TX 77082, USA.*

[6]*Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.*

[7]*Shell Global Solutions International B.V., Kessler Park 1, 2288 GS, Rijswijk, The Netherlands.*

Summary

This study reports the ability of one hyperthermophilic and two thermophilic microorganisms to grow anaerobically by the reduction of chlorate and perchlorate. Physiological, genomic and proteome analyses suggest that the Crenarchaeon *Aeropyrum pernix* reduces perchlorate with a periplasmic enzyme related to nitrate reductases, but that it lacks a functional chlorite-disproportionating enzyme (Cld) to complete the pathway. *Aeropyrum pernix*, previously described as a strictly aerobic microorganism, seems to rely on the chemical reactivity of reduced sulfur compounds with chlorite, a mechanism previously reported for perchlorate-reducing *Archaeoglobus fulgidus*. The chemical oxidation of thiosulfate (in excessive amounts present in the medium) and the reduction of chlorite result in the release of sulfate and chloride, which are the products of a biotic–abiotic perchlorate reduction pathway in *Ae. pernix*. The apparent absence of Cld in two other perchlorate-reducing microorganisms, *Carboxydothermus hydrogenoformans* and *Moorella glycerini* strain NMP, and their dependence on sulfide for perchlorate reduction is consistent with the observations made on *Ar. fulgidus*. Our findings suggest that microbial perchlorate reduction at high temperature differs notably from the physiology of perchlorate- and chlorate-reducing mesophiles and that it is characterized by the lack of a chlorite dismutase and is enabled by a combination of biotic and abiotic reactions.

Received 27 August, 2015; accepted 27 August, 2015. *For correspondence. E-mail Bart.Lomans@shell.com; Tel. (+3170) 447 5754; Fax (+3170) 447 5974.

Introduction

Both dissimilatory reduction of perchlorate (ClO_4^-) and chlorate (ClO_3^-) are microbial traits that occur under oxygen-free conditions, yielding energy for growth and maintenance. The microbial reduction of chlorine oxyanions, namely of chlorate applied as herbicide, was first reported in 1928 (Aslander, 1928). Microorganisms that grow by the reduction of chlorate or perchlorate mainly affiliate with the Proteobacteria and have a mesophilic and facultative anaerobic lifestyle (Coates and Achenbach, 2004).

The functional difference between perchlorate- and chlorate-reducing microorganisms is reflected by the enzymes involved. Respective microorganisms use a perchlorate reductase [reducing perchlorate to chlorate and chlorite (ClO_2^-)] or a chlorate reductase (reducing chlorate to chlorite), both followed by an enzyme disproportionating chlorite to dioxygen and chloride, called chlorite dismutase (Cld) (van Ginkel *et al.*, 1996; Kengen *et al.*, 1999; Danielsson Thorell *et al.*, 2003). Perchlorate and chlorate reductases are two distinct classes of enzymes that differ in structural, biochemical and evolutionary aspects (Nilsson *et al.*, 2013).

Interests in the physiology of chlorate- and perchlorate-reducing microorganisms got particularly stimulated by their potential role in the biological remediation of sites heavily contaminated with man-made perchlorate. Chlorate and perchlorate, both of health concern when released to the environment, have been manufactured by the chemical industry for a wide range of applications

(Parker, 2009). However, the presence of chlorate and perchlorate on Earth and thus the development of enzymes reducing chlorine oxyanions may date back already to pre-anthropogenic eras (Michalski *et al*., 2004; Kounaves *et al*., 2010; Rao *et al*., 2010). Geophysical studies of recent years have demonstrated several (photo)chemical mechanisms of natural chlorine oxyanion formation that lead to a continuous introduction of chlorate and perchlorate on Earth and Mars (Catling *et al*., 2010; Rao *et al*., 2010). Ancient chlorine oxyanion deposition on Earth has therefore possibly influenced the evolution of early prokaryotic enzymes reducing chlorine oxyanions used for energy conservation. The degree of homology of perchlorate reductases with nitrate reductases suggests an evolutionary relatedness of these enzymes (Bender *et al*., 2005; Clark *et al*., 2013). The substrate ambiguity of numerous enzymes in the DMSO enzyme family (using chlorate and perchlorate, next to the canonical substrates) may have evolved in ancestral enzymes already. Recent studies confirmed that perchlorate reduction can also be found beyond the bacterial domain of life in Archaea (Liebensteiner *et al*., 2013; Oren *et al*., 2014). However, in both chlorate- and perchlorate-reducing mesophiles, horizontal transfer of genes coding for key enzymes also seems to play a major role for the acquisition of the trait (Melnyk *et al*., 2011; Clark *et al*., 2013). The related nitrate reductases have a wide dispersal over diverse groups of prokaryotes. Also, here, a complex evolution of these enzymes involving several mechanisms, amongst which horizontal gene transfer, was described (Jones *et al*., 2008).

Recently, perchlorate-reducing and chlorate-reducing capabilities of thermophilic (Balk *et al*., 2008; Nepomnyashchaya *et al*., 2012; Alves *et al*., 2013) and hyperthermophilic (Liebensteiner *et al*., 2013) microorganisms were described. In the current study, we examined hyperthermophilic and thermophilic microorganisms for the ability to grow by the reduction of chlorate and perchlorate. The Crenarchaeon *Aeropyrum pernix*, a microorganism that has thus far been known for its strictly aerobic lifestyle, was identified as the first member of its phylum that is able to grow by the reduction of chlorate and perchlorate. It extends microbial perchlorate reduction up to 90°C (and possibly to 100°C). This physiological characteristic of *Ae. pernix* was investigated in more detail by genomic and proteomic analyses. Similarly, two thermophilic Firmicutes, *Carboxydothermus hydrogenoformans* and strain NMP, related to *Moorella glycerini*, showed the capability to grow by perchlorate reduction. The presented work enlarges the current knowledge on perchlorate-reducing microorganisms at high temperatures and confirms the characteristic important role of sulfur compounds for this trait, in the absence of Cld.

Results

Screening for perchlorate and chlorate reduction in thermophilic microorganisms

In this study, we examined the ability of hyperthermophilic and thermophilic microorganisms to reduce and grow by the reduction of the chlorine oxyanions perchlorate and chlorate. Selected microorganisms exhibit optimum growth temperatures between 55°C and 90°C and belong to phylogenetically diverse phyla; some also carry genes resembling the ones of functional chlorite dismutase (Cld). The group of test organisms consisted of *Caldibacillus debilis* Tf, *Thermoplasma volcanium* GSS1, *Thermus scotoductus* SE-1, *Marinithermus hydrothermalis* T1, *Sulfolobus solfataricus* P2, *Carboxydothermus hydrogenoformans* Z-2901, *Moorella glycerini* NMP and *Aeropyrum pernix* K1.

Three out of the eight microorganisms tested were able to grow efficiently using chlorine oxyanions as electron acceptors. These include the hyperthermophile *Ae. pernix* (Fig. 1), which was previously described as a strict aerobe (Sako *et al*., 1996). By monitoring routinely added sulfur components (thiosulfate, sulfate) in the medium (DSM 820), it was demonstrated that, similar to *Archaeoglobus fulgidus* (and sulfide), perchlorate reduction by *Ae. pernix* resulted in a concomitant oxidation of thiosulfate (compare Fig. 2A and B), present in the medium described for that archaeon. In addition, two Gram-positive bacteria of the phylum Firmicutes, namely the strict anaerobe *Car. hydrogenoformans* (strain Z-2901) (Svetlichny *et al*., 1991) and strain NMP, affiliated with the type strain of *Mo. glycerini* (strain JW/AS-Y6) (Slobodkin *et al*., 1997), were also able to reduce perchlorate (Figs S1A and S2) and chlorate (Fig. S1B) in the presence of sulfide.

Perchlorate and chlorate reductase (PcrAB/ClrABC) genes in the studied microorganisms

No genes that closely resemble known perchlorate reductases (PcrAB) or chlorate reductases (ClrABC) (Clark *et al*., 2013) are found in the genome of *Ae. pernix*. However, a putative Nar-type reductase (alpha subunit: APE_1288.1, ref: NP_147849.2) is encoded in the genome, which is also found in the second described *Aeropyrum* species, *Ae. camini* (100% coverage, 95% identity). APE_1288.1 has a high sequence similarity with the periplasmic alpha subunit of the functionally characterized Nar-type reductase (pNar) of *Pyrobaculum aerophilum* (97% coverage, 59% identity) (ref: WP_011009509.1). Similar to this enzyme, APE_1288.1 carries a twin-arginine translocation (TAT) signal sequence, which is indicative for a periplasmic location of the catalytic subunit. The pNar of *P. aerophilum* was

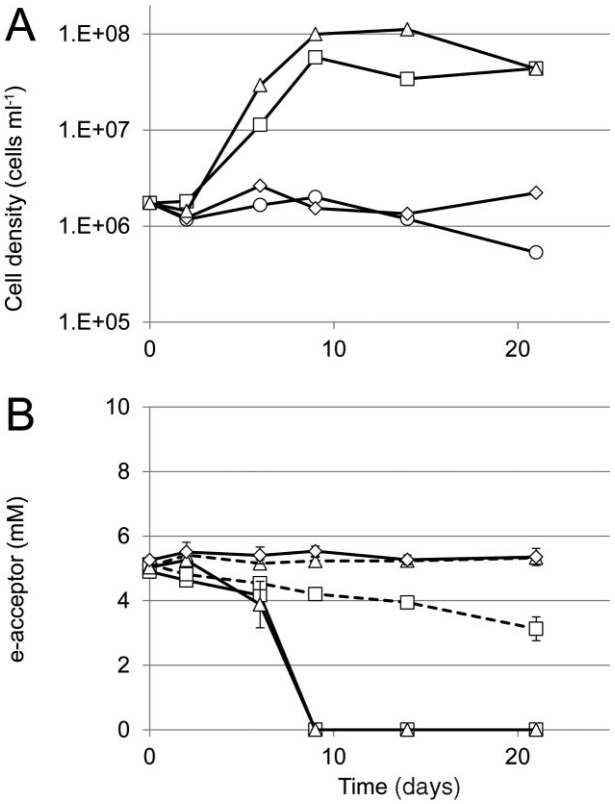


Fig. 1. Growth (A) and electron acceptor utilization (B) of *Aeropyrum pernix* in anaerobic medium (DSM medium 820) at 90°C containing either perchlorate (triangles), chlorate (squares), nitrate (diamonds) or no additional e-acceptor (circles); solid lines indicate biological experiments (2% inoculum) and dashed lines (uninoculated) chemical controls; plotted are means ($n = 2$) ± range bars in (B). Medium contained peptone (5 g l^{-1}) and yeast extract (1 g l^{-1}) as growth substrates.

reported earlier to use chlorate, besides nitrate as substrate (Afshar *et al*., 2001); perchlorate was not tested as substrate with this enzyme.

The genomes of both *Car. hydrogenoformans* (Wu *et al*., 2005) and *Mo. glycerini* NMP also lack genes that closely resemble known perchlorate reductases (PcrAB) or chlorate reductases (ClrABC), but harbour genes for enzymes that belong to the DMSO II enzyme family, most closely resembling respiratory nitrate reductases. The alpha subunit of the nitrate reductase of *Car. hydrogenoformans* (Chy_2082, YP_360901.1) is predicted to be located outside of the cell, using PRED-TAT (Bagos *et al*., 2010). This characteristic has also been found for the putative perchlorate-reducing enzymes of *Ae. pernix* as mentioned above and *Ar. fulgidus* (Liebensteiner *et al*., 2013). The best enzyme candidate for the reduction of chlorate and perchlorate in strain NMP (also predicted to have an extracellular catalytic subunit) has a high similarity with the nitrate reductase of *Car. hydrogenoformans* (alpha subunits: 99% coverage, 73% identity) and with a molydopterin oxidoreductase in *Moorella thermoacetica* ATTC 39073 (alpha subunits: 99% coverage, 93% identity).

Chlorite dismutase (Cld) genes in the studied microorganisms

In the hyperthermophile *Ae. pernix*, a protein (APE_0237.1; NP_147071.2) is encoded in the genome (Kawarabayasi *et al*., 1999) that belongs to the same protein family as functional chlorite dismutases (Cld) (Pfam06778), but the overall similarity with respective enzymes is very low. Additionally, there was no Cld activity observed in *Ae. pernix* cell extracts or whole cells (grown with perchlorate) upon chlorite injection. A closer examination of this Cld-like protein in *Ae. pernix* revealed the absence of earlier defined signature residues present in functional Cld (Ile88, Trp97, Leu122, Arg127, Glu167 – position refers to *Nitrobacter winogradskyi*) (Mlynek *et al*., 2011).

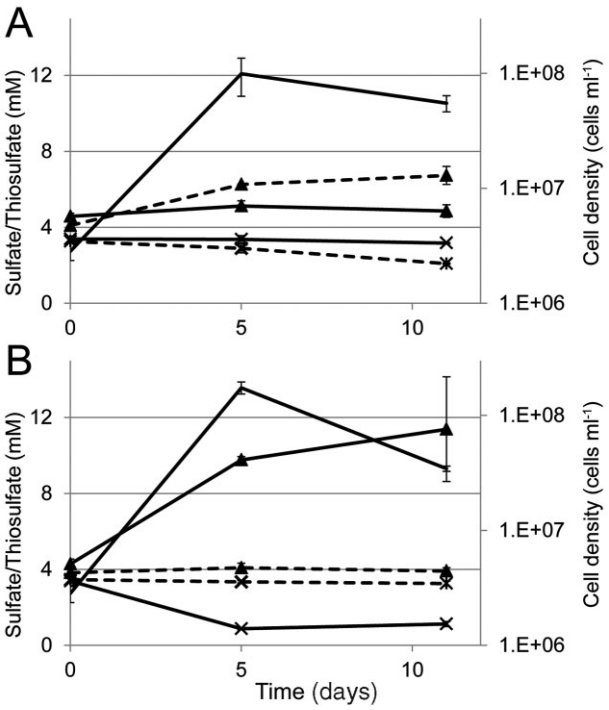


Fig. 2. Oxidation of thiosulfate during growth of *Aeropyrum pernix* with either oxygen (A) or perchlorate (B). Primary *y*-axis shows thiosulfate (crosses) and sulfate concentrations (triangles); cell densities (solid lines/no symbols) are plotted against secondary *y*-axis. Dashed lines indicate change of respective sulfur compounds in (uninoculated) chemical controls. Perchlorate and oxygen concentrations are not displayed. Means ($n = 2$) ± range (bars). Note that the expected stoichiometry where 2 chlorite molecules react with 1 thiosulfate molecule to form 2 sulfate molecules (Fig. 3) was roughly met in the experiment [all available perchlorate (7.9 mM) was depleted after 5 days]. Also, the activity of the cultures in Fig. 2 was higher compared with that in Fig. 1.

Table 1. The expression of selected proteins of *Aeropyrum pernix* grown with oxygen, or anaerobically with chlorate or perchlorate as electron acceptors. An extended table listing differentially abundant proteins (five times more) can be found in Table S1.

Protein	Locus tag	Spectral counts					
		Oxygen		Chlorate		Perchlorate	
Cld-like protein	APE_0237.1	18	19	21	18	43	40
Heme–copper oxidase subunit II	APE_0792.1	20	17	2	0	0	1
Heme–copper oxidase subunit I + III	APE_0793.1	6	5	0	0	0	0
Sulfate adenylyltransferase	APE_1197.1	40	42	3	0	6	1
Nitrate reductase, alpha subunit	APE_1288.1	641	683	778	837	497	492
Nitrate reductase, beta subunit	APE_1294.1	199	186	264	275	146	152
Nitrate reductase, gamma subunit	APE_1297	14	17	21	23	8	9
Molybdopterin oxidoreductase, iron–sulfur binding subunit	APE_2605.1	25	23	21	16	3	0
Molybdopterin oxidoreductase, membrane subunit	APE_2607	18	21	18	18	1	0
Molybdopterin oxidoreductase, molybdopterin binding subunit	APE_2610	298	285	203	165	22	5

In the case of the two bacterial strains, neither the draft genome of strain NMP nor the genome of *Car. hydrogenoformans* encode any protein resembling known chlorite dismutases. Additionally, no chlorite dismutase activity was detected with whole cells or cell-free extracts of strain NMP; *Car. hydrogenoformans* was not tested.

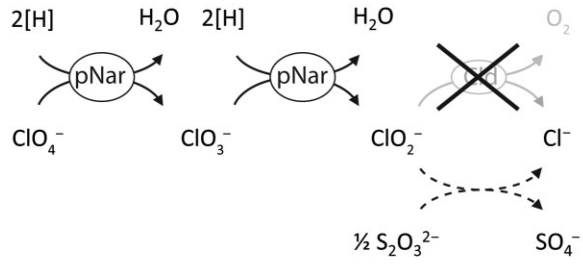
Proteome analysis of Ae. pernix

To obtain more understanding of the perchlorate-reducing metabolism in *Ae. pernix*, a proteome analysis was conducted. The presence and abundance of proteins in cells grown with oxygen, chlorate or perchlorate were compared. The proteome analysis of *Ae. pernix* cells grown with oxygen, chlorate and perchlorate resulted in coverage of *c.* 60% of the protein-coding genes. The best candidate for perchlorate and chlorate reduction, the aforementioned putative Nar-type reductase (alpha subunit: APE_1288.1), was surprisingly abundant at about similar levels under all three growth conditions (Table 1).

APE_0237.1, the chlorite dismutase family protein of *Ae. pernix*, had a low number of total spectral counts, which remained in the same range under all different growth conditions (Table 1). However, the fate of biologically formed chlorite does not only depend on the presence of a chlorite-disproportionating enzyme (Liebensteiner *et al.*, 2013; Martinez-Espinosa *et al.*, 2014), but may, due to its high chemical reactivity, also be determined by reducing chemicals in the medium (Liebensteiner *et al.*, 2014a). In the cultures of *Ae. pernix*, the chemical reactivity of chlorite seemed to coincide with the oxidation of thiosulfate (Fig. 2).

Oxidation of sulfur compounds by chlorine oxyanions

The role that reduced sulfur compounds play in the biological reduction of chlorate and perchlorate in microorganisms that lack a chlorite-disproportionating enzyme was reported previously (Liebensteiner *et al.*, 2013) and seems to be crucial for perchlorate reduction in *Ae. pernix* as well (Fig. 3). Enzymes related to the sulfur metabolism of the archaeon showed differential expression in chlorate-, perchlorate- and oxygen-grown cells (Table 1). A putative ATP sulfurylase (APE_1197.1) is significantly more abundant in aerobically grown cells. The function of that enzyme is not completely clear. As thiosulfate has a positive effect on aerobic growth (Sako *et al.*, 1996), the enzyme might play a role in thiosulfate oxidation to sulfate (Fig. 2). The ability of heterotrophic microorganisms to gain energy from



1) $ClO_4^- + 4[H] \rightarrow ClO_2^- + 2H_2O$ $\Delta G^{0'} = -448.8$ kJ biological ——

2) $ClO_2^- + \frac{1}{2}S_2O_3^{2-} + \frac{1}{2}H_2O \rightarrow Cl^- + SO_4^{2-} + H^+$ $\Delta G^{0'} = -617.1$ kJ chemical - - -

Fig. 3. Proposed mechanism of perchlorate reduction in *Aeropyrum pernix* and thermodynamics of biologically (Eq. 1; solid lines) and chemically mediated branches (Eq. 2; dashed lines) of this metabolism under standard conditions (pH 7, 25°C). In mesophilic perchlorate-reducing microorganisms, Eq. 1 is commonly catalysed by perchlorate reductases (Pcr); however, a periplasmic nitrate reductase (pNar) in *Ae. pernix* may carry out the same reaction. In the absence of a functional chlorite dismutase (Cld), biologically formed chlorite is abiotically reduced with thiosulfate (Cld) in *Ae. pernix* (Eq. 2).

the oxidation of thiosulfate in the presence of oxygen has been reported for several microorganisms (Sorokin, 2003). Also, for *Ae. pernix* cultures grown with oxygen, growth-stimulating effects of thiosulfate and its biological oxidation were demonstrated before (Sako *et al*., 1996). The strongly increased abundance of a molybdopterin oxidoreductase related to tetrathionate reductase enzymes (APE_2605.1, APE_2607, APE_2610) in oxygen- compared to perchlorate-grown cells (Table 1) seems to be related to that. Tetrathionate reductase has been shown to catalyse thiosulfate conversion to tetrathionate (Whited and Tuttle, 1983). The molybdopterin oxidoreductase subunits are repressed when cells were grown with chlorate or perchlorate respectively (Table 1). This might be attributed to an inability of transferring electrons derived from thiosulfate oxidation to electron acceptors other than oxygen. Also, a potentially faster chemical reactivity between chlorite and thiosulfate (Liebensteiner *et al*., 2014a) than between oxygen and thiosulfate (Fig. 2A, dashed lines) may compete with the biological oxidation of thiosulfate, and combined with the above-mentioned aspect, it can be the cause of lower abundances of a putatively thiosulfate-oxidizing enzyme (APE_2605.1, APE_2607, APE_2610).

For the two perchlorate-reducing members of the Firmicutes tested in this study, it seems probable that complete perchlorate reduction proceeds in a similar manner as described for *Ar. fulgidus* and *Ae. pernix*, where the absence of Cld is compensated by chemical chlorite elimination, involving reduced sulfur compounds.

Metabolic flexibility in Ae. pernix

Besides perchlorate reduction, there are indications for an even broader metabolic flexibility in *Ae. pernix*. The increased abundance of a putative arsenite oxidase in presence of oxygen (but in the absence of arsenite) (Table S1) may indicate a pre-adapted metabolic preference.

Additionally, the putative tetrathionate reductase encoded in the genome (which was discussed earlier) may get expressed under anaerobic conditions in case tetrathionate is present. This would possibly enable another anaerobic respiration pathway next to perchlorate reduction in *Ae. pernix*.

Discussion

In the current study, we examined hyperthermophilic and thermophilic microorganisms for the ability to grow by the reduction of chlorate and perchlorate. The presumed strictly aerobic Crenarchaeon *Ae. pernix*, and two thermophilic Firmicutes, *Car. hydrogenoformans* and strain NMP, related to *Mo. glycerini*, showed the capability to grow by perchlorate reduction. Unlike *Ae. pernix* and *Car. hydrogenoformans*, strain NMP was not capable of reducing chlorate.

The difficulty of strain NMP to grow with chlorate may be related to the kinetics of the perchlorate-reducing enzyme combined with the lack of a functional Cld. Higher specific activities of perchlorate-reducing enzymes (including pNar) with chlorate compared with perchlorate were shown earlier (Kengen *et al*., 1999; Steinberg *et al*., 2005; Martinez-Espinosa *et al*., 2014). Thus, it seems possible that chlorate reduction exceeds the abiotic reduction rates of chlorite with sulfide (in case chlorate is offered as electron acceptor instead of perchlorate), causing the accumulation of toxic chlorite, which terminates chlorate reduction.

Properties and localization of perchlorate and chlorate reductases

The predicted periplasmic-localized subunit of Nar-type reductase (pNar) from *Ae. pernix*, APE_1288.1, has a high sequence similarity to the periplasmic alpha subunit of the functionally characterized pNar of *P. aerophilum*, which was reported earlier to use chlorate, besides nitrate as substrate. While the V_{max} was slightly higher with chlorate compared with nitrate, the K_m was about 2.5-fold increased with chlorate (Afshar *et al*., 2001). Despite the fact that perchlorate was not tested as a substrate for *P. aerophilum*'s pNar enzyme, the pNar's capability of reducing perchlorate next to nitrate, chlorate and bromate in another archaeon, *Haloferax mediterranei*, was recently demonstrated (Martinez-Espinosa *et al*., 2014).

A common characteristic of hyperthermophilic and thermophilic perchlorate-reducing microorganisms known thus far is the periplasmic location of putative perchlorate-reducing enzymes. In the respective microorganisms (*Ae. pernix*, *Car. hydrogenoformans* and strain NMP), perchlorate reduction seems to be catalysed by enzymes resembling Nar-type nitrate reductases. Nar-type nitrate reductase can reduce chlorate (Moreno-Vivian *et al*., 1999) but are commonly located in the cytosol, where they form toxic chlorite upon chlorate exposure. However, in some microorganisms, the catalytic subunit of Nar-type reductases and related enzymes are located outside the cell (Martinez-Espinosa *et al*., 2007). Such pNar enzymes, which have demonstrated activity towards perchlorate and chlorate (Martinez-Espinosa *et al*., 2014), enable microorganisms to grow by the reduction of chlorine oxyanions even in the absence of a chlorite dismutase (the key enzyme of chlorate- and perchlorate-reducing mesophiles) (Liebensteiner *et al*., 2013; Oren *et al*., 2014).

Other microorganisms tested in this study, which were incapable of reducing chlorate and perchlorate (see the

Experimental procedures section), had no putative Nar-type reductases encoded in their genomes that exhibit N-terminal TAT signal peptides.

Nitrate reductases of the Nar-type as well as perchlorate and chlorate reductases are normally negatively regulated by oxygen (Cabello *et al*., 2004; Bender *et al*., 2005; Lindqvist *et al*., 2012). However, in this study, the best candidate for perchlorate and chlorate reduction, the aforementioned putative pNar-type reductase, was surprisingly abundant at about similar levels under all three growth conditions. Former proteomic studies of *Ae. pernix* grown with oxygen have also resulted in high expression levels of the Nar-type complex (Palmieri *et al*., 2009). However, *Ae.* pernix is not able to grow by nitrate reduction (Sako *et al*., 1996). Transcriptomic analyses of *P. aerophilum* demonstrated that its pNar is also expressed independently of the presence or absence of oxygen and nitrate (Cozen *et al*., 2009). Considering the close relatedness of pNar of *P. aerophilum* with the putative nitrate reductase in *Ae. pernix*, the similarity in the regulation of the two enzymes seems plausible. Based on the observation made by Palmieri and colleagues (2009), the authors debated whether *Ae. pernix* was a real strict aerobe. The microorganism's ability to grow in the absence of oxygen shown in this study experimentally verifies their earlier hypothesis.

Chlorite conversion

The Cld-like protein in *Ae. pernix* lacks the earlier defined signature residues that are present in functional Cld (Mlynek *et al*., 2011). From the broad diversity of assigned Cld-like proteins in bacterial and archaeal microbes, only a few carry the respective signature residues and for a small number of these chlorite-disproportionating activity was confirmed (Maixner *et al*., 2008; Mlynek *et al*., 2011). Additionally, some Cld-like proteins may play a role other than that of a chlorite-disproportionating enzyme, like in heme biosynthesis (Mayfield *et al*., 2013).

Although earlier studies reported chlorite-disproportionating activities in perchlorate-grown members of the Firmicutes (Balk *et al*., 2008; 2010), neither the draft genome of strain NMP nor the genome of *Car. hydrogenoformans* encode any protein resembling known chlorite dismutases. As such, the mechanism for complete perchlorate reduction observed in this study seems to differ from the other reported Firmicute perchlorate reducers.

Meso- versus thermophilic perchlorate and chlorate reduction

The current study broadened the knowledge on high temperature perchlorate reduction and strengthened the idea that classical microbial chlorate and perchlorate reduction is primarily found in mesophilic microorganisms. There is consistency in the observation that all up-to-now described hyperthermophiles and thermophiles [with one exception, *Moorella perchloratireducens* (Balk *et al*., 2008)] lack chlorite-disproportionating activity. Similar to *Ar. fulgidus*, *Ae. pernix*, *Car. hydrogenoformans* and strain NMP also couple the complete reduction of chlorate and perchlorate to the oxidation of sulfur compounds. In *Ae. pernix*, chlorite is reduced to chloride anions by the chemical oxidation of available thiosulfate to sulfate, whereas in *Car. hydrogenoformans* and strain NMP sulfide in the medium proved essential for the initiation of perchlorate reduction.

Substrate ambiguity of evolutionary-related enzymes, like nitrate reductases and perchlorate reductases, seems to enable the reduction of chlorine oxyanions beyond classical pathways found in mesophiles (employing chlorite dismutase) (Liebensteiner *et al*., 2014b). A broadened substrate range of these, in particular periplasmic-oriented, molybdenum enzymes, may have competitive advantages for microorganisms possessing these enzymes. Especially in the frame of the early co-occurrence of nitrate, (chlorate) and perchlorate on Earth (Navarro-Gonzalez *et al*., 2001; Michalski *et al*., 2004; Kounaves *et al*., 2010), this consideration seems intriguing.

Summarizing, this study expands the diversity of perchlorate-reducing microorganisms growing at high temperatures and reports the first Crenarchaeon growing by this metabolism. The results so far suggest that microbial perchlorate reduction at extremely elevated temperatures is characterized by the lack of chlorite dismutase and is enabled by periplasmic perchlorate-reducing enzymes, followed by abiotic chlorite elimination. In contrast to mesophiles, perchlorate reduction in thermophiles can occur through this abiotic chlorite elimination due to the higher reactivity of chlorite at elevated temperatures. The occurrence of the perchlorate metabolism as demonstrated for microorganisms in this study is likely restricted to natural redox transition environments (such as hydrothermal vents, high temperature microbial mats) as well as natural anaerobic environments that are contaminated by human activity where both the more oxidized chlorine oxyanions and the more reduced sulfur compounds are available.

Experimental procedures

Strains, media and cultivation

A selection of thermophilic and hyperthermophilic microorganisms were purchased from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures – and cultivated according to the recommendations of

the supplier (media, substrates, growth temperatures); *Ts. scotoductus* SE-1, DSM 8553 (DSM medium 878, 65°C); *Ma. hydrothermalis* T1, DSM 14884 (DSM medium nr. 973, 70°C); *Cal. debilis* Tf, DSM 16016 (DSM medium nr. 220, 55°C); *Car. hydrogenoformans* Z-2901, DSM 6008 (DSM medium nr. 507, 70°C); *Ta. volcanium* GSS1, DSM 4299 (DSM medium nr. 398, 60°C); *S. solfataricus* P2, DSM 1617 (DSM medium 182; 70°C) and *Ae. pernix* K1, DSM 11879 (DSM medium nr. 820, 90°C).

The main substrates of the above-mentioned media were yeast extract (1 g l^{-1}) and tryptone (1 g l^{-1}) for *Ts. scotoductus* and *Ma. hydrothermalis*; peptone from casein (15 g l^{-1}) and peptone from soy meal (5 g l^{-1}) for *Cal. debilis*; pyruvate (20 mM) for *Car. hydrogenoformans*; glucose (5 g l^{-1}) and yeast extract (1 g l^{-1}) for *Ta. volcanium*; yeast extract (1 g l^{-1}), casamino acids (1 g l^{-1}), yeast extract (1 g l^{-1}) and peptone (5 g l^{-1}) for *Ae. pernix*.

Due to enhanced evaporation at elevated temperatures, also aerobic cultivations were performed in 120-ml serum bottles (filled with 50 ml of medium) sealed with butyl stoppers. Prior to autoclaving, the headspace gas was replaced with air, stoppered and sealed.

Next to these above-mentioned cultures, also strain NMP was included in this study. This microorganism was isolated in our laboratory (on perchlorate and methanol) from an enrichment previously obtained from an underground gas storage site (Balk *et al*., 2008) and turned out to be affiliated with *Mo. glycerini* (Slobodkin *et al*., 1997) (99% sequence identity, 96% coverage). In contrast to the type strain of *Mo. glycerini*, strain NMP was not able to use glycerol as substrate. The medium used for cultivating strain NMP was described earlier (Stams *et al*., 1993); a bicarbonate/CO_2 and phosphate-buffered medium containing yeast extract (0.2 g l^{-1}) and sulfide (1.25 mM) at neutral pH. Methanol or formate was used as substrate for the growth of strain NMP at 60°C.

Several stocks for media preparations were prepared anaerobically, separately autoclaved and added to the media after sterilization. These included electron acceptors for anaerobic growth studies (sodium chlorate, sodium perchlorate), electron donors (sodium formate, methanol) and media additives (sodium sulfate, sodium thiosulfate, sodium bicarbonate, sodium sulfide). Vitamin solutions and the sodium pyruvate stock (for *Car. hydrogenoformans*) were filter-sterilized and added to the medium after autoclaving.

Chlorate and perchlorate reduction trials

Growth studies on chlorine oxyanions were conducted in 120-ml bottles containing 50 ml of oxygen-free medium (see above) using either chlorate or perchlorate as electron acceptors (5 mM each). Microorganisms grown under standard conditions were used as inoculum (5%). The headspace gas for anaerobic media was a N_2/CO_2 (80/20; v/v; 1.5 bar) gas mixture (for strain NMP, *Car. hydrogenoformans* and *Ta. volcanium*) or pure N_2 (for anaerobic incubation of *Ae. pernix*, *S. solfataricus*, *Cal. debilis*, *Ma. hydrothermalis* and *Ts. scotoductus*). Growth study lasted up to 3 weeks; potential growth was investigated visually and by quantifying the reduction of chlorate and perchlorate in the media.

In the trial determining sulfur fluxes during perchlorate reduction of *Ae. pernix* (Fig. 2), the concentration of sulfate in the medium was lowered to 4 mM to increase the analytical accuracy for additionally formed sulfate. The standard medium used for growing *Ae. pernix* (DSM nr. 820) contained 4 mM sodium thiosulfate and 23 mM sodium sulfate.

All experiments throughout the study were performed with biological duplicate.

Chromatographic analyses and Cld activity

Oxyanions were measured by high-performance liquid chromatography (HPLC) equipped with an Ion Pac AS22 column (4 × 250 mm) and an ED 40 electrochemical detector (Dionex, Sunnyvale, CA). The eluent was carbonate-bicarbonate buffer (1.29 g l^{-1} $Na_2CO_3{\cdot}10H_2O$ and 0.12 g l^{-1} $NaHCO_3$) and the analyses were conducted with a flow rate of 1.2 ml min^{-1} at 35°C. Sodium iodide (NaI) was used as an internal standard.

Methanol and fatty acids were analysed on an HPLC system comprising a Varian column (MetaCarb 87H Guard 4.6 × 50 mm, Middelburg, The Netherlands) and a UV and refractive index detector. The eluent contained sulfuric acid at a concentration of 10 mM and L-arabinose was used as internal standard. The analyses were performed at 30°C with a flow rate of 0.8 ml min^{-1}. The software ChromQuest was used for analysing the chromatograms later.

For *Ae. pernix* and strain NMP, the potential activity of a chlorite-disproportionating enzyme was tested in crude extracts and cell suspensions by using a Clark electrode detecting the formation of oxygen (Wolterink *et al*., 2002). Cell-free extracts were prepared as described earlier (Liebensteiner *et al*., 2013) and assays performed in anaerobic phosphate buffer and under oxygen-free conditions at 60°C. Control experiments were included, substituting cell-free extracts (or whole cells) with anaerobic water or leaving out the addition of chlorite as substrate. As a positive control, cell-free extract of a known chlorate-reducing microorganism, *Alicycliphilus denitrificans* strain BC (Weelink *et al*., 2008), was used at 30°C.

Genome sequencing and proteome analysis

Paired-end sequencing of genomic DNA of strain NMP was performed at Baseclear (Leiden, Netherlands). A de novo assembly was done using the de novo assembler Ray (Boisvert *et al*., 2012). Pilon (Walker *et al*., 2014) and manual curation was used for assembly improvement afterwards. The assembled scaffolds were annotated by using a Semantic Annotation Pipeline for Prokaryotes (SAPP). This annotation included (amongst others) predicted rRNA genes and predicted proteins, and InterProScan was used for protein functional analysis. It was stored in an RDF data model. PRED-TAT was used to predict the subcellular location of proteins of interest (Bagos *et al*., 2010).

The proteome analyses of *Ae. pernix* cells grown with oxygen, and anaerobically with chlorate or perchlorate, were performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Lasonder *et al*., 2002). For all three conditions, independent duplicates of 500 ml of cell suspensions were grown until the late exponential phase and harvested by centrifugation. Ultrasonication and chemical

disintegration using 4% SDS in PBS followed by centrifugation were used to obtain cell-free extracts. Prior to loading the samples on a SDS-PAGE, an incubation step of 95°C for 10 min was included. As a control of sample quality, an equal amount of total protein was separated by SDS-PAGE on a 10-well SDS-PAGE 10% Bis-Tris Gel (Mini Protean System, Bio-Rad, USA) for 90 min at a constant voltage of 120 V using Tris–SDS as running buffer. Label-free quantitative proteomics type experiments were carried out to identify differentially expressed proteins under different growth conditions (oxygen, chlorate and perchlorate). Equal amounts of the protein extracts were loaded onto a Novex 4–12% Bis-Tris–SDS page gel (Invitrogen) and electrophoresed for 5 min at 200 V constant voltage using MES-SDS as running buffer. For each lane, a single band containing all proteins was cut out and treated for reduction and alkylation using 20 mM dithiothreitol and 40 mM iodoacetamide in 50 mM ammonium bicarbonate. Digestion was performed by incubating the samples overnight at 37°C with trypsin at a 1:20 enzyme–protein ratio. Peptides were diluted with 5% formic acid and 5% dimethylsulphoxide and subjected to nano-LC-MS/MS using a Thermo Easy-nLC 1000 and an Orbitrap Q-Exactive Plus instrument (Thermo Fisher Scientific). Each peptide sample was auto-sampled and separated over a 25 cm analytical column (75 μm inner diameter) in-house packed with ReproSil-Pur C18 AQ 5-μm reversed phase material (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with a 60-min gradient from 5% to 40% acetonitrile in 0.6% acetic acid. The effluent from the column was directly electrosprayed into the mass spectrometer. Full MS spectra were acquired in the positive ion mode from *m*/*z* 300–1200 at a resolution of 70 000 after accumulation of 3×10^6 ions within a maximal injection time of 250 ms. A top20 method was used to acquire MS^2 spectra at a resolution of 17 500 after accumulation of 1×10^5 ions within a maximal injection time of 50 ms. Precursor ions were isolated with a 2.5 *m*/*z* window and fragmented with a normalized higher-energy collisional dissociation (HCD) energy of 28. Only multiply charged ions were selected and the dynamic exclusion time was set to 30 s. Raw data were analysed using Proteome Discoverer 1.4 (Thermo Fisher Scientific), and Mascot 2.2 (Matrix Science) was used as search engine. A database containing all protein entries of *Ae. pernix* listed in Uniprot was used to search the data. Search settings used were as follows: 5 ppm for parent ions, 0.02 Da for fragment ions, trypsin as proteolytic agent, carbamidomethyl cysteine as fixed modification and methionine oxidation as variable modification. Scaffold 3.0 (Proteome Software) was used to merge all search results. Filtering of the data was done by setting the minimum protein threshold to 99%, the minimum peptide count to 2 and the minimum peptide threshold to 95%.

The genome of *Ae. pernix* is publicly available and encodes 1752 genes, from which 1700 genes are predicted to be protein-coding (Kawarabayasi *et al*., 1999; Yamazaki *et al*., 2006).

The raw proteome analysis resulted in the identification of 993 different proteins (with at least two unique peptides identified). A likelihood ratio G-test for independence (Sokal and Rohlf, 1994) with a null hypothesis of equal protein distribution between the different conditions was applied. The null hypothesis was rejected in case of differential expression of a gene at levels of significance of $p \leq 0.05$ or $p \leq 0.01$. Proteins five times more abundant in one condition compared with another are listed in Table S1. Proteins that possibly play an important role under different growth conditions are discussed in more detail in the text (Table 1).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Reduction of perchlorate (triangles) (A) and chlorate (diamonds) (B) by *Carboxydothermus hydrogenoformans* Z-2901 in sulfide-reduced medium at 70°C. Pyruvate was used as electron donor (not measured) and acetate accumulated as product (crosses). Increase of biomass is indicated by protein concentration (dashed line) over time. Data points are means of $n = 2$.

Fig. S2. Reduction of perchlorate (ClO_4^-, triangles) by *Moorella glycerini* strain NMP at 60°C. Methanol (circles) (A) or formate (squares); the dashed line represents perchlorate reduction in absence of sulfide (methanol was not quantified in this setup) (B) were used as substrates and acetate (crosses) accumulated over time. Complete reduction of perchlorate resulted in formation of chloride anions (Cl^-, diamonds). Plotted data are means of $n = 2$.

Table S1. Proteins of *Aeropyrum pernix* that were found to be at least five times more abundant under one growth condition compared with another ('>>>'). Different levels of significance are indicated (**$p \leq 0.05$ and ***$p \leq 0.01$).