Microbial (Per)chlorate Reduction in Hot Subsurface Environments

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Chapter 1

General introduction and thesis outline

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Microbial redox reactions

Elements on Earth, such as carbon, nitrogen or sulfur are chemically and biologically transformed from their most reduced (methane, ammonia, sulfide) to their most oxidized states (carbon dioxide, nitrate, sulfate) and vice versa. Such processes, if enzymatically mediated, yield energy that microorganisms use for anabolic functions like growth, motility and maintenance. The metabolic and phylogenetic diversity of microorganisms that mediate sulfur, carbon or nitrogen biotransformations has received a lot of attention and much understanding in how microorganisms participate in global biogeochemical cycles has been gained. Some of the more recent and spectacular findings in these areas were for instance the anaerobic oxidation of methane and ammonium.

At a time when no oxygen was yet present on Earth, life existed based on the generation of energy from anaerobic respiration processes. The microbial reduction of metals and elemental sulfur is estimated to stand at the evolutionary beginning of life around 3.5 billion years ago (1). A long time after, namely 2.5-2.1 billion years ago, the great oxygenation event on Earth took place (2). The cause of it was the evolution of oxygenic photosynthetic microorganisms resulting in the release of free dioxygen to the atmosphere.

The physiology of anaerobic microorganisms is characterized by different respiratory processes such as CO_2 reduction to methane, sulfur and sulfate reduction or the reduction of metals (e.g. Fe^{3+}, Mn^{4+}) and nitrate. These different forms of microbial respiration occur in diverse anaerobic environments (that differ in temperature, pH, salinity etc.) and phylogenetically distant groups of microorganisms.

Besides oxygenic photosynthesis other anaerobic processes have been described that produce oxygen enzymatically, namely by the disproportionation of nitrogen and chlorine oxyanions (3, 4). It was speculated whether these metabolic routes may have existed already in ancestral life forms and if their occurrence might even have preceded the evolution of photosynthesis (3, 5). Such considerations are particularly tempting in context of the growing evidence for the deposition of chlorine and nitrogen oxyanions since early Earth (6, 7).

Particularly the yet expanding diversity of microorganisms that grow by the reduction of chlorate (ClO_3^-) and perchlorate (ClO_4^-) [together termed (per)chlorate] (5, 8) may indicate that this metabolism is still underestimated in its ecological and phylogenetic distribution.

This thesis presents the outcome of a study investigating chlorate- and perchlorate-reducing microorganisms, their occurrence in hot environments and the deep subsurface. It gives insight in a novel variation of the metabolism known from mesophilic bacteria and interprets the results also in terms of industrial applications based on microbial chlorate and perchlorate reduction.

In this chapter the source of perchlorate and chlorate will be discussed, followed by an overview of (per)chlorate-reducing microorganisms and the current knowledge of their metabolism. The final section will give a brief overview on oil reservoirs and oil production.

Inorganic chlorine oxyanions

Human-made chlorate has been extensively used as herbicide and in the paper and pulp industry, where it is converted to chlorine dioxide. Chlorine dioxide and hypochlorite are common disinfectants and bleaching agents. They are very reactive and form chlorine compounds of different oxidation states, like chlorite, chlorine anions and chlorate. The production of chlorate in North America was 2.1 million tons in year 2000 and around 3 million tons worldwide (9). However, the chlorine oxyanion most widely dispersed in nature is perchlorate. Both, anthropogenic and natural sources of (per)chlorate are found in the environment (10). Perchlorate has been manufactured for more than 100 years, mainly for its use as explosive and rocket propellant in the form of ammonium perchlorate (11). Especially after WWII an increased demand in perchlorate raised its production to an estimate of 18 million kg per year in the United States only (9). The current production of perchlorate is difficult to determine, since perchlorate is a classified strategic compound in the United States (11).

Former disposal practices in the aerospace, military and chemical industry are the main cause for perchlorate found in groundwater and surface waters. It was reported that 15.9 million kg of perchlorate were released to the environment since the 1950s (12), causing a threat to the environment and human health due to the toxicity of the compound. This raised also interests in the (biological) remediation of perchlorate-polluted sites and drove scientific research on (per)chlorate-reducing bacteria. The use of respective microorganisms was discussed for the treatment of contaminated waste (13) and ground waters (14). Furthermore, (per)chlorate reducers were proposed for the *in-situ* bioremediation of soils (15).

Especially research of the last decade led to much evidence and insight on the natural formation of perchlorate. In contrast to the anthropogenically caused pollutions, natural formation and deposition of perchlorate involves much lower concentrations of perchlorate and is not locally concentrated (6). The most significant natural accumulation of perchlorate on Earth is found in the Atacama Desert in Chile, where it is co-deposited with nitrate (16). The formation of sizable depositions in the Atacama Desert and the recently discovered perchlorate accumulations on Mars are both of atmospheric origin. The atmospheric formation and introduction on Earth was also proposed for chlorate, which was detected in caliches and soils,

groundwater and precipitation samples (17).
Several mechanisms have been proposed for the natural formation of perchlorate such as electrochemical discharge reactions (18), the oxidation of chloride by ozone (19), and photochemically-mediated processes in the atmosphere (20). While there is still diverging opinions about the most significant mechanisms for natural perchlorate formation there is consensus about its permanent deposition on Earth, most probably from a stratospheric source. Perchlorate accumulates only in arid environments (e.g. Atacama Desert, Antarctic dry valleys) which is likely attributed to the inactivity of microorganisms in the absence of water (6). Elsewhere on Earth perchlorate is thought to be biologically reduced to chloride anions.
Perchlorate has been found in groundwater samples from pre-anthropogenic times and some estimates about when natural perchlorate formation and deposition on Earth started, range up to millions of years ago (6, 21). Taking an average deposition rate of perchlorate on Earth of 3.6 g/km^2/yr (6) then around 1.8 million kg of perchlorate are deposited on Earth every year (also considering the water surface). This rough estimate of natural perchlorate deposition even exceeds the anthropogenic release of perchlorate reported by Xu et al. (12).
This permanent introduction of perchlorate on Earth since pre-anthropogenic ages has represented a valuable source of energy for microorganisms and may have affected the evolution of (per)chlorate-reducing enzymes.

(Per)chlorate-reducing microorganisms

The chlorinated oxyanions perchlorate (ClO_4^-) and chlorate (ClO_3^-), contain chlorine in an oxidized form (+VII; +V). They are chemically stable (especially perchlorate), their salts are highly soluble in water (in M range) and the high redox potential (Table 1) makes them ideal electron acceptors for microorganisms, comparable to those of oxygen or nitrate respiration.
In the early 20th century, the first scientific observations of microbial reduction of chlorate were reported, but it took another 50 years before the first axenic (per)chlorate-reducing bacterium was isolated and described (13). Particularly research on model microorganisms, such as *Wolinella succinogenes* HAP-1, *Ideonella dechloratans*, *Azospira oryzae* GR-1, *Dechloromonas agitata* CKB and *Dechloromonas aromatica* RCB has resulted in more insight in the physiology and genetics of (per)chlorate reduction in the following decades (4, 22-25).
Almost 100 strains of (per)chlorate-reducing microorganisms have been obtained over the last 40 years, although the number of publicly deposited organisms is much lower. The vast majority of (per)chlorate-reducing bacteria are facultative anaerobes affiliated to the phylum of Proteobacteria, predominantly belonging to

Table 1: Standard reduction potentials ($E^{0'}$) of selected redox couples.

N_2O/N_2	+ 1355 mV
ClO_2^-/Cl^-	**+ 1199 mV**
$2NO/N_2O$	+ 1175 mV
O_2/H_2O	+ 820 mV
ClO_4^-/ClO_3^-	**+ 788 mV**
Fe^{3+}/Fe^{2+}	+ 772 mV
ClO_3^-/ClO_2^-	**+ 709 mV**
MnO_2/Mn^{2+}	+ 380 mV
NO_2^-/NH_4^+	+ 440 mV
NO_3^-/NO_2^-	+ 430 mV
NO_2^-/NO	+ 350 mV
HSO_3^-/HS^-	- 110 mV
CO_2/CH_4	- 240 mV
SO_4^{2-}/HSO_3^-	- 516 mV

Reduction potentials (circumneutral pH; 25°C) were retrieved from Thauer et al. (26) and Wolterink (27). In bold are redox couples involved in perchlorate reduction.

the class of β-Proteobacteria. Apart from that two (per)chlorate-reducing members of the Gram-positive Firmicutes were identified recently that couple (per)chlorate reduction to growth (28, 29). Most of the isolated (per)chlorate-reducing bacteria are mesophiles (30), although (per)chlorate reduction is also thermodynamically favorable at elevated temperatures (31). (Per)chlorate-reducing bacteria are mostly strict respirers, coupling the reduction of (per)chlorate to the oxidation of acetate as electron donor. In addition, other organic electron donors have been reported to enable (per)chlorate reduction, such as alcohols (28, 29), organic acids (4, 24), aromatic (25, 32) and aliphatic hydrocarbons (33). Inorganic electron donors like hydrogen, ferrous iron, zero-valent iron (34) or sulfide, thiosulfate and elemental sulfur (35) are also used by (per)chlorate-reducing microbes. (Per)chlorate reduction has been reported to occur at heterotrophic and autotrophic conditions (36). Often (per)chlorate reducers are able to utilize nitrate or oxygen besides perchlorate and chlorate. (Per)chlorate-reducing microorganisms have been described and isolated

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from a diverse range of environments, comprising pristine and hydrocarbon-polluted soils, aquatic sediments, paper mill waste sludge, farm animal waste lagoons (24, 30, 37, 38), sea water and saline lake water samples (39), activated sludge (4, 23, 35), digester sludge (22), an underground gas storage site (28, 29) and from a bioelectric reactor (40). Latest results presented in this thesis show that (per)chlorate-reducing microorganisms can further be found in submarine hot springs (41), marine solfataric thermal vents (42) and terrestrial hot springs (43).

The metabolism of microbial (per)chlorate reduction in known (per)chlorate-reducing microorganisms is based on the action of perchlorate reductase (Pcr), which reduces perchlorate (via chlorate) to chlorite, followed by the disproportionation of chlorite by chlorite dismutase to chloride and oxygen (Fig. 1). Chlorate-reducing microorganisms that cannot reduce perchlorate employ an enzyme that is genetically and structurally different to Pcr, namely chlorate reductase (Clr) (44-46).

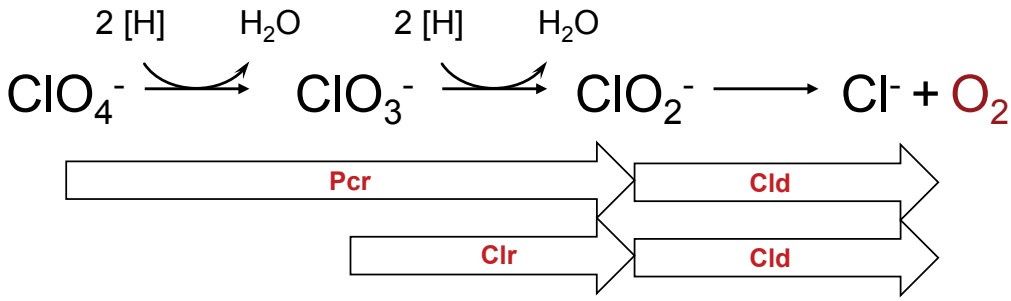

Figure 1: Complete microbial (per)chlorate reduction involving perchlorate reductase (Pcr) and chlorite dismutase (Cld) in perchlorate-reducing microorganisms or chlorate reductase (Clr) combined with chlorite dismutase in chlorate reducers. The final step (the disproportionation of chlorite) forms dioxygen under anoxic conditions (and in the absence of light).

Oxygen formed under anaerobic conditions by the action of Cld is subsequently reduced by a terminal oxidase (47) or used by oxygenases for the activation of recalcitrant substrates. This allows for instance the *de facto* aerobic degradation of hydrocarbons based on anaerobic (per)chlorate reduction and has been demonstrated for the degradation of aromatic and aliphatic compounds, reaching growth rates comparable to the oxidation of respective hydrocarbons with oxygen (32, 33).

(Per)chlorate reduction is a metabolism mainly occurring under neutrophilic conditions and low salinities (30), but recently also halophilic (per)chlorate-reducing microorganisms, such as *Marinobacter vinifirmus* and members of the Halobacteriacea were described (8, 48). Not much is known about their (per) chlorate-reducing physiology so far. However, the fact that Halobacteriaceae carry a gene resembling those of known Cld in their genome may suggest that a

chlorite-disproportionating enzyme is involved. However, the respective protein is only distantly related to functional Cld that have been described up until now, not even sharing conserved key residues (49). This indicates that the full diversity of enzymes catalyzing complete (per)chlorate reduction is not yet discovered.
Within the current study, (per)chlorate reducers were identified that belong to the hyperthermophilic phyla of the Eury- and Crenarchaeota. Besides the only known thermophilic (per)chlorate reducer (29) other thermophilic members of the Firmicutes with this trait are described in this thesis. All (per)chlorate-reducing microorganisms described in this work differ considerably from the "classical (per)chlorate reducers" in respect to their physiology, which seems to lack an enzymatic disproportionation step.
These latest findings and the recently grown awareness of an ages-long introduction of chemically formed perchlorate on Earth, show the yet underestimated diversity of mechanisms for microbial (per)chlorate reduction and their distribution over the tree of life.

Oil Reservoir Environment

Next to the fundamental motivation for a deepened knowledge of the anaerobic respiration pathway of (per)chlorate, interests from the oil and gas industry were drivers for this project. Oil and gas occurs and is produced from different systems with extremely varying environmental conditions (with respect to temperature, formation water composition and salinity, formation rock type, pressure, depth, etc.). These systems range from the heavily biodegraded bitumen outcrops and low temperature shallow oil sands to the deeply buried high pressure/high temperature oil and gas reservoirs. Upon deep burial, oil and gas are generated from kerogen through a process of thermal cracking (50). The generated oil and gas migrates from the deeper buried source rock upwards through different formations till it encounters a "trap" that is formed by a reservoir rock (sandstone or limestone) and a cap rock (Fig. 2 - left). Cap rocks are geological structures from shale, clay, anhydrite or salt with a dense and fine texture, resulting in high capillary pressures of the pore network that therefore prevents the further passage of the oil and gas (50). Due to density differences, migrating gas accumulates directly under this cap rock creating a gas cap, whereas an aquifer is usually underlying the oil leg (Fig. 2 - left).

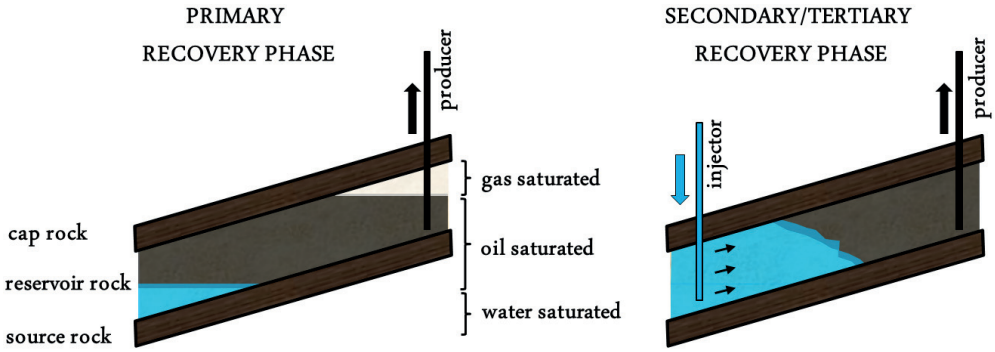

Figure 2: Schematic overview of an oil reservoir during primary and secondary/tertiary recovery phase. Gas- (white), water- (blue) and oil-saturated zones (grey) are indicated, as well as the fluid flow from the injecting to the producing well.

Besides oil, the reservoir rock also contains connate water, which is the remnant of the water originally in place before it got expelled by the upward migrating oil. These pockets with connate water and the interface between oil leg and aquifers underlying oil reservoirs are the locations in which most of the naturally occurring microbes and microbial activity are assumed to reside (51). As oil reservoirs are highly reduced environments that are deprived of oxygen, microbial energy generation can only be based on anaerobic respiration processes. Naturally occurring microbial respiratory processes such as CO_2 reduction to methane, sulfur and sulfate reduction or the reduction of metals (e.g. Fe^{3+}, Mn^{4+}) likely play a key role in the formation of the biodegraded oil sands (as found in Canada).

Upon discovery, oil from reservoirs is produced either through primary recovery solely relying on aquifer pressure support (Fig. 2 - left), but more often through water injection (secondary recovery, Fig. 2 - right) or water and chemical injection (tertiary recovery, Fig. 2 - right). It is during these periods of water injection to the reservoir that the conditions established over geological times are drastically changing. Injection of sulfate-containing seawater at offshore locations in sulfate-depleted environments often results in the formation of sulfide by sulfate-reducing prokaryotes causing reservoir souring. Reservoir souring poses many problems to oil producing facilities such as metal(sulfides) precipitation, corrosion, toxicity, H_2S-containing export gas and crude that lead to rising production costs and deferment of production.

Over the last decades, much research has been directed towards the development of strategies to mitigate reservoir souring. One of the strategies developed, the injection of nitrate, relies on *in-situ* microbial respiratory processes by nitrate-reducing communities that outcompete and inhibit the sulfate-reducing prokaryotes. An

adequate impact of nitrate on the sulfate-reducing community is, however, debated for both low and hot temperature oil reservoirs. For both scenarios this is thought to be associated with the zonation of different functional groups of microorganisms (SRP, NRP, etc.) throughout the reservoir (Chapter 2).

During oil production, much of the original oil in place remains in the reservoir resulting in recovery factors between 40-60%. Recent studies have indicated that Microbial Enhanced Oil Recovery (MEOR) might be achieved by improved sweep efficiency through stimulation of indigenous microbial communities.

The applied objective of the scientific work described in this thesis was to identify and investigate the potential of microorganisms reducing chlorate and perchlorate at high temperatures in order to develop improved strategies for souring mitigation and microbial enhanced oil recovery in oil reservoirs. Such processes are regarded to be most sustainable if they are based on the activity of microorganisms that are indigenous to these mostly hot subsurface environments.

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Outline of the thesis

The research described in this thesis gives insight in microbial (per)chlorate reduction at high temperatures. A new (per)chlorate-reducing pathway in hyperthermophilic archaea and thermophilic bacteria is described and the findings are discussed from a biochemical, evolutionary and applied perspective.

Chapter 2 gives an introduction on microorganisms indigenous to oil reservoirs, covering different functional groups (iron, manganese and nitrate reducers). The occurrence of (per)chlorate-reducing microorganisms in the deep subsurface is discussed based on latest findings and analyses of (meta)genomic datasets. The broad diversity of genes related to the ones coding for functional chlorite dismutase is analyzed and the recent discovery of perchlorate *sensu lato* is briefly described in context of oil reservoirs. Finally industrial applications based on microbial (per) chlorate reduction are discussed, such as bioremediation, reservoir souring mitigation and microbial enhanced oil recovery.

Chapter 3 reports the ability of *Archaeoglobus fulgidus* to grow by the reduction of chlorate and perchlorate. This finding extended microbial (per)chlorate reduction to the hyperthermophilic archaeal life and discovered a metabolism that differs notably from the one known from mesophilic bacteria. Due to absence of chlorite dismutase, the biological reduction of perchlorate and chlorate in *A. fulgidus* is dependent on the chemical reduction of chlorite mediated by sulfide in the medium. This forms oxidized sulfur compounds that can be reduced back by the archaeon, creating a "sulfur loop" driving (per)chlorate reduction.

Chapter 4 gives more insight in the (per)chlorate-reducing metabolism of *A. fulgidus*. The strict dependence on dissolved sulfide, the temporary (chemical) formation of elemental sulfur (S^0) and the biological reduction of S^0 during (per)chlorate reduction confirmed the earlier proposed model of a "sulfur loop" physiologically right. Additionally findings relevant for souring mitigation are reported, such as the formation of nitrite by *A. fulgidus* under increased redox conditions and the difficulties of the microorganism to resume with sulfate reduction after long-time exposure to perchlorate.

Chapter 5 describes a bacterial consortium growing at high temperature that reduces perchlorate coupled to acetate oxidation, a substrate that is abundant in hot oil reservoirs. Community analysis and physiological observations indicate a potentially syntrophic degradation of perchlorate, linked to chlorite elimination by reaction with sulfur compounds. Several sulfur/sulfate reducers and the most

probable candidate for perchlorate reduction, a microorganism remotely related with the genus *Thermanaeromonas* were identified.

Chapter 6 reports the capability of the hyperthermophilic Crenarchaeon, *Aeropyrum pernix* to grow by chlorate and perchlorate reduction. The previously as strict aerobe known microorganism lacks chlorite dismutase and employs chemical scavengers (thiosulfate) to reduce chlorite to chloride. Similarly, two Firmicutes are described here that grow by (per)chlorate reduction. Their physiology and genomic information draw a picture of (per)chlorate reduction resembling the ones of *A. pernix* and *A. fulgidus*, lacking chlorite dismutase.

Chapter 7 gives a short overview on the knowledge on classical (per)chlorate reduction. The results of this thesis are discussed in an integrative way and compare the classical metabolism with the here newly discovered (per)chlorate reduction *sensu lato* lacking chlorite dismutase. The diversity of microorganisms that are possibly able to grow by such mechanisms receives special attention, also in respect to evolution and future applications for the oil industry.

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Chapter 2

Microbial redox processes in deep subsurface environments and the potential application of (per)chlorate in oil reservoirs

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2

Abstract

The ability of microorganisms to thrive under oxygen-free conditions in subsurface environments relies on the enzymatic reduction of oxidized elements, such as sulfate, ferric iron or CO_2, coupled to the oxidation of inorganic or organic compounds. A broad phylogenetic and functional diversity of microorganisms from subsurface environments has been described using isolation-based and advanced molecular ecological techniques. The physiological groups reviewed here comprise iron-, manganese- and nitrate-reducing microorganisms. In the context of recent findings also the potential of chlorate and perchlorate [jointly termed (per)chlorate] reduction in oil reservoirs will be discussed. Special attention is given to elevated temperatures that are predominant in the deep subsurface. Microbial reduction of (per)chlorate is a thermodynamically favorable redox process, also at high temperature. However, knowledge about (per)chlorate reduction at elevated temperatures is still scarce and restricted to members of the Firmicutes and the archaeon *Archaeoglobus fulgidus*. By analyzing the diversity and phylogenetic distribution of functional genes in (meta)genome databases and combining this knowledge with extrapolations based on earlier-made physiological observations, we speculate on the potential of (per)chlorate reduction in the subsurface and more precisely in oil fields. In addition, the application of (per)chlorate for bioremediation, souring control and microbial enhanced oil recovery are addressed.

Keywords: oil reservoirs, deep subsurface, (per)chlorate reduction, anaerobic redox processes, MEOR, reservoir souring

Introduction

Microorganisms inhabit subsurface environments hundreds of meters below Earth's surface where oxygen is most often lacking. The development of the first modern oil wells in the 19th century opened the "gate to the deep biosphere" and not long after that scientists discovered the first microbes thriving in these environments (1-3). Particularly the studies of Tausson, 1925 (4, 5) and ZoBell, 1945 (6) gained detailed insight into the microbial oxidation of hydrocarbons by indigenous subsurface microbes. A large number of studies in the following decades tightened the concept of an active and diverse microbial subsurface community. The development of improved anaerobic culturing techniques during the second half of the 20th century resulted in another step forward in the identification of anaerobes and their physiology (7, 8). These indigenous subsurface microbes were isolated and often deposited in publicly accessible strain collections. A major driver for investigating the microbiology of oil reservoirs has been the biogenic *in-situ* formation of hydrogen sulfide from sulfate, causing souring. The detrimental effects associated with the formation of hydrogen sulfide (high toxicity, sulfide stress cracking, corrosion, precipitation of metal sulfides) increase the production and refinery costs of petroleum (9) and have created a generally negative image of microorganisms in oil fields from the beginning of modern oil recovery (10). However, particular microorganisms indigenous (or introduced) to the subsurface may have characteristics that are desirable during oil recovery, and it might be beneficial to stimulate these further *in-situ*. The most prominent example is the mitigation of souring by nitrate-reducing communities in oil fields (11, 12). Additionally, growing effort is spent on the development of new strategies for microbial enhanced oil recovery (MEOR), or other processes (e.g. conversion of coal to methane) that use the "help of microorganisms" for increasing hydrocarbon recovery.

Subsurface microbiology

The developments in molecular biology made it possible to obtain a deeper insight into the microorganisms that inhabit oil reservoirs. Numerous studies have been conducted which describe the bacterial and archaeal community structure of produced waters using the 16S rRNA gene marker (clone libraries, DGGE, pyrosequencing) (13-15). The genomes of an increasing number of subsurface microorganisms have been sequenced and the advances of next generation DNA sequencing technologies have made metagenomic analyses on samples from the subsurface and oil reservoirs possible (16-18). The computational processing and comparison of the steadily growing amount of information in databases will provide a detailed picture of the subsurface microbiota. Nevertheless, cultivation and isolation of microorganisms is indispensable for the characterization of novel enzymes and metabolic pathways and

will deepen the interpretability of future sequencing data information.
The authenticity of indigenous microbes isolated from oil fields (and subsurface environments in general) is controversial. Oil fields that have not been treated with secondary recovery methods may be considered pristine, however already the drilling into the formation is a potential source of "microbial contaminations". Magot (19) emphasized the additional risk of contamination during sampling and processing of subsurface material. Especially for cultures that differ considerably in growth requirements from the original *in-situ* conditions the autochthonous character is often questioned (19). Nevertheless, an unexpected high diversity of aerobic microorganisms was observed in several oil deposits (coal beds and oil sands), where oxygen was assumed to be very limited (17). The study further demonstrated that the presence of the respective aerobes was not attributed to anthropogenically caused contaminations.
Microorganisms thriving in the subsurface are phylogenetically and physiologically diverse. Here, we focus on microbially-mediated redox reactions involving terminal electron acceptors that allow energy conservation and growth when coupled to the oxidation of inorganic or organic electron compounds. The microbial reduction of iron, manganese, nitrate and (per)chlorate are discussed in this review. Sulfate reducers, methanogens, fermentative and aerobic microorganisms isolated from oil reservoirs are not covered but these groups of microorganisms were extensively reviewed earlier (8, 20).

Manganese- and iron-reducing microorganisms

Microbial ferric iron reduction [Fe(III)] is estimated to have evolved around 3.5 billion years ago and is considered to be one of the oldest respiratory processes on Earth (21). A relative broad diversity of microorganisms uses ferric iron as electron acceptor. Several iron reducers can also reduce manganese(IV) [Mn(IV)] or other metals (22).
Dissolved or chelated Fe(III) and Mn(IV) are good electron acceptors yielding relatively high amounts of energy. However, the standard redox potential of the redox couple Fe(III)/Fe(II) (+770 mV) is only relevant at low pH where Fe(III) is soluble (Table 1 in Chapter 1). In general, produced water from oil reservoirs is in the range of circumneutral pH. Therefore the concentrations of dissolved Fe(III) is very low and practically unavailable for microbial utilization (22); same is the case for Mn(IV) and Mn(III). Moreover, the predominant form of Fe(III) and Mn(IV) in subsurface environments is bound in solid minerals (23). Their use as terminal electron sink for microbial redox reactions is hence, associated with less favorable redox potentials and a lower accessibility (24). Some microorganisms have physiological adaptations that enable them to utilize insoluble forms of

ferric iron as electron acceptors. Several such mechanisms have been described in mesophilic Gram-negative bacteria, particularly for *Geobacter sulfurreducens* and *Shewanella putrefaciens* (22, 25-27). The physiology of Gram-positive and archaeal iron reducers is much less understood (28). The first archaeal ferric iron reductase was isolated and characterized from *Archaeoglobus fulgidus* (29, 30). Its potential role in energy conservation was discussed, but dissimilatory growth on ferric iron has never been observed (29).

For respiration with ferric iron, microbes use organic and inorganic compounds as electron donors (30). A limited number of strains with the ability to reduce soluble and insoluble forms of ferric iron were isolated from oil reservoirs (31, 32) (Table 1). Microbial iron reduction also occurs at high temperature and is wide-spread over the bacterial and the archaeal domain of life. (30).

The first thermophilic Fe(III)-/Mn(IV)-reducing microorganism isolated from an oil field was *Deferribacter thermophilus* (33) (Table 1). Slobodkin and co-workers isolated a number of other thermophilic strains from an oil reservoir (34). These belonged to the genera *Thermoanaerobacter*, *Thermotoga*, and *Thermococcus* and they were able to reduce ferric iron. With this finding they concluded that the reduction of ferric iron may be a common trait for energy conservation among anaerobic thermophiles in oil reservoirs.

Another oil field isolate that derived from a moderately hot oil field, *Geoalkalibacter subterraneus*, grows by the reduction of Fe(III), Mn(IV), nitrate or elemental sulfur and trimethylamine-N-oxide (35).

Table 1: Microorganisms isolated from oil field environments, that are able to grow by the reduction of nitrate, Fe(III) and/or Mn(IV).

Species	Strain	Growth in °C [optima]	Electron acceptors	Electron donors	Source	Lit.
Arcobacter sp.	FWKO B	15-40	nitrate	sulfide, hydrogen, formate	produced brine, oil field (Canada)	(38)
Deferribacter thermophilus	BMA^T	50-65 [60]	nitrate, Mn(IV), Fe(III)	hydrogen, malate, acetate, citrate, pyruvate, lactate, succinate, valerate	production water, North sea oil field (UK)	(33)
Denitrovibrio acetiphilus	N2460^T	4-40 [35-37]	nitrate	acetate	oil field environment (oil refinery)	(39)
Garciella nitratireducens	MET79^T	25-60 [55]	nitrate	lactate, pyruvate, malate, fumarate and others	oil field separator, oil field (USA)	(40)
Geoalkalibacter subterraneus	Red1^T	30-50 [40]	Fe(III), Mn(IV), nitrate	formate, acetate, propionate, lactate, butyrate, isobutyrate, succinate, fumarate, valerate, isovalerate, citrate, salicylate, octanoate, palmitate, glycerol, hydrogen and others	produced water, oil field (USA)	(35)

Geobacillus lituanicus	N-3^T	55-70 [55-60]	nitrate	yeast	oil field (Lithuania)	(41)
Geobacillus subterraneus	34^T	45-65	nitrate	acetate	formation water, oil field (China)	(42)
Marinobacter hydrocarbonoclasticus (formerly *M. aqueolei*)	VT8	13-50 [30]	nitrate	acetate, succinate, citrate	produced fluid, oil field (Vietnam)	(43)
Petrobacter succinatimandens	4BON^T	35-60 [55]	nitrate	formate, fumarate, pyruvate, succinate, ethanol, yeast extract	production water, oil field (Australia)	(44)
Shewanella putrefaciens (formerly *Alteromonas putrefaciens*)			Fe(III)	hydrogen, formate	produced water, oil storage tanks (Canada)	(31)
Sulfurimonas sp. (formerly *Thiomicrospira* sp.)	CVO	5-35	nitrate, nitrite, N_2O	sulfide, elemental sulfur	produced brine, oil field (Canada)	(38)
Thermoanaerobacter acetoethylicus	SL 26, S128	40-80 [65]	Fe(III)	peptone, hydrogen	formation water, oil field (Russia)	(34)
Thermoanaerobacter brockii	M739	35-85 [65]	Fe(III)	peptone, hydrogen	formation water, oil field (Russia)	(34)
Thermococcus sibiricus	MM 739^T	40-88 [81]	Fe(III)	peptone, hydrogen	formation water, oil field (Russia)	(34)
Thermotoga subterranea	SL-1	50-75 [70]	Fe(III)	peptone, hydrogen	formation water, oil field (France)	(34)

Iron-reducing microorganisms, next to oil reservoirs, have also been isolated from other hot environments such as marine and terrestrial hydrothermal vents, hot freshwater springs and geothermally heated soils [reviewed in Lovley et al. (22) and Slobodkin (30)]. Microbial iron reduction has been reported to occur up to 121°C and at salinities 10-times higher than that of sea water (36, 37). Such environmental conditions are also common to hot oil reservoirs.

In turn, the oxidation of ferrous iron in anaerobic environments is an important microbially-mediated process, probably innate to nitrate reducers in general (45, 46). At circumneutral pH, Fe(II) and Mn(II) are several magnitudes more soluble than their oxidized counterparts [Fe(III), Mn(III), Mn(IV)] (24). To which extent the injection of nitrate in oil fields may result in the oxidation of *in-situ* deposited metal(hydr-)oxides is unknown.

A novel microbial enhanced oil recovery (MEOR) strategy was proposed dosing dissolved ferrous iron together with nitrate (47). The microbial *in-situ* formation of solid forms of ferric iron by the action of nitrate-reducing microorganisms could eventually result in improved sweep efficiencies.

Nitrate-reducing microorganisms

The injection of nitrate during water flooding is applied for souring mitigation purposes, diminishing the biogenic *in-situ* formation of hydrogen sulfide by sulfate-reducing prokaryotes (SRP).

In oil fields, nitrate is reduced by microorganisms to dinitrogen gas (denitrification) or ammonia, using inorganic and organic electron donors (12). The first step in denitrification and dissimilatory nitrate reduction to ammonia is the reduction of nitrate to nitrite. This conversion is catalyzed by nitrate reductases of the respiratory Nar-type (with the catalytic subunit located in the cytoplasm for bacteria and periplasm for archaea, respectively) and the Nap-type reductases (catalytic subunit in the periplasm) (48). Both types of nitrate reductases are found in microorganisms thriving in oil fields (49, 50).

Strain CVO and strain FWKO B, related to the genus *Sulfurimonas* (formerly *Thiomicrospira*) and *Arcobacter* respectively, are chemolithoautotrophic nitrate-reducing mesophiles both isolated from produced fluids (38). Strain FWKO B couples the oxidation of sulfide, hydrogen or formate and strain CVO the oxidation of sulfide and elemental sulfur to the reduction of nitrate. Whether these microorganisms can couple the reduction of nitrate also to the oxidation of ferrous iron is not known.

One of the heterotrophic nitrate reducers isolated from oil reservoirs is *Deferribacter thermophilus* (33). This thermophile in addition to nitrate can also reduce Fe(III) and Mn(IV) (see above).

Geobacillus is a prominent genus associated with nitrate reduction at elevated

temperature in oil reservoirs (42, 51, 52). *Geobacillus* species can utilize a broad range of carbon sources. Some isolates are also able to degrade (long-chain) alkanes in the presence of oxygen. (51, 53).
The thermophilic nitrate-reducing oil field isolate *Denitrovibrio acetiphilus* couples the reduction of nitrate to acetate oxidation (39). *Marinobacter hydrocarbonoclasticus* (synonym *Marinobacter aqueolei*), isolated from an oil reservoir, is a mesophilic bacterium that can grow by the reduction of nitrate and degrades oil compounds under aerobic conditions (43).
Numerous thermophilic nitrate reducers have been isolated from other hot environments, like *Thermovenabulum ferriorganovorum* from a hydrothermal vent (54), *Garciella nitratireducens* from an oil field separator (40) or *Caldinitratiruptor microaerophilus* from a hot spring (55).
Ferroglobus placidus was isolated from the vicinity of a hydrothermal vent and is one of the few hyperthermophilic nitrate reducers. It is also able to reduce thiosulfate and Fe(III). This archaeon can also couple the oxidation of aromatic compounds to Fe(III) reduction (56, 57).
However, to our knowledge no hyperthermophilic nitrate-reducing microorganism has ever been isolated from oil reservoirs up until now. Despite an extensive search on metagenomic resources for hyperthermophilic nitrate reducers, microorganisms like *Ferroglobus placidus* and *Pyrobaculum aerophilum* are very rarely found to be associated with hydrocarbon resources. Examples include the high temperature (102°C) water samples coming from Bass Strait oil reservoirs where the aforementioned nitrate reducers were detected to be present at very low abundance (0.02% and 0.01% respectively) (MG-RAST ID 4550335.3). Assuming that these microorganisms are indeed present, the absence of nitrate in the water samples as it was reported in the chemical analysis might explain their minor role in this system. Ideally, microbes detected at low abundance should be reported with caution as metagenomics algorithms can sometimes struggle to distinguish between rare microbes and false positives. At the same time, as metagenomic datasets are usually incomplete, failure to detect certain taxa or genes should not be interpreted that they are absent from these particular environments.

(Per)chlorate reducers in the deep biosphere?!

(Per)chlorate reduction is a well-studied dissimilatory reductive pathway performed under anaerobic conditions (58). The complete reduction of perchlorate (ClO_4^-) involves the action of a perchlorate reductase (Pcr), that reduces perchlorate to chlorate (ClO_3^-) and further to chlorite (ClO_2^-) followed by the disproportionation of chlorite to oxygen and chloride by a chlorite dismutase (Cld). Microorganisms that carry enzymes which only reduce chlorate but that are incapable of reducing perchlorate

are called chlorate reducers (59). The respective enzyme, chlorate reductase (Clr) is an enzyme that differs from Pcr in genetic, structural and evolutionary aspects (60-62).

Most (per)chlorate-reducing microbes favor neutrophilic conditions (63) and low salt concentrations. However, some microorganisms have been reported to cope with high salinities during (per)chlorate reduction. *Dechloromarinus chlorophilus* and *Arcobacter* sp. strain CAB can grow at salinities of up to 5% and 3%, respectively (58, 64). Actively perchlorate-reducing enrichment cultures were reported at salinities of up to 11% (65). Recently, *Marinobacter vinifirmus* and members of the Halobacteriaceae have shown to reduce (per)chlorate at salinities beyond 10% sodium chloride (66, 67).

The vast majority of (per)chlorate-reducing bacteria are mesophilic facultative anaerobes affiliated to the phylum of Proteobacteria, predominantly belonging to the class of β-Proteobacteria (63). Acetate is a common substrate for (per)chlorate reducers, but other organic electron donors were also reported to sustain (per)chlorate reduction, such as alcohols (68, 69), organic acids (70, 71), aromatic hydrocarbons (72, 73) and aliphatic hydrocarbons (74). Inorganic electron donors like hydrogen, ferrous iron, zero-valent iron (75) or thiosulfate and elemental sulfur (76) are also used by (per)chlorate-reducing microbes. For a broad range of mesophilic (per) chlorate-reducing bacteria it was demonstrated that the oxidation of sulfide could be coupled to chlorate and/or perchlorate reduction, resulting in the accumulation of elemental sulfur but not promoting growth (77). The authors also reported the oxidation of sulfide to sulfate by *Dechloromarinus anomalous* strain NSS during the reduction of chlorate.

"Classical (per)chlorate reduction"

Up until now no (per)chlorate-reducing microbes have been isolated from oil reservoirs. Here, we discuss a computational analysis to aid to get insight into the occurrence of (per)chlorate reducers in oil fields.

A crucial necessity for classical (per)chlorate-reducing microorganisms is the presence of a chlorite-disproportionating enzyme, named chlorite dismutase, that avoids accumulation of the toxic intermediate chlorite. Hundreds of proteins that resemble functional chlorite dismutases (Cld) are encoded in phylogenetically diverse groups of prokaryotes (78, 79). However, the actual number of proteins that are able to catalyze the disproportionation of chlorite to chloride is not known.

Up until now all publicly available genomes of *Geobacillus* species carry a gene encoding a Cld-like protein (pfam06778). Also the genomes of two oil field isolates, *Bacillus cereus* Q1 and *Geobacillus thermodenitrificans* NG80-2 harbor the gene of this Cld-like protein, which is highly conserved within the genus *Geobacillus* (>70%

identity over ca. 250 amino acids length). However, its similarity with functionally efficient chlorite dismutases is low (max. 24% over full length). The function of this particular protein is unknown, neither has any *Geobacillus* sp. ever been reported to grow by (per)chlorate reduction.

Another subgroup of a Cld-family protein is found in the Halobacteriaceae (>50% homology among different species) and only very remotely related to functional chlorite dismutases (max. 23% amino acid sequence identity). Even though this protein group has not been further characterized, several members of the Halobacteriaceae (e.g. *Haloferax mediterranei* and *Haloarcula marismortui*) are able to grow by the reduction of perchlorate and chlorate (67). Microorganisms that belong to the genus *Haloferax* and *Halorubrum* (both Halobacteriaceae) were also isolated earlier from oil fields (80, 81); their ability for (per)chlorate reduction has never been tested. The halobacterial group of Cld-like proteins lacks key residues (Ile88, Trp97, Leu122, Arg127, Glu167 – position refers to *Nitrobacter winogradskyi*) that were identified for functionally active chlorite dismutases (79). Based on the same key residues 119 sequences (harbored in 112 microorganisms) were identified from the IMG and related ones from the NCBI database as potentially functional Cld (ranging from lengths of 123-288 amino acids); a selected set is displayed in Figure 1. These sequences belong to a phylogenetically diverse group of mesophiles. Cld-like proteins were assigned according to the earlier proposed lineage I and II (62, 79).

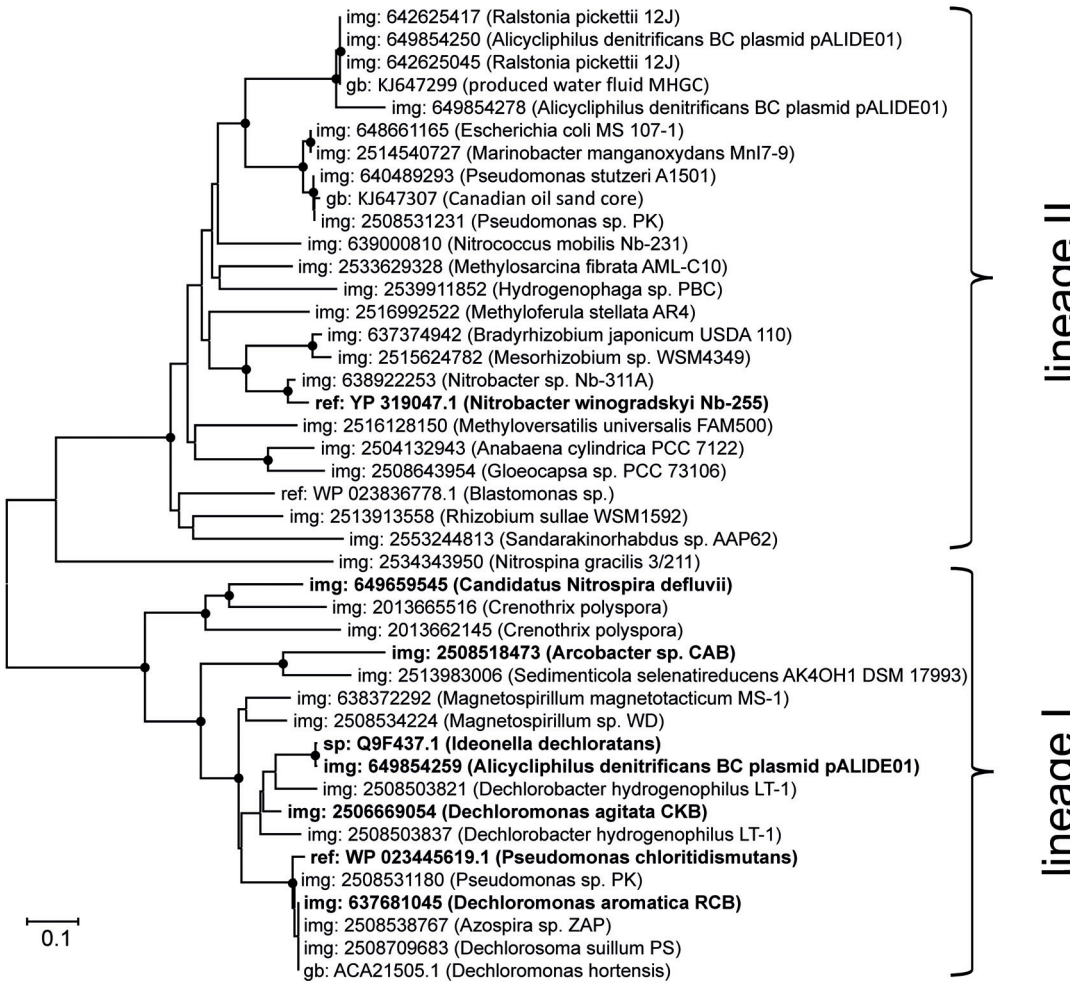

Figure 1: Phylogeny of selected protein sequences that carry key residues of functional chlorite dismutases (Cld) [earlier defined by Mlynek et al. (79)] Accession numbers and the respective microorganism (or metagenome dataset) where sequences derive from are added. Sequences in bold belong to functionally efficient chlorite dismutase enzymes and have been investigated in more detail. The bar illustrates substitutions per site. The phylogenetic tree was constructed using the Neighbor-Joining method including bootstrap values (for 500 replicates). Bootstrap values above 70% are indicated by nodes at the respective branches. Evolutionary distances of the tree were computed using the Poisson correction method; the scale bar indicates amino acid substitutions per site.

The Cld-like protein of *Sedimenticola selenatireducens* for instance has a sequence identity of 65% (with 92% coverage) with the chlorite dismutase of *Arcobacter* sp. CAB, a known marine (per)chlorate reducer (64). *S. selenatireducens* is an

anaerobic selenate-respiring microbe isolated from estuary sediments (82). Although this microorganism has also a perchlorate reductase encoded in its genome (gb: ATZE00000000.1) it is not able to grow by the reduction of perchlorate (82).
Marinobacter manganoxydans, a halophilic microorganism isolated from a deep-sea hydrothermal vent harbors another Cld-like protein with respective key residues (79). *M. manganoxydans* is the only genome-sequenced *Marinobacter* species (in total 11; via IMG database) that has a Cld-like protein encoded (gb: EHJ03506.1). *Marinobacter* spp. are ubiquitously found at different depths of the ocean and have been described from oil fields as well (43, 83). Members of the genus *Marinobacter* are able to grow with hydrocarbons as sole carbon and energy source (84). Under anoxic conditions these microorganisms can grow by the reduction of nitrate, using the membrane-bound Nar-type reductase. Studies on the Nar-type reductase of *M. hydrocarbonoclasticus* strain 617 have demonstrated the enzyme's ability to catalyze chlorate reduction as well (85); a trait known for Nar-type reductases in general (86). Another member of the same genus *Marinobacter vinifirmus*, was reported to grow by the reduction of nitrate and perchlorate recently (66). The genome of *M. vinifirmus* has not yet been sequenced, however a Cld similar to the one in *M. manganoxydans* is possibly involved in the complete reduction of perchlorate.
Two putative chlorite dismutase sequences, one deriving from a produced water sample (MHGC) (gb: KJ647299) (17), and another from a Canadian oil sand core (gb: KJ647307) were retrieved from metagenomic databases (Fig. 1). These partial sequences show resemblance (identity >50%) with the functional chlorite dismutase of *N. winogradskyi* (ref: YP_319047.1), carrying the key residues of functional lineage II Cld (79). Sequence KJ647299 (186 amino acids length) is identical to proteins encoded in *Ralstonia picketti* (Rpic_1480), *Cupriavidus metallidurans* CH34 (Rmet_6340) and *Alicycliphilus denitrificans* BC (Alide_4606); and almost identical to another protein of *A. denitrificans* BC (Alide_4635; 99% identity, 91% query coverage) (Fig. 1). *A. denitrificans* strain BC is a known chlorate-reducing bacterium. However, another protein in this microorganism was proposed as functional chlorite dismutase (Alide_4615) (87). The proteins Alide_4606 and Alide_4635 on the other hand are both part of a transposon flanking functional enzymes responsible for chlorate reduction located on a plasmid (62). Just like the functional Cld of *A. denitrificans* BC the above-mentioned proteins are encoded next to Cupin 2 domain genes (Alide_4607 and Alide_4634), which might suggest a functional connection between Cld/Cld-like genes and Cupin 2 genes (62). Alide_4606 and Alide_4635 have high resemblance with the functional Cld of *N. winogradskyi*.
Sequence KJ647307 (with a length of 131 amino acids) is identical to a Cld-like protein encoded in *Pseudomonas stutzeri* A1501 (PST_3351) and very closely related to a hypothetical protein in *Pseudomonas chloritidismutans* (NCBI ref. seq.: WP_023445505.1; 99% identity, 94% query coverage) (88) (Fig. 1). Several strains

of the genus *Pseudomonas* are able to reduce chlorate (62, 89), probably indicating that this trait is more often found in the respective taxon. However, similar to KJ647299 and *A. denitrificans* the resemblance of sequence KJ647307 is not related to the proposed chlorite dismutase of *P. chloritidismutans* (ref: WP_023445619.1).
Due to the ubiquitous distribution of some above discussed microorganisms (e.g. *Marinobacter* spp.) it is likely that they are regularly introduced in off-shore oil reservoirs during the secondary recovery stage of oil recovery. Even in high temperature oil reservoirs some of these mesophilic prokaryotes may survive in the well-bore region where temperatures are lowered by the injected water. Metagenomic analysis on produced fluid samples from oil reservoirs, however, seems to indicate that some of the above-mentioned microorganisms (*Pseudomonas*, *Marinobacter*, *Arcobacter*, *Geobacillus*, etc.) might also be indigenous to oil reservoirs.
Even though thermodynamic calculations do not exclude (per)chlorate reduction under elevated temperatures (90), (hyper)thermophilic (per)chlorate reducers have not been described until recently. The isolation of a thermophilic (per)chlorate-reducing member of the phylum Firmicutes (69), *Moorella perchloratireducens* and the recently discovered (per)chlorate-reducing capability of the hyperthermophilic archaeon *Archaeoglobus fulgidus* extended the range of this trait to high temperatures (91). These findings broadened the diversity of (per)chlorate-reducing prokaryotes considerably. Besides the phylogenetic diversity, also the ecological background of (per)chlorate reducers might be wider than previously expected. Whereas *M. perchloratireducens* was isolated from an underground gas storage, the type strain of *A. fulgidus* (strain VC-16) originates from a submarine hot spring (92). *A. fulgidus* strains are, however, also frequently found in subsurface environments like oil reservoirs and deep aquifers, and they are considered to be main contributors to souring in high temperature oil reservoirs (93, 94).

(Per)chlorate reduction *sensu lato* in the subsurface

Some microorganisms appear to grow by (per)chlorate reduction without the involvement of a chlorite dismutase. In the absence of a functional Cld an alternative mechanism may enable microorganisms to completely reduce (per)chlorate to chloride anions. In *A. fulgidus* the lack of a chlorite-disproportionating enzyme seems to be overcome by the abiotic scavenging of chlorite formed in the periplasm with naturally occurring or microbially generated sulfide (91). There is ample evidence that this chemical chlorite elimination (using sulfide) forms sulfur fractions of higher oxidation states and enables the continuous biological reduction of (per)chlorate. In turn these sulfur compounds are partially reduced back regenerating reducing power for an ongoing (per)chlorate reduction (Fig. 2 and Chapter 3 and 4).
Similar mechanisms for the biological reduction of (per)chlorate coupled to growth

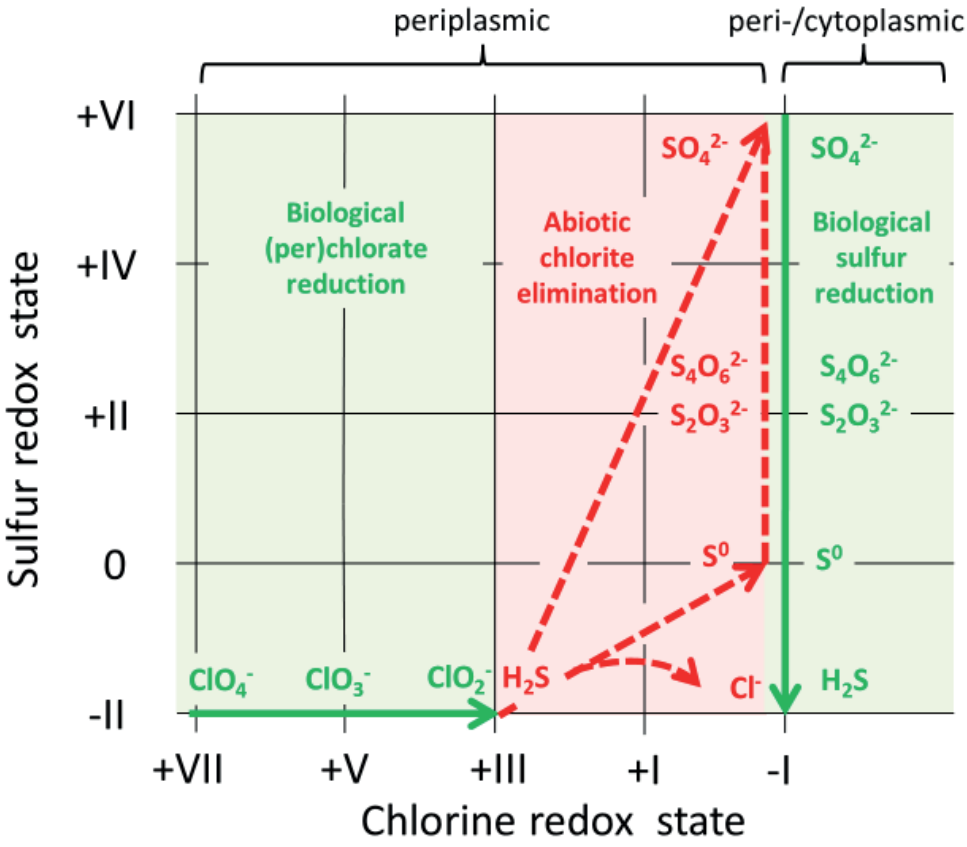

Figure 2: Complete (per)chlorate reduction by *Archaeoglobus fulgidus* VC-16 involving a biotic-abiotic reaction loop depending on sulfur. Biological processes are illustrated with green arrows indicating the direction of a respective reaction, whereas dashed red arrows stand for abiotic reactions. Chlorine and sulfur compounds relevant for the complete reduction of perchlorate are shown.

may also occur in reduced subsurface environments (e.g. oil reservoirs) and other microorganisms (besides *A. fulgidus*). Such a "(per)chlorate reduction *sensu lato*" involves an enzyme reducing (per)chlorate, followed by an abiotic chlorite detoxification step.

Several characterized molybdenum enzymes of the DMSO reductase family have shown to be rather unspecific in their substrate range. For some enzymes of this group the reduction of chlorate was demonstrated (besides the canonical function) by biochemical tests (95-97); especially Nar-type reductases seem to reduce chlorate at high rates (86, 98). The activity for enzymes of the DMSO family towards perchlorate has often not been assessed. An exception is the Nar-type enzyme of *Marinobacter hydrocarbonoclasticus* strain 617, which has a very low activity with perchlorate (85).

For the reduction of (per)chlorate *sensu lato* involving chemical chlorite scavenging the periplasmic localization of the functional enzymes will be of crucial importance to prevent the accumulation of toxic chlorite levels in the cell. Under these conditions chlorite would be better accessible for potential scavengers and thus probably enable continuous (per)chlorate reduction (in the absence of a functional Cld).

2

Application of (per)chlorate in the oil business

Similar to nitrate reduction, the reduction of (per)chlorate involves energetically more-favorable redox couples, compared to e.g. sulfate reduction or methanogenesis (Table 1 in Chapter 1).

The two-step reduction of perchlorate to chlorite via chlorate and its subsequent disproportionation liberates molecular oxygen (99). Microbial (per)chlorate reduction is therefore a metabolism potentially forming molecular oxygen under *de facto* anaerobic non-phototrophic conditions. This light-independent *in-situ* oxygen production is exceptional and offers innovative possibilities for applications in the oil recovery business. Unlike oxygen, which is soluble in the mM-range, (per)chlorate is soluble in the M-range.

In a previous study, it was demonstrated that oxygen generated by chlorite dismutase could even be utilized by other microbes living in a consortium with a (per)chlorate-reducing bacterium (100). Given the fact that aerobic processes are energetically more favorable compared to anaerobic ones, the *in-situ* formation of oxygen under anoxic conditions could have promoting effects on both growth yields and rates and thereby allow bioconversion of compounds that are barely degradable by anaerobic without oxygen.

Bioremediation

Man-made perchlorate pollution of soils and drinking water resources is a threat to human health and has therefore caused a considerable rise in attention over the last decades. In the 1970s ideas came up to use (per)chlorate-reducing microbes for the purification of (per)chlorate-containing industrial waste waters, as well as for the remediation of (per)chlorate-polluted soils (101). The advances of *in-situ* bioremediation of perchlorate-polluted soils have been extensively discussed in the books of Gu and Coates (102) and Stroo and Ward (103). A comprehensive review on *ex-situ* treatment of perchlorate-containing streams is provided by Sutton (104).

Besides the bioremediation of toxic perchlorate contamination, the potentially remediating effect of (per)chlorate reduction on the co-degradation of recalcitrant organic pollutants (e.g. hydrocarbons) in anaerobic soil layers was proposed (100). The feasibility and extend of *in-situ* biodegradation of hydrocarbon-polluted sites

often relies on the supply of oxygen. Latter can be supplied by the injection of compressed air or pure oxygen into deeper anaerobic soil layers but this is associated with high costs and a limited oxygen penetration of the soil body. (Per)chlorate-reducing bacteria form oxygen under anaerobic conditions when the intermediate chlorite is disproportionated during the reduction of perchlorate and chlorate. Even though oxygen release from the cell has never been observed in cultures growing on (per)chlorate, experiments with washed cell suspensions form and release molecular oxygen upon the addition of chlorite (71, 100).

For the bioremediation of recalcitrant organic compounds, the concept assumes that (per)chlorate reduction results in formation of molecular oxygen under anaerobic conditions, which might set on the action of oxygenases, involved in the degradation of pollutants (105, 106) or be utilized as a terminal electron acceptor. Both would thermodynamically be favored over anaerobic degradation and thus they enable higher growth rates and faster degradation rates of pollutants (107). This has already been demonstrated with studies on bacterial isolates that reduce (per)chlorate coupled to the oxidation of different aromatic and aliphatic hydrocarbons in pure culture (73, 74). The respective microorganisms had comparable growth rates with chlorate and oxygen during hydrocarbon oxidation.

The broad-scale injection of perchlorate or chlorate to contaminated soils, probably combined with bioaugmentation of (per)chlorate-reducing microbes remains an interesting but debatable strategy due to the potential toxic effects of chlorine oxyanions. The *in-situ* applicability of chlorite injections at hydrocarbon-contaminated sites, already containing microorganisms growing on (per)chlorate is tempting (100) but can also be questioned. Limitations may be associated with the high toxicity of chlorite already at low concentrations and the high chemical reactivity of chlorite with reduced soil or iron particles (108).

Reservoir Souring Mitigation

Reservoir souring had long been considered to occur only due to abiotic subsurface processes (109-111). When the role of sulfate-reducing bacteria was acknowledged (112, 113), this resulted in efforts directed to develop strategies to mitigate microbial reservoir souring. So far several strategies have been proposed for souring control: nitrate injection, sulfate removal and biocide injection. Although probably most effective in the majority of cases, sulfate removal is only scarcely applied for souring mitigation purposes. This is due to the high investment and operational cost associated with sulfate removal units. Application of biocide is used by oil and gas companies to achieve microbial control in their surface production and processing facilities, but it is generally debated whether it is effective to control reservoir souring as its effect does not extend sufficiently deep into the reservoir formations. Nitrate injection is

the most widely accepted and used strategy to control microbial reservoir souring, especially effective in homogeneous hot reservoirs (Fig. 3 – right panel) and to a somewhat lower extend also in highly heterogeneous hot reservoirs (Fig. 3 – middle panel). Nitrate is considered to be effective in controlling reservoir souring by: 1) the competitive exclusion of sulfate-reducing bacteria by more efficient nitrate-reducing bacteria (competing over the same electron donating compounds; volatile fatty acids, BTEX, other hydrocarbons, etc.), 2) inhibition of the dissimilatory sulfite reductase, a key enzyme in the sulfate reduction pathway, by nitrite (an intermediate in reduction of nitrate) (114), and 3) the oxidation of generated sulfide by nitrate-reducing sulfide-oxidizing bacteria (12).

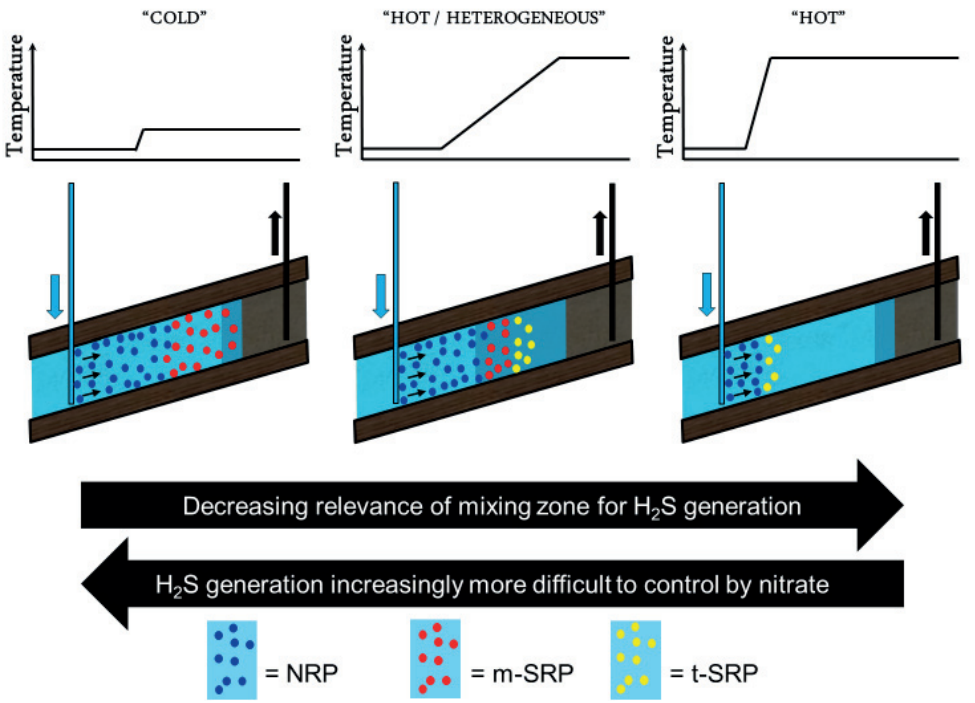

Figure 3: Schematic representation of water flood with nitrate injection in cold, heterogeneous hot, and (homogeneous) hot oil reservoirs. The difference in the thermal gradient (graph) and the size of the injection/formation water mixing zone (dark blue) determine zones that are dominated by nitrate- (NRP, blue dots), mesophilic and (hyper)thermophilic sulfate-reducing prokaryotes (m-SRP and t-SRP, red and yellow dots respectively). Nitrate injection does provide protection against souring in the vicinity of the injector well bore in hot reservoirs (right panel), but this protection becomes significantly more challenging in the deeper nitrate-depleted parts of the low-temperature reservoir exposed to sulfate-containing injection or formation water (left panel). Reservoir souring is often also strongly dependent on the mixing of nutrients from the formation and injection water and therefore tends to be more extensive in heterogeneous reservoirs (middle panel).

The effectiveness of nitrate injection to control souring is, however, questionable for e.g. low-temperature reservoirs. Nitrate might provide protection against souring in the vicinity of the injector well bore, but not in the deeper nitrate-depleted parts of the low-temperature reservoir exposed to sulfate-containing injection or formation water (115, 116). This will result in the development of zones that are dominated either by nitrate- and sulfate-reducing communities (Fig. 3 – left panel). Therefore the success of nitrate injection to control souring in low temperature reservoirs is linked to how deep nitrate can be delivered into the reservoir.

Depending on the type and abundance of nitrate- and sulfate-reducing microbes, nitrate might also be less effective in high temperature reservoirs with heterogeneous permeability distribution as these systems might have become depleted for nitrate and tend to have larger high temperature mixing zones where nitrate might not be able to prevent growth of more temperature tolerant sulfate-reducing microbes (Fig. 3 – middle panel). This seems to be confirmed with the fact that although hyperthermophilic nitrate-reducing microorganism do exist (e.g. *Pyrobaculum aerophilum*), these microorganisms have not yet been identified in significant numbers in samples from oil reservoirs (contrarily to hyperthermophilic sulfate reducers).

The limitations of above-mentioned strategies are the driver to seek for alternative mitigation strategies. Based on findings related to (per)chlorate reduction by *A. fulgidus* (91) and sulfide oxidation by mesophilic (per)chlorate-reducing Proteobacteria (77) we propose that (per)chlorate injection could be a good addition or alternative to that of nitrate for souring mitigation in both high and low temperature reservoirs. Whereas the biological oxidation of sulfide to elemental sulfur seems to be a characteristic innate to classical (per)chlorate reducers of low temperature optimum, the oxidation of sulfide during (per)chlorate reduction at higher temperatures such as that of *A. fulgidus*, is chemically mediated (Chapter 4).

In contrast to a mesophilic (per)chlorate-reducing community that does not directly affect the sulfate-reducing community and would have to be established first, (per) chlorate reduction by *A. fulgidus* appears to have a direct negative impact on the microorganism's sulfate-reducing capability (Chapter 3 and 4). The increased expression of stress proteins in *A. fulgidus* when exposed to (per)chlorate, indicates that the inhibition might be linked to redox stress from the chlorite produced as intermediate.

Given the ability of *A. fulgidus* to reduce (per)chlorate and the fact that (per) chlorate reduction seems to interfere with sulfate reduction, (per)chlorate injection could provide control in high(er) temperature zones where that is not feasible by nitrate. The fact that *Azospira suillum* only oxidized sulfide coupled to (per)chlorate reduction, but not nitrate (which is normally also used together with organic electron donors), (77) may indicate the different impact of (per)chlorate compared to nitrate

during souring mitigation interventions. In other words the alternating use of nitrate and (per)chlorate possibly combined with biocides could profit from complementary effects that avoid scavenging of nitrate/(per)chlorate in the vicinity of the injector well bore and thereby extend the impact of both nitrate as well as (per)chlorate deeper into the reservoir.

2

Microbial Enhanced Oil Recovery (MEOR)

The efficiency of oil recovery from oil reservoirs is very often limited due to the geological structure of the oil-bearing formation and the oil characteristics. Although a matrix, piston-wise displacement of the target oil is intended (Fig. 4A), the actual displacement is often highly unstable due to fingering of water in oil (because of viscosity differences) (Fig. 4B) or preferred flow through high permeable zones (Fig. 4C) or fractures (Fig. 4D).

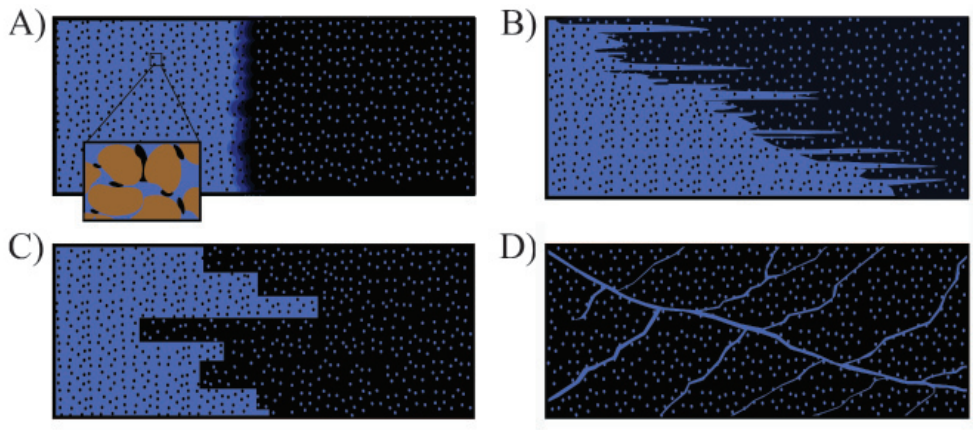

Figure 4: Schematic representation of oil displacement in a petroleum reservoir. Ideal matrix piston-wise (stable) displacement leaving low residual oil levels (A) with the close-up showing the residual oil (black blobs) attached to sand grain particles (brown), unstable displacement showing fingering of water into the oil phase (B), unstable displacement due to thief zones (C) and unstable displacement through fractures (especially for carbonates) (D). Oil phase is indicated in black, water in blue. Small black blobs indicate residual oil after being flooded, small blue blobs indicate connate water before being flooded.

Microbial Enhanced Oil Recovery (MEOR) had already been proposed at the advent of modern oil production (117). Although several MEOR trials have been reported and hundreds of patents are filed, the process often lacks reproducibility or remains unproven (118, 119). Moreover, most of the MEOR trials are in fact well stimulation rather than "full-field" MEOR treatments. Many driving mechanisms for MEOR were postulated, of which the *in-situ* generation of biosurfactants received lots of attention. Convincing evidence that *in-situ* microbes will be able to generate sufficient

amounts of effective surfactant in a full-field setting in order to increase the capillary number sufficiently such that residual oil is indeed mobilized is, however, still lacking. A critical analysis of the proposed mechanistic drivers for MEOR revealed that only the plugging of high-permeability zones (aka conformance control), seemed to be most plausible (120). In order to be feasible for a field-wide application, an MEOR process based on conformance control would have to rely on the stimulation of indigenous microbes (avoiding requirement of injecting microbes) utilizing part of the hydrocarbon fraction (or *in-situ* commonly occurring volatile fatty acids) as electron donor. The reduction of (per)chlorate in the subsurface might liberate highly oxidative chlorine intermediates (or even oxygen) in a *de facto* anaerobic environment. Reactive chlorine oxyanions (such as chlorite) and oxygen will either chemically or biologically oxidize (in)organic compounds (e.g. sulfide, ferrous iron, hydrocarbons etc.) in the vicinity of the (per)chlorate reducer. The availability of oxygen is also a pre-requisite for the oxygenation of hydrocarbons by mono- and dioxygenases. This may yield "activated hydrocarbons" that are subsequently more easily degradable by (other) microorganisms. The presence of oxygen would enable facultative prokaryotes to switch from a lower-efficiency anaerobic "lifestyle" to a more efficient microaerophilic metabolism, generating more biomass. We therefore propose that injection of (per)chlorate alone or in combination with nitrate and phosphates (if the latter proves to be limiting), might be able to sufficiently stimulate the indigenous microbial community to achieve conformance control and thereby enhance oil recovery. Further research is needed to show the effectiveness of (per) chlorate injection for MEOR.

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Chapter 3

(Per)chlorate reduction by the hyperthermophilic archaeon *Archaeoglobus fulgidus*: An interplay of biotic and abiotic reactions

adapted from:
Martin G. Liebensteiner, Martijn W.H. Pinkse, Peter J. Schaap, Alfons J.M. Stams, Bart P. Lomans (2013) Archaeal (per)chlorate reduction at high temperature; an interplay of biotic and abiotic reactions. Science 340:85-87.

Abstract

Perchlorate and chlorate anions exist in the environment from natural and anthropogenic sources, where they can serve as electron acceptors for bacteria. We performed growth experiments combined with genomic and proteomic analyses of the hyperthermophile *Archaeoglobus fulgidus* that show (per)chlorate reduction extends into the archaeal domain of life. The (per)chlorate reduction pathway in *A. fulgidus* relies on molybdo-enzymes that have similarity with bacterial enzymes; however, chlorite is not split into chloride and oxygen, as occurs in bacteria, but evidence strongly suggests that it is eliminated by an interplay of abiotic and biotic redox reactions involving sulfur compounds. Biological (per)chlorate reduction by ancient archaea may have prevented accumulation of perchlorate in early terrestrial environments and consequently given rise to oxidizing conditions on Earth, before the occurrence of oxygenic photosynthesis.

Keywords: hyperthermophilic (per)chlorate reduction, deep subsurface, *Archaeoglobus*, sulfur

(Per)chlorate reduction by *Archaeoglobus fulgidus*

Perchlorate and chlorate anions [together referred to as (per)chlorate] in the environment have long been considered as arising mainly from anthropogenic activities, namely the production of perchlorate-containing rocket propellants and ammunitions followed by environmental pollution (1). Recent findings, however, indicate that perchlorate is continuously formed naturally in the atmosphere, with proposed mechanisms ranging from photochemically triggered processes (2) to electrical-discharge-based reactions and ozone oxidation of chlorides (3). Such natural sources make perchlorate an ubiquitous compound on Earth, though sizeable accumulations tend to be limited to certain arid environments, like the Atacama desert in Chile (4). Perchlorate deposits also exist on Mars (5). It has been proposed that the lack of perchlorate accumulation elsewhere on Earth might be due to microbial activity (4), which is supported by the widespread occurrence of bacteria that can use perchlorate and chlorate as terminal electron acceptor for growth (6).

(Per)chlorate-reducing bacteria described so far belong mainly to the bacterial phylum of Proteobacteria (7). The widely accepted pathway of biological (per) chlorate reduction consists of a two-step reduction from perchlorate, via chlorate to chlorite followed by a disproportionation to molecular oxygen and chloride (8). This metabolism relies on the action of a perchlorate reductase (Pcr) and a chlorate reductase (Clr), two functions that in perchlorate reducers are often performed by a single enzyme (9); it further requires a chlorite dismutase (Cld) to form molecular oxygen and chloride. Microbial formation of molecular oxygen under anaerobicity is a biochemical rarity.

Because the reported mechanisms for natural perchlorate generation on Earth seem to have existed already during preanthropogenic times (1, 2), the appearance of biological reduction of (per)chlorate may have been an important event in Earth's history. One indication of an ancient origin for reduction of (per)chlorate would be its occurrence in microorganisms that thrive in environments resembling those of early Earth. *Archaeoglobus fulgidus*, a hyperthermophilic archaeon fulfils this criterion. *A. fulgidus* was isolated from marine hot vents close to Vulcano island in Italy (10), but has since then been found in many extreme subsurface environments, such as hot oil reservoirs or geothermal formations (11, 12). It is considered to be a major contributor to sulfate reduction and sulfide formation at high temperature. *A. fulgidus* strain VC-16 is the best studied sulfate-reducing archaeon. Its genome contains many oxidoreductases genes with unknown function (13).

We demonstrate that *A. fulgidus* strain VC-16 as well as *A. fulgidus* strain Z (14), (Fig. S1) can grow with perchlorate or chlorate as electron acceptors (Fig. 1, and

Table S1) coupled to lactate oxidation. Strain VC-16 coupled (per)chlorate reduction also to fatty acids oxidation (butyrate, capriate, palmitate) and carbon monoxide utilization (results not shown); traits that have earlier been demonstrated coupled to sulfate reduction (15, 16). Our finding extends (per)chlorate reduction into the archaeal domain of life and high temperature environments.

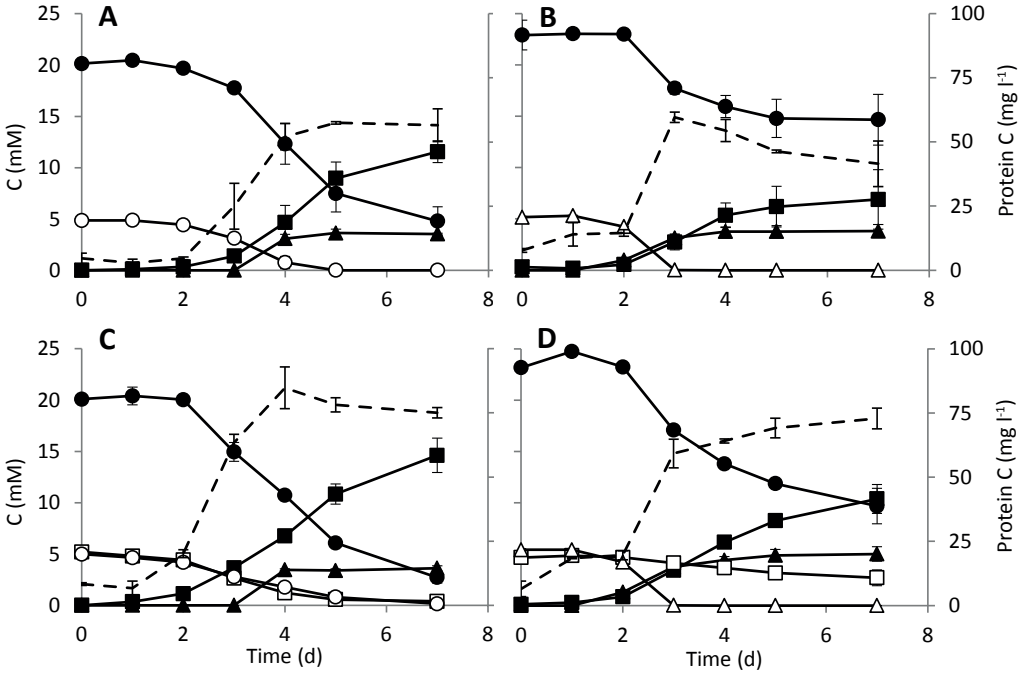

Figure 1: (Per)chlorate reduction by *A. fulgidus* strain VC-16. Experiments were performed with [(C) and (D)] and without addition of sulfate [(A) and (B)] as an alternative electron acceptor. The reduction of perchlorate (open circle), chlorate (open triangle) and sulfate (open square) is coupled to the oxidation of lactate (filled circle), producing acetate (filled square) and formate (filled triangle). Growth is represented by the protein concentration (dashed line), plotted against the secondary y-axis (right). Inocula used were exponentially growing perchlorate- (A, C) and chlorate-reducing (B, D) cultures, respectively. In the absence of either electron acceptor or electron donor (lactate) no growth and substrate conversion were observed (Table S1 and Fig. S4); means ± range (bars), *n=2*.

Enzyme assays showed chlorate-reducing activity in cell-free extracts and suspensions of *A. fulgidus* VC-16 that were comparable with activities found in some mesophilic (per)chlorate-reducing bacteria (Table S2). An activity towards perchlorate could not clearly be identified, which is similar to an earlier study on the perchlorate reductase of *Azospira oryzae* strain GR-1 (9). No chlorite dismutase activity could be detected either. Consistent with this, no genes similar to known

chlorite dismutases were identified in the genome of *A. fulgidus*. On the other hand no accumulation of chlorite was observed, suggesting an alternative mechanism of chlorite conversion.

Perchlorate- and chlorate-grown cells of *A. fulgidus* are still able to use sulfate as electron acceptor. When sulfate and perchlorate are present together in the cultures, both are used simultaneously, whereas sulfate reduction is delayed if chlorate is present (Fig. 1C and D). Simultaneous perchlorate and sulfate reduction in a single culture is intriguing from an energetic viewpoint. The midpoint potentials of redox couples involved in (per)chlorate reduction are high, while those involved in sulfate reduction are low (ClO_4^-/ClO_3^- $E^{0'} = + 0.788$ V; ClO_3^-/ClO_2^- $E^{0'} = + 0.709$ V; ClO_2^-/Cl^- $E^{0'} = + 1.199$ V versus SO_4^{2-}/HSO_3^- $E^{0'} = - 0.516$ V; HSO_3^-/HS^- $E^{0'} = - 0.110$ V). During the reduction of (per)chlorate the redox potential is locally increasing, but the overall redox state remained low in all the cultures throughout the entire experiment (<-200 mV; indicated by resazurin in the medium and a redox electrode).

Sulfide is normally omitted from media when growing (per)chlorate reducers but is used to establish a low-redox-potential required for growth of strict anaerobes, like *A. fulgidus*. Similar to sulfate reduction, (per)chlorate reduction in *A. fulgidus* requires reduced conditions that are established by the addition of sulfide to the medium and even pronounced by sulfide formation if sulfate is present as well (Fig.1C and D).

Proteome analysis of cells grown with either perchlorate or chlorate shows that in comparison with sulfate-grown cells, there is an increased abundance of a large number of proteins that are associated with redox and oxygen stress (Table S3). We hypothesize that in the presence of (per)chlorate these proteins play a crucial role in creating and maintaining a low intracellular redox potential, which is a basic requirement for dissimilatory sulfate reduction to take place. The differential expression of stress proteins reflects differences in redox-stress conditions. Potentially destructive redox stress is also indicated by the increased abundance of enzymes involved in cell repair (Table S3). The higher Gibbs free energy change when coupling the oxidation of a substrate to (per)chlorate instead of sulfate (Table S4) was not visible in terms of enhanced growth rate and/or higher growth yield of *A. fulgidus* strain VC-16. The mass balances at the 3 growth conditions resulted in similar protein concentrations, as a measure of biomass (Table S1). These growth yields reflect high maintenance costs when growing with (per)chlorate.

The proteome of VC-16 cells grown with (per)chlorate also contains proteins encoded by three gene clusters that are annotated as molybdopterin oxidoreductases (cluster I: AF0157-AF0160; cluster II: AF0173-AF0176; cluster III: AF2384-AF2386) (Table 1).

Table 1: Expression of key proteins involved in (per)chlorate reduction by *A. fulgidus* strain VC-16.

	Spectral counts					
Function	**Perchlorate**		**Chlorate**		**Sulfate**	
molybdo-oxidoreductase I (α-subunit: AF0159)	568	712	261	300	20	16
molybdo-oxidoreductase II (α-subunit: AF0176)	1039	1190	640	766	112	69
molybdo-oxidoreductase III (α-subunit: AF02384)	1305	1392	1703	2154	349	190
ATP sulfurylase (AF1667)	952	1033	741	834	1769	1515
APS reductase (α-subunit: AF1670)	2399	1906	1856	1375	3121	2871
sulfite reductase (α-subunit: AF0423)	255	328	265	450	317	283

Growth was performed with perchlorate, chlorate and sulfate as electron acceptor. Columns indicate biological duplicates. The locus tag numbers are in parentheses. The full set can be found in the Supplementary Materials (Table S3).

Cluster II is the best candidate for the reduction of (per)chlorate. Its catalytic subunit encoded by AF0176, has 31% identity (on protein level) and a query coverage of 98% with the alpha-subunit of the characterized perchlorate reductase of *Dechloromonas agitata* (17). Cluster I and cluster III are most likely involved in the metabolism of sulfur-based substrates: Cluster I carries a conserved domain in its alpha-subunit for a tetrathionate reductase (cld02758), while that of cluster III carries a conserved domain for thiosulfate-, sulfur-, and polysulfide reductases (cd02755) (18, 19).
Structure-based modeling suggested that AF0176 possibly is the catalytic subunit of a periplasmic NarG-type nitrate reductase (20). However, NarG key residues found in AF0176 can often be identified in the alpha-subunit of characterized perchlorate reductases (PcrA) as well (Fig. S2). Thus far, no reports mention the ability of *Archaeoglobus* species to respire with nitrate. Nitrate reductase and perchlorate reductase both belong to the type II DMSO family of enzymes, and share the conserved domain cd02750, indicating the high level of similarity between these enzymes. In addition, perchlorate reductases are known to reduce nitrate as well (9). The apparent involvement of the three molybdopterin oxidoreductases in (per)

chlorate reduction suggests the following model. (Per)chlorate reduction performed by AF0174-0176 results in the formation of chlorite, which reacts abiotically with sulfide. The reactivity of sulfide with chlorite triggers a cascade of subsequent reactions forming higher oxidized sulfur compounds (21, 22). Depending on the conditions in the medium all sulfur might eventually be converted to sulfate (Fig. S3). The accumulation of oxidized sulfur fractions requires the action of enzymes regenerating reduced sulfur compounds to continuously detoxify chlorite. These conversions can be catalyzed by AF0157-0159, a tetrathionate reductase-like enzyme, and AF2384-2386, an enzyme similar to thiosulfate-, sulfur-, and polysulfide reductases. Our findings give rise to a hypothesis where (per)chlorate reduction by *A. fulgidus* relies strictly on a sulfur-based cycle that is driven by both biotic and abiotic processes (Fig. 2). In nature these reduced conditions might also be accomplished by co-existing sulfate-reducing organisms or alternative reductants.

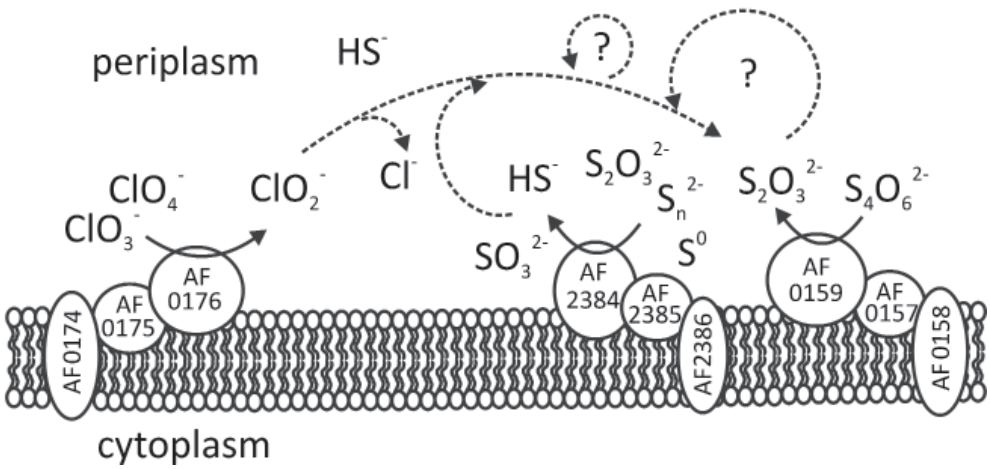

Figure 2: Proposed pathway and enzymatic machinery for the reduction of (per)chlorate coupled to sulfur compounds in *A. fulgidus*. Reductases involved in the reduction of (per)chlorate (AF0174-176) and tetrathionate (AF0157-159) and an enzyme similar to thiosulfate-, sulfur-, and polysulfide reductases (AF2384-2386). Interactions between biological and chemical processes are illustrated by solid and dashed lines, respectively.

In our model complete conversion of sulfide to sulfate is not essential, but the enzymes needed to activate and reduce sulfate are constitutively present (23) (Table 1). The chemical destruction of chlorite formed during biological (per)chlorate reduction by *A. fulgidus* is thus an alternative to biological disproportionation.

Because the (per)chlorate reduction mechanism in *A. fulgidus* does not involve chlorite dismutase, a key enzyme in known (per)chlorate-reducing bacteria, the coupling of biotic processes to the abiotic removal of the produced chlorite is a

necessity. The fact that a strict anaerobic archaeon is able to reduce and grow with (per) chlorate as electron acceptor suggests that this metabolism may be more widespread in the prokaryotic world. Furthermore, if perchlorate was produced in Earth's early atmosphere, biological mechanisms utilizing this compound may have evolved at a very early time, potentially even before oxygen-generating photosynthesis. The utilization of (per)chlorate together with other compounds such as nitrogen-oxo compounds may depict the first entry of highly oxidative compounds into microbial metabolism, and thus could have contributed to the rise of life adapted to more oxidizing conditions on Earth (24).

3

Materials and Methods

Strains and substrate utilization tests

The type strain of *A. fulgidus*, VC-16 (DSM4304^T) (10) and strain Z (DSM4139) (14) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The medium used throughout the study was DSM 399, recommended by DSMZ. The amounts of sodium bicarbonate and sodium sulfide added to the medium were slightly modified compared to the reference (final concentration of $NaHCO_3$, 4 g l^{-1} and $Na_2S.9\ H_2O$, 0.3 g l^{-1}). These compounds were added to the medium from a sterile stock solution after autoclaving. The recommended trace element solution was supplemented with 3 mg l^{-1} Na_2WO_4. Unless stated otherwise, electron donors and acceptors were added from 1 M heat-sterilized stock solutions. The pH of the medium was set to 6.5 (at 80°C, which was the incubation temperature). Growth experiments were performed in butyl-rubber stoppered, 120 ml serum bottles, containing 50 ml medium and a N_2/CO_2 (80:20; v/v) atmosphere of 1.5 bar in the headspace. Anaerobic cultivations were conducted according to standard methods. Multiple transfers were required to get stable growth of *A. fulgidus* with the novel electron acceptors, chlorate and perchlorate. Experiments were performed in duplicates. Cultures were inoculated with cells (1%) grown under the same respective conditions (3 subsequent transfers on the same substrates). Growth experiments were performed using lactate (20 mM) as electron donor and carbon source and perchlorate ($NaClO_4$), chlorate ($NaClO_3$) or sulfate (Na_2SO_4) (5 or 10 mM) as electron acceptors. The chemical stability of perchlorate and lactate under test conditions is illustrate in Fig. S5. The redox potential in the medium was monitored with resazurin and by using a redox electrode (Qis, Oosterhout, The Netherlands).

Analytical methods

Oxyanions were measured on a HPLC equipped with an Ion Pac AS22 column

(4x250 mm), using an ED 40 electrochemical detector (Dionex, Sunnyvale, CA). The eluent contained 1.29 g l^{-1} Na_2CO_3.10 H_2O and 0.12 g l^{-1} $NaHCO_3$, and the analysis was conducted with a flow rate of 1.2 ml min^{-1} at 35°C. Sodium bromide (NaBr) was used as an internal standard.
Lactate and fatty acid concentrations were quantified with a HPLC system using a Varian column (MetaCarb 87H Guard 4.6x50 mm, Middelburg, The Netherlands), connected to a UV and Refractive Index (RI) detector. Sulfuric acid at a concentration of 10 mM was used as eluent and sodium crotonate as internal standard. The flow rate was 0.8 ml min^{-1} and analyses were carried out at 30°C. The data generated were analyzed by the software ChromQuest.
Biomass yields were determined by measuring the protein content, rather than the optical density in order to avoid interference with precipitating sulfur (fractions). The protein content in cell-free extracts was quantified with a Bradford assay after cell disruption using sonication (5 cycles: 30 sec sonication at 40 kHz followed by 30 sec pause during incubation on ice). Bovine serum albumin was used as standard.

Enzyme activities

All steps for obtaining cell suspensions and cell-free extracts for enzyme activity measurements were performed in an anaerobic chamber with a N_2/H_2 (96:4; v/v) atmosphere, circulated over a palladium catalyst to remove traces of oxygen. Cells were harvested by centrifuging cultures in the late exponential phase, followed by re-suspending the pellets in phosphate buffer or PIPES buffer (50 mM), both set to a pH of 6.5 at 80°C. After one washing step the procedure was repeated. To obtain cell-free extracts cell suspensions were disrupted by ultrasonic disintegration and centrifuged for 10 min at 16000 x g. The supernatants and the cell suspensions were transferred to serum bottles, flushed with N_2, and kept on ice (9). The oxidation of reduced methylviologen (0.5 mM in 50 mM phosphate buffer, pH 6.5 at 80°C) was measured in response to the addition of cell-free extracts (cell suspensions) and perchlorate, chlorate and nitrate at 578 nm wavelength. Potential electron acceptors were added at a final concentration of 5.3 mM to the cuvette (Hellma GmbH, Müllheim, Germany). The solutions used for these trials were prepared anaerobically and added with gas-tight syringes to the cuvettes that were flushed with nitrogen prior to use.
A Clark-type electrode was used for the determination of chlorite dismutase activities (Yellow Spring Instruments, Yellow Springs, OH) (25). The activity was measured at 70°C in a 50 mM phosphate buffer (pH 6.5). Sodium chlorite was injected with a gas-tight syringe from an anaerobic stock solution (final concentration 0.2-1 mM). Enzyme activities are expressed in units (U); one unit is defined as the amount of enzyme required to convert 1 µmol of substrate per minute, and are related to the

protein content determined, as described above. In all assays control experiments were included, omitting either the addition of cell free extracts or the addition of substrates.

Proteome analysis

The differential protein abundances in cultures growing with different electron acceptors were investigated with LC-MS/MS (26). Cells grown with lactate and perchlorate, chlorate or sulfate as electron acceptors were compared. For each of the 3 conditions independent duplicates of 500 ml cell suspensions were grown until the late exponential phase and harvested by centrifugation. After disintegration (using 4% SDS) combined with ultrasonic treatment, samples were centrifuged to get cell-free samples. The protein concentrations were determined and for each growth condition an equal amount of total protein was separated by SDS-PAGE on a 10 well PAGE® Novex 4-12% Bis-Tris Gel (Invitrogen, Bleiswijk, NL) for 30 min at a constant voltage of 200 V using MES-SDS as running buffer. The gel was stained with Coomassie Blue (Colloidal Blue Staining Kit, Invitrogen) after which each lane was cut into 25 equal slides using a grid cutter (Gelcompany, San Francisco, CA). Gel pieces were reduced with 10 mM dithiotreitol (30 min at room temperature), alkylated with 20 mM iodoacetamide (60 min at room temperature in the dark) and digested with sequencing-grade trypsin overnight at 37°C. After digestion, formic acid and DMSO were added (both 5% v/v) to increase peptide recovery. Protein digests were analyzed on a reversed-phase nano-HPLC coupled to a LTQ-Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany). An Agilent 1200 series HPLC system was equipped with an in-house packed capillary trapping column (100 µm ID x 20 mm length) and analytical column (50 µm ID and 300 mm length) filled with Reprosil Pur 120 C18-AQ (Dr. Maisch, Ammerbuch-Entringen, Germany) (27).
Trapping was performed at 5 µl min^{-1} for 10 min in solvent A (0.1 M acetic acid), and a linear gradient from 0 to 40% solvent B (0.1 M acetic acid in 8:2 v/v acetonitrile:water) for 40 min at a flow rate of 100-150 nl min^{-1} was used to elute the peptides.
The column effluent was directly electro-sprayed in the ion source of a LTQ-Orbitrap Velos (Thermo Fisher, Bremen, Germany), which was programmed to operate in data-dependent mode, automatically switching between MS and MS/MS. Survey full-scan MS spectra were acquired from m/z 400 to 1500 in the Orbitrap analyser at a resolution of 30000 at m/z 400 after accumulation of ions to a target value of 1 x 10^6. The twenty most intense multiply charged ions above a set threshold of 5000 were fragmented in the linear ion trap using collision-induced dissociation (CID) after accumulation to a target value of 1 x 10^4. The isolation width was set to 2.5 amu, the normalized collision energy at 35% and dynamic exclusion was 90 sec. All

raw data files were processed into peaklists using Proteome Discoverer 1.2 (Thermo Fisher).

Bioinformatics

The genome of *A. fulgidus* VC-16 was published by Klenk et al., 1997 (13) and is publicly available via the NCBI website. MS/MS spectra were searched using the Open Mass Spectrometry Search Algorithm (OMSSA) (28). The spectra (for each condition between 66000 and 94000) were searched against a peptide database derived from the predicted *A. fulgidus* VC-16 proteome, and a decoy database constructed from reversing the predicted *A. fulgidus* VC-16 proteome. Peptide spectrum matches were performed with OMSSA (28) with the following parameters: a precursor ion tolerance of 0.2 Da, a fragment ion tolerance of 0.3 Da, a missed cleavage allowance of up to and including 2, fixed carbamide methylation, variable oxidation of methionine and deamination of glutamine and asparagine. The E-value threshold was set iteratively from the false discovery rate (FDR) and was set to 0.01, in which case the FDR is expected to be below 5%. For FDR calculation, top hit spectral matches to peptides in the reversed database were taken and the number of false positives was divided by the number of total positives. Relative abundance of each protein within the total pool of proteins was estimated by spectral counting (29).

Significant differences in spectral counts were investigated by applying the likelihood ratio G-test for independence (30) with the null hypothesis of equal protein distribution between the three conditions. The null hypothesis was rejected when a specific gene was differentially expressed at a level of $p \leq 0.05$.

A rather wide set of proteins was at least 3-times more abundant when cells were grown under perchlorate-/chlorate-reducing conditions compared with under sulfate-reducing conditions (Table S3). Those inferred to be fundamentally involved in metabolizing (per)chlorate and accumulating intermediates during the process are displayed in Table 1 of the article. The proteome analysis of perchlorate- and chlorate-grown cells did not reveal any significant difference in the expression profiles, thus they are henceforth discussed together in the comparison with cultures grown under sulfate-reducing conditions. The complete proteomic data set generated is accessible via The Proteomics Identifications Database (PRIDE), accession number 1-20130122-51500. Multiple-sequence alignments were made using ClustalX2 (31). Gene-cluster identification was done via the microbes online database, which is based on the work of Price et al., 2005 (32). For the subcellular location of archaeal proteins Pred-Signal was used (33).

Acknowledgements

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Supplemental Material

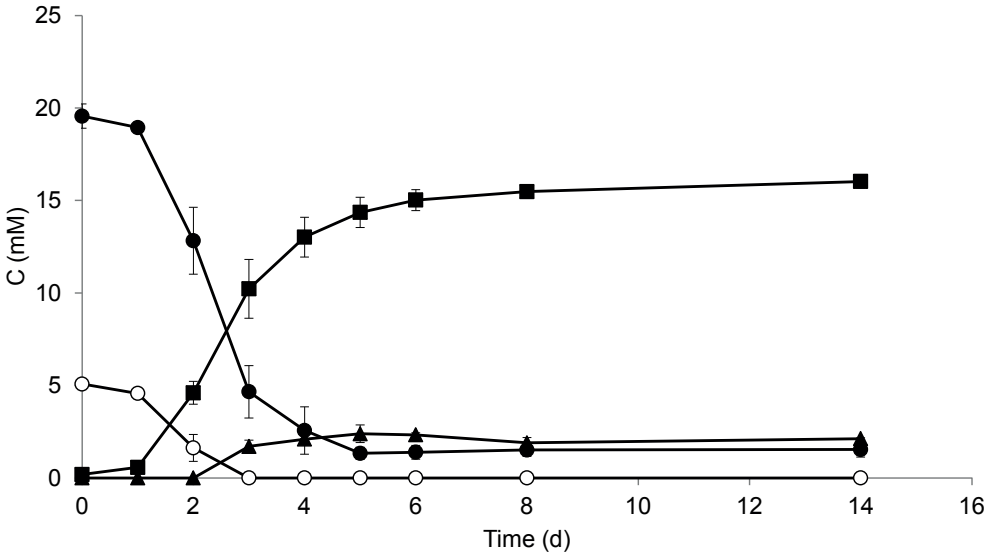

Figure S1: Perchlorate reduction by *A. fulgidus* strain Z. The reduction of perchlorate (open circle), is coupled to the oxidation of lactate (filled circle), accumulating acetate (filled square) and formate (filled triangle). Inocula used were exponentially growing perchlorate-reducing cultures. In the absence of either electron acceptor or electron donor no growth or conversion was observed; $n=2$, means ± range (bars).

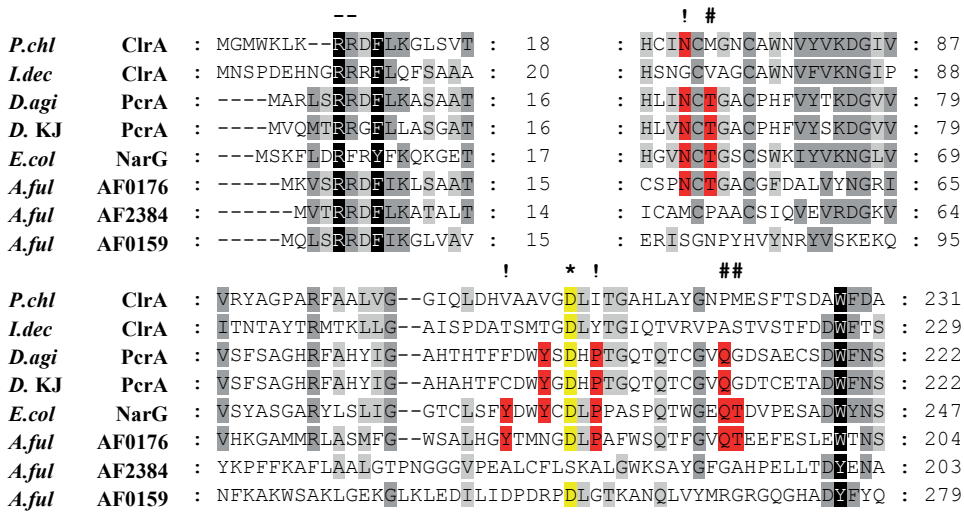

Figure S2: Multiple sequence alignment of the catalytic alpha-subunits of characterized chlorate reductases (ClrA), perchlorate reductases (PcrA), nitrate reductase (NarG) and the molybdopterin-binding subunits of the oxidoreductases of *A. fulgidus*, *Pseudomonas chloritidismutans* (P.chl), *Ideonella dechloratans* (I.dec), *Dechloromonas agitata* (D.agi), *Dechlorosoma* sp. KJ (D. KJ), and *Escherichia coli* (E.col). Key residues of NarG according to Dridge et al., 2006 (1) are in red. Exclamation mark indicates residues that are adjacent to the Mo cofactor; the asterisk marks the Mo ligand and the hash key the residues dictating the putative substrate entry channel. The twin-arginine motif in the putative leader sequence is indicated with hyphens.

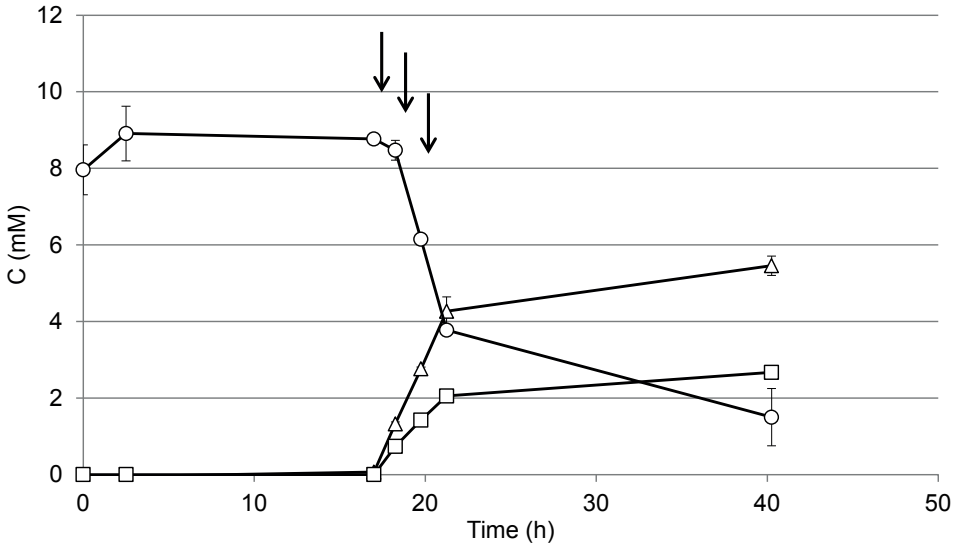

Figure S3: Chemical reactivity of chlorite with sodium sulfide at 80°C. The relatively thermal-stable chlorite (open circle) reacts with injected sulfide (arrows), forming sulfate (open square) and chloride (open triangle), according to the stoichiometry: $2ClO_2^- + S^{2-} \rightarrow SO_4^{2-} + 2Cl^-$. Injections of 1.25 mM sodium sulfide are indicated by arrows; in phosphate buffer (50 mM, pH = 6.5); *n=3* (error bars, s.d.).

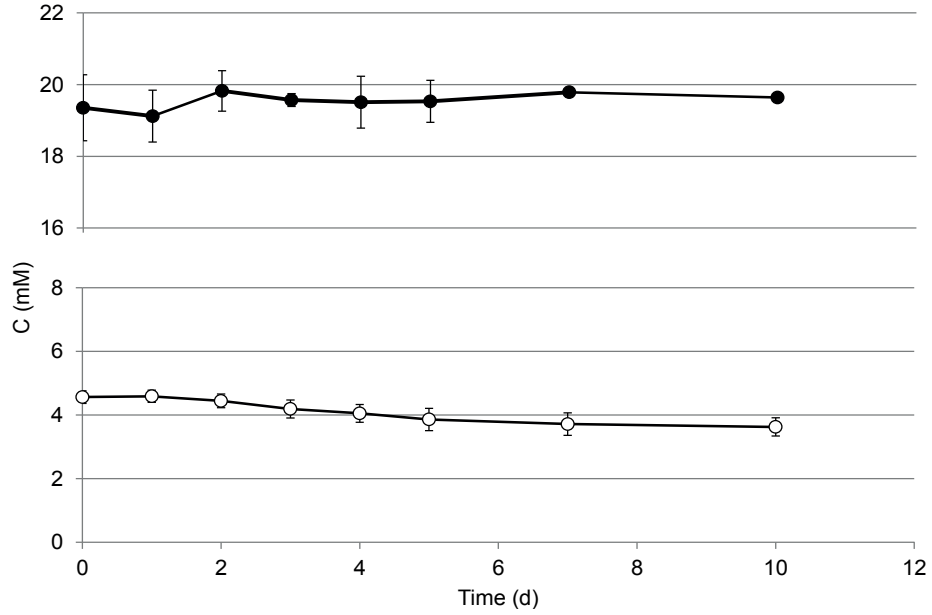

Figure S4: Biological controls with *A. fulgidus* strain VC-16 exposed to lactate (filled circle) or perchlorate only (open circle). Exponentially growing perchlorate-reducing cultures were used as inocula; *n=2*, means ± range (bars).

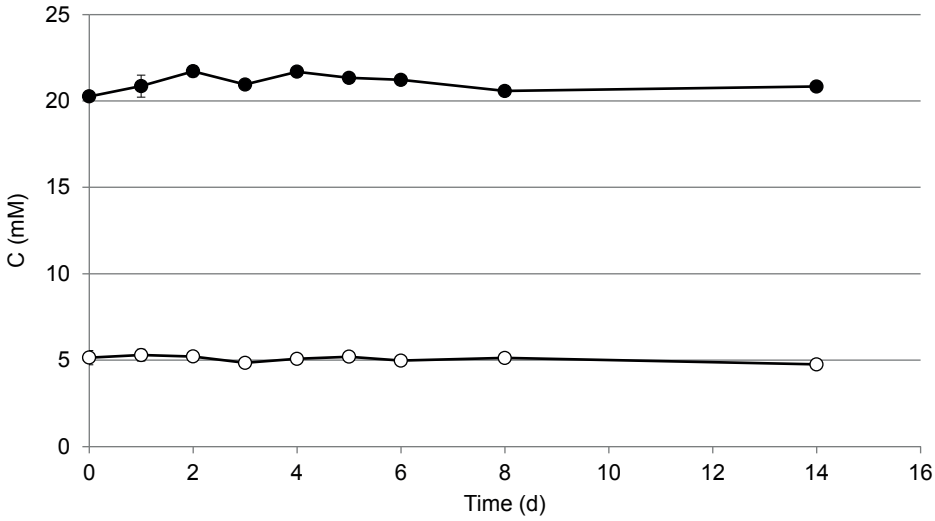

Figure S5: Abiotic stability of perchlorate (open circle) and lactate (filled circle) under cultivation conditions (reduced DSM 399 Medium, 80°C); *n=2*, means ± range (bars).

Table S1: *A. fulgidus* strain VC-16, growing under three different redox conditions. Start and end-point measurements (t=0 and t=9 days) were performed for substrates and products, as well as for the growth yield (expressed in the protein content). No conversion or turbidity was observed when lactate was offered without any electron-acceptor. "NM" stands for not measured; *n=2*, means ± range.

Conditions		**Start**	**End**	**Difference**
Sulfate reduction	sulfate (mM)	9.6 ± 0.1	1.4 ± 0.1	-8.2
	lactate (mM)	18.5 ± 0.1	8.4 ± 0.6	-10.1
	acetate (mM)	0	4.6 ± 0.0	4.6
	formate (mM)	0	1.3 ± 0.1	1.3
	protein yield (mg l^{-1})			107.3 ± 16.3
Chlorate reduction	chlorate (mM)	10.4 ± 0.1	0	-10.4
	lactate (mM)	19.3 ± 0.1	5.2 ± 0.2	-14.1
	acetate (mM)	0	9.0 ± 0.2	9.0
	formate (mM)	0	2.8 ± 0.0	2.8
	protein yield (mg l^{-1})			73.0 ± 6.6
	perchlorate (mM)	9.7 ± 0.3	0	-9.7

Perchlorate reduction	lactate (mM)	20.0 ± 0.5	2.3 ± 0.4	-17.7
	acetate (mM)	0	10.6 ± 0.7	10.6
	formate (mM)	0	3.1 ± 0.1	3.1
	protein yield (mg l^{-1})			98.6 ± 5.4
Lactate (without e-acceptor)	lactate (mM)	19.3 ± 0.9	19.6 ± 0.1	0.3
	acetate (mM)	0	0	0
	formate (mM)	0	0	0
	protein yield (mg l^{-1})			NM

Table S2: Specific enzyme activities determined in crude extracts (cell-free extracts) of (per)chlorate-reducing microorganisms [µmol mg^{-1} min^{-1}]. Cell-free extracts of *A. fulgidus* derived from cells grown with perchlorate (*) or chlorate (**); incubations using cell-free extracts or electron acceptors (ClO_4^-, ClO_3^-, ClO_2^-) alone were used as controls; *n=3* (± s.d.).

	Pcr	Clr	Cld	Reported e-acceptors	Ref.
*A. ful.**	ND	0.27 ± 0.05	ND	ClO_4^-, ClO_3^-, SO_4^{2-}, SO_3^{2-}	this study
*A. ful.***	ND	0.29 ± 0.06	ND		
A. ory.	0.15	0.39	145	ClO_4^-, ClO_3^-, NO_3^-, O_2, Mn(IV)	(2), (3)
D. hor.	+	3.12	155	ClO_4^-, ClO_3^-, NO_3^-, O_2	(4)
A. den.	NM	0.30-0.40	5.7-22	ClO_3^-, NO_3^-, NO_2^-, O_2	(5)
P. chl.	ND	9	134	ClO_3^-, O_2	(6)

***A. ful.*, *Archaeoglobus fulgidus* VC-16; *A. ory.*, *Azospira oryzae* GR-21; *D. hor.*, *Dechloromonas hortensis* MA-1; *A. den.*, *Alicycliphilis denitrificans* BC; *P. chl.*, *Pseudomonas chloritidismutans* AW-1; ND, not detectable; NM, not measured; +, degree of activity not reported**

Table S3: Proteins of *A. fulgidus* strain VC-16 with at least 3-times higher abundance when growing under perchlorate- and chlorate-reducing conditions, compared to sulfate-reducing conditions.

		Spectral counts							
	Locus tag	**Perchlorate**		**Chlorate**		**Sulfate**		**Function**	**Cluster**
	AF0035	36	18	31	16	3	5	mannosephosphate isomerase	
	AF0062	57	57	55	97	12	13	signal transduction protein	
	AF0066	7	8	8	8	1	1	AbrB family transcriptional regulator	
	AF0080	23	23	24	24	5	2	acetylornithine aminotransferase	
	AF0092	7	23	21	18	1	1	sulphate ABC transporter ATP-binding protein	
	AF0094	10	19	27	24	3	1	molybdate ABC transporter periplasmic substrate-binding protein	
	AF0099	14	10	14	9	3	3	PilT protein domain-containing protein	
	AF0113	11	14	10	9	1	1	5-formaminoimidazole-4-carboxamide-1-(beta)-D-ribofuranosyl 5'-monophosphate synthetase	
	AF0152	216	285	195	179	10	8	copper-transporting ATPase P-type	
	AF0154	60	97	76	58	3	3	iron permease FTR1	
	AF0155	74	112	86	106	19	15	iron-sulphur cluster-binding oxidoreductase	1
	AF0156	23	22	32	15	2	2	ferredoxin	1
	AF0157	24	46	13	18	1	1	molybdopterin oxidoreductase (Fe-S binding subunit)	2
	AF0158	26	34	18	14	1	1	molybdopterin oxidoreductase (membrane subunit)	2
	AF0159	568	712	261	300	20	16	molybdopterin oxidoreductase (mo-binding subunit)	2
*	AF0160	2	16	5	15	1	2	TtrD	2
*	AF0161	9	35	11	17	1	2	molybdenum cofactor biosynthesis protein A	2
	AF0163	38	50	32	27	1	1	unknown function	
	AF0168	29	33	34	14	2	1	arsenical resistance operon repressor	3
	AF0169	51	59	46	45	1	1	unknown function	3

	AF0170	156	177	154	249	1	1	sensory box protein	4
	AF0171	25	33	34	32	1	1	oxidoreductase molybdopterin binding protein	4
*	AF0172	1	1	1	1	1	1	unknown function	4
	AF0173	24	28	23	32	1	1	reductase assembly protein	5
	AF0174	69	81	46	54	2	2	molybdopterin oxidoreductase (membrane-subunit)	5
	AF0175	157	233	125	167	9	15	molybdopterin oxidoreductase (Fe-S binding subunit)	5
	AF0176	1039	1190	640	766	112	69	molybdopterin oxidoreductase (mo-binding subunit)	5
	AF0177	18	88	92	140	3	4	tungsten formylmethanofuran dehydrogenase subunit E	
	AF0179	8	17	7	20	1	2	unknown function	
	AF0183	18	18	21	7	1	1	NAD(FAD)-dependent dehydrogenase	
	AF0184	125	222	203	183	41	34	XRE family transcriptional regulator	6
	AF0185	39	75	59	51	4	5	nifU protein	6
	AF0186	110	191	107	177	11	6	class-V aminotransferase	6
	AF0187	17	20	23	11	1	1	DsrE family protein	6
*	AF0188	2	3	1	1	1	1	SirA family protein	6
	AF0204	8	11	10	14	1	1	polyprenyl synthetase	
	AF0212	39	31	31	55	2	2	histidinol dehydrogenase	
	AF0253	54	38	43	45	10	9	GMP synthase subunit B	
*	AF0265	8	10	20	16	2	3	molybdenum cofactor biosynthesis protein B	
	AF0343	7	10	26	8	1	1	tryptophan repressor binding protein (wrbA)	
	AF0361	21	24	23	34	1	5	UDP-glucose 4-epimerase	
	AF0374	12	15	15	11	3	3	p-nitrophenyl phosphatase	
	AF0395	20	38	30	30	1	2	NADH oxidase	
	AF0409	81	162	92	144	13	12	LL-diaminopimelate aminotransferase	
	AF0411	36	17	25	25	2	1	cysteinyl-tRNA synthetase	
	AF0435	23	19	17	12	2	3	enoyl-CoA hydratase	
	AF0444	20	25	33	26	5	6	phosphoglycolate phosphatase	

	AF0461	13	9	15	12	1	3	Sam Hydroxide Adenosyltransferase	
	AF0480	15	31	25	19	5	4	fuculose-1-phosphate aldolase	
	AF0508	76	79	99	215	11	15	pyridoxal biosynthesis lyase PdxS	7
	AF0509	33	43	49	35	6	8	glutamine amidotransferase subunit PdxT	7
*	AF0510	23	15	12	28	27	16	putative permease	7
	AF0529	5	15	12	8	1	1	transcriptional regulator TrmB	
	AF0550	19	9	12	22	2	3	N-ethylammeline chlorohydrolase	
	AF0555	79	69	169	69	19	17	Peroxiredoxin family protein	8
	AF0556	12	14	21	12	1	2	SirA family protein	8
	AF0557	89	84	133	183	3	3	flavoprotein reductase	8
*	AF0558	65	61	135	98	23	34	unknown function	8
*	AF0563	2	2	1	2	2	2	DsrE family protein	9
	AF0564	69	103	65	95	9	2	class-V aminotransferase	9
	AF0565	39	75	59	51	4	5	nifU protein	9
*	AF0566	116	212	191	164	43	39	XRE family transcriptional regulator	9
	AF0588	20	14	16	17	4	3	7-cyano-7-deazaguanine tRNA-ribosyltransferase	
	AF0590	50	37	40	38	5	9	ATP phosphoribosyltransferase	
	AF0628	89	62	68	77	5	7	multifunctional 3-isopropylmalate dehydrogenase/D-malate dehydrogenase	
	AF0632	13	14	12	6	1	1	nifU protein	
	AF0651	9	9	10	8	2	2	AsnC-type transcription regulator	
	AF0656	32	39	37	41	8	5	antibiotic maturation protein	
	AF0665	5	13	4	5	1	1	O-sialoglycoprotein endopeptidase	
	AF0713	27	16	22	20	3	3	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	
	AF0728	28	38	35	41	1	1	cobalt transport protein CbiM	10
*	AF0729	1	1	1	1	1	1	cobalt transport protein CbiN	10
	AF0730	11	20	24	9	2	3	cobalt transporter	10

*	AF0731	22	51	67	80	11	14	cobalt ABC transporter ATP-binding protein	10
*	AF0732	23	29	56	15	6	4	cobalamin biosynthesis precorrin-8W decarboxylase	10
	AF0802	13	18	17	8	1	2	unknown function	
	AF0822	8	14	6	17	1	1	branched-chain amino acid ABC transporter ATP-binding protein	
	AF0851	38	18	14	25	3	1	carbohydrate kinase	
	AF0887	19	17	23	32	2	2	ribose ABC transporter ATP-binding protein	
	AF0901	4	5	5	4	1	1	NTPase	
	AF0917	5	6	6	6	1	1	2-phospho-L-lactate transferase	
*	AF0930	1	9	8	7	1	1	molybdenum cofactor biosynthesis protein A	11
	AF0931	24	25	22	15	3	1	molybdopterin biosynthesis protein MoeA/LysR substrate binding-domain-containing protein	11
	AF0933	73	75	40	81	8	10	branched-chain amino acid aminotransferase	12
*	AF0934	3	4	2	1	6	4	amino acid-binding ACT domain-containing protein	12
*	AF0935	6	5	6	7	1	2	homoserine dehydrogenase	12
	AF0936	24	37	31	26	5	3	peptidase A24B, FlaK domain protein	12
	AF0942	12	9	12	11	1	1	stationary phase survival protein SurE	
	AF0957	31	22	22	25	4	2	2-isopropylmalate synthase	
*	AF0958	11	13	5	7	3	2	branched-chain amino acid ABC transporter ATP-binding protein	13
*	AF0959	5	7	8	8	2	1	branched-chain amino acid ABC transporter ATP-binding protein	13
*	AF0960	1	3	4	6	1	2	branched-chain amino acid ABC transporter permease	13
*	AF0961	13	5	9	11	6	8	branched-chain amino acid ABC transporter permease	13
*	AF0962	15	22	22	54	8	7	branched-chain amino acid ABC transporter substrate-binding protein	13

*	AF0963	7	6	22	5	1	2	enoyl-CoA hydratase	13
	AF0964	14	14	13	12	1	1	acyl-CoA dehydrogenase	13
*	AF0965	19	14	24	21	5	13	putative nucleic-acid-binding protein containing a Zn-ribbon	13
*	AF0966	18	30	35	38	3	8	protein associated with acetyl-CoA C-acyltransferase	13
	AF0967	50	35	50	74	4	10	acetyl-CoA acetyltransferase	13
*	AF0968	32	21	27	45	2	10	acetyl-CoA acetyltransferase	13
	AF0976	7	14	7	8	1	1	acetyl-CoA synthetase	
	AF0997	11	6	6	11	1	1	N-ethylammeline chlorohydrolase	
	AF1030	48	39	30	45	4	2	bipolar helicase	
	AF1046	4	11	8	5	1	1	PAS sensor protein	
	AF1097	36	28	31	27	2	3	mannose-6-phosphate isomerase	
	AF1099	17	19	28	25	2	2	fumarate hydratase	
	AF1125	12	18	9	21	3	1	heme biosynthesis protein (nirJ-1)	
	AF1132	39	29	30	29	9	9	phosphopyruvate hydratase	
	AF1146	70	60	33	45	9	6	3-phosphoglycerate kinase	
	AF1157	191	89	110	103	25	18	phosphoribosylamine-glycine ligase	
	AF1186	12	11	7	11	1	1	inosine-5'-monophosphate dehydrogenase-like protein VIII	
	AF1265	40	38	66	92	11	11	metal-dependent hydrolase	
	AF1277	20	17	15	14	1	3	glutamine amidotransferase	
	AF1290	13	17	25	17	1	3	cobalamin adenosyltransferase	
	AF1293	19	48	31	34	1	2	acyl-CoA dehydrogenase	
	AF1316	60	54	42	104	10	7	threonine synthase	
	AF1336	4	6	4	4	1	1	cobalamin biosynthesis protein	
	AF1343	22	17	26	13	4	4	beta-lactamase domain protein	
	AF1354	115	192	169	208	18	20	DSBA oxidoreductase	
	AF1368	11	14	11	12	3	3	hydrogenase expression/ formation protein (hypB)	
	AF1400	11	13	20	14	1	1	adenosylcobinamide amidohydrolase, putative	
	AF1446	30	32	30	42	10	10	unknown function	

	AF1452	17	9	14	11	1	2	signal-transducing histidine kinase
	AF1489	52	68	46	55	13	9	indolepyruvate ferredoxin oxidoreductase subunit alpha
	AF1494	28	22	30	17	3	3	glucose-6-phosphate isomerase
	AF1504	34	36	52	28	6	6	unknown function
	AF1523	7	8	11	4	1	1	unknown function
	AF1535	30	35	30	23	3	4	ferredoxin-thioredoxin reductase catalytic subunit
	AF1536	37	35	39	25	4	4	glutaredoxin
	AF1554	61	75	79	58	11	14	thioredoxin reductase
	AF1644	5	6	7	6	1	1	tungsten formylmethanofuran dehydrogenase subunit F
	AF1647	18	18	17	12	1	1	nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase
	AF1650	41	35	45	69	6	8	tungsten formylmethanofuran dehydrogenase subunit B
	AF1671	51	101	84	131	9	9	coenzyme F390 synthetase
*	AF1692	5	4	6	6	1	1	endonuclease III
	AF1724	7	18	7	21	1	2	dinitrogenase reductase activating glycohydrolase
	AF1732	212	284	159	223	53	52	glyceraldehyde-3-phosphate dehydrogenase
	AF1736	32	32	27	70	5	4	3-hydroxy-3-methylglutaryl-coenzyme A reductase
	AF1751	16	11	19	42	3	2	cofactor-independent phosphoglycerate mutase
	AF1764	9	19	13	16	3	3	dCMP deaminase
	AF1958	10	11	7	21	1	1	2-hydroxyglutaryl-CoA dehydratase subunit alpha
	AF1963	74	108	78	112	18	15	aconitase
	AF1965	10	23	28	17	2	1	Holliday junction-type resolvase
	AF1985	116	77	98	107	18	20	ketol-acid reductoisomerase
	AF2000	52	41	41	58	11	9	S-adenosyl-L-homocysteine hydrolase
	AF2021	165	151	156	159	25	28	rod shape-determining protein MreB

AF2035	23	13	17	19	2	1	seryl-tRNA synthetase	
AF2049	7	9	8	9	2	1	polynucleotide 5'-hydroxyl-kinase	
AF2062	17	8	7	11	1	1	signal recognition particle receptor	
AF2065	48	46	41	41	9	10	translation initiation factor IF-6	
AF2074	28	28	42	23	5	7	thiamine phosphate pyrophosphorylase	
AF2093	35	55	45	81	6	6	unknown function	
AF2153	9	7	9	11	2	2	metal dependent hydrolase	
AF2185	40	41	37	40	10	8	succinyl-CoA synthetase subunit alpha	
AF2186	44	35	27	47	4	5	succinyl-CoA synthetase subunit beta	
AF2191	12	15	15	9	3	3	CcmE/CycJ protein	
AF2200	12	16	19	16	1	2	mutator protein MutT	
AF2211	16	22	36	23	5	5	HIT family protein	
AF2255	241	307	155	201	45	36	alanyl-tRNA synthetase	
AF2256	8	10	7	6	1	1	F420-dependent oxidoreductase	
AF2265	17	20	19	13	4	4	imidazoleglycerol phosphate synthase subunit H	
AF2268	28	33	27	17	2	4	acetoacetate decarboxylase-like protein	
AF2290	43	37	22	26	6	4	acetylpolyamine aminohydrolase	
AF2314	9	15	26	14	3	3	methylated DNA protein cysteine methyltransferase	
AF2317	9	12	12	12	1	1	ribonuclease P protein component 3	
AF2325	9	7	7	6	1	1	unknown function	
AF2366	16	21	25	27	5	5	aspartate aminotransferase	
AF2372	10	16	9	9	1	2	extragenic suppressor (suhB)	
AF2381	14	17	19	17	2	4	iron-sulfur cluster binding protein	
AF2384	1305	1392	1703	2154	349	190	molybdopterin oxidoreductase (mo-binding subunit)	14
AF2385	169	315	307	371	37	38	molybdopterin oxidoreductase (Fe-S binding subunit)	14

*	AF2386	24	40	34	48	9	8	molybdopterin oxidoreductase (membrane-subunit)	14
	AF2388	23	48	31	33	4	7	cation diffusion facilitator family transporter	
	AF2410	51	57	40	59	12	7	unknown function	

Proteins listed were at least three times more abundant under perchlorate- and chlorate-reducing conditions compared to sulfate-reducing conditions, unless marked with an asterisk. Enzyme complexes likely involved in the proposed model of (per)chlorate reduction (Fig. 2) are highlighted in yellow. Proteins playing a role in redox stress (response) processes and cell maintenance are marked blue. The potential co-expression of genes is indicated on the right.

Table S4: Gibbs free energy changes under standard conditions ($\Delta G^{0'}$) for the complete oxidation of lactate coupled to the reduction of sulfate (1), chlorate (2) and perchlorate (3).

(1) $2lactate^- + 3SO_4^{2-} \rightarrow 6HCO_3^- + 3HS^- + H^+$ $\Delta G^{0'} = -255$ kJ/reaction

(2) $1lactate^- + 2ClO_3^- \rightarrow 3HCO_3^- + 2Cl^- + 2H^+$ $\Delta G^{0'} = -1578$ kJ/reaction

(3) $2lactate^- + 3ClO_4^- \rightarrow 6HCO_3^- + 3Cl^- + 4H^+$ $\Delta G^{0'} = -3013$ kJ/reaction

The differences in free energy releases between perchlorate-, chlorate- and sulfate-reduction coupled to complete lactate oxidation. However, in the current study the accumulation of end products was observed under all three conditions (acetate, formate). The amount of lactate oxidized and the formation of fermentation products during (per)chlorate reduction varied among different experiments (Fig. 1 and Table S1). These differences in stoichiometry may be caused by the redox stress or the involvement of sulfur compounds in the (per)chlorate reduction of *A. fulgidus* (Fig. 2).

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Chapter 4

Coupling (per)chlorate reduction to the sulfur metabolism: A case study of *Archaeoglobus fulgidus*

adapted from:
Martin G. Liebensteiner, Alfons J.M. Stams, Bart P. Lomans (2014) (Per)chlorate reduction at high temperature: Physiological study of *Archaeoglobus fulgidus* and potential implications for novel souring mitigation strategies, Int. Biodeter. Biodegr. *in press*.

Abstract

The recent finding that *Archaeoglobus fulgidus* is able to couple (per)chlorate reduction to growth expanded this trait to the hyperthermophilic range of life. This sulfate-reducing archaeon is considered to be one of the major contributors to souring in hot oil reservoirs. Therefore, it is important to study its physiology in depth, particularly in view of novel souring mitigation strategies. *A. fulgidus* does not possess the classical (per)chlorate reduction pathway, as it lacks the key enzyme chlorite dismutase. The microorganism rather seems to couple (per)chlorate reduction to sulfur metabolism. Growth experiments show the strict necessity of sulfur compounds to sustain perchlorate reduction. Furthermore, the chemical formation of elemental sulfur was observed during perchlorate reduction, a compound that is biologically reduced again. Additional experiments showed that tetrathionate, but not elemental sulfur and polysulfide, serve as electron acceptor for growth by *A. fulgidus*. Taken together these results provide further evidence for the importance of chemical and biological redox reactions involving sulfur compounds during (per)chlorate reduction. In non-reduced media also nitrate could be reduced by *A. fulgidus*, although this was not coupled to growth. This observation and the fact that *A. fulgidus* had prolonged adaptation phases on sulfate after long-lasting growth on perchlorate are interesting aspects for the development of new souring mitigation strategies using nitrate and/or (per)chlorate.

Keywords: (per)chlorate reduction, oil reservoirs, souring mitigation, *Archaeoglobus*, elemental sulfur, nitrate

Introduction

Microbial reservoir souring is caused by the formation of sulfide by sulfate-reducing prokaryotes (SRP) that are indigenous to oil reservoirs or were introduced during secondary oil recovery processes. Sulfide formation is associated with a set of negative effects (such as metal precipitation, corrosion, toxicity, H_2S-containing export gas and crude, etc.) that lead to rising production costs during oil recovery (1). To avoid or limit the detrimental impact of reservoir souring, various mitigation strategies are being applied in the oil industry. Sulfate removal from injection water is up until now probably the most effective method for souring control (provided that sulfate is not present in formation water), however, it is not often applied for due to high costs associated with the installation and operation and legislation issues concerning the disposal of reject streams. A more rarely applied option for souring mitigation is the extensive dosing of biocides, as its effect (deeper) in the reservoir is debated.

The most successfully applied strategy for souring mitigation in oil reservoirs is the dosage of nitrate to the injection water, a treatment that affects souring in different ways (2): a) heterotrophic nitrate-reducing prokaryotes (hNRP) outcompete SRP for available electron donors (3), b) inhibition of sulfite reductase by nitrite (4, 5) and c) sulfide removal by autotrophic NRP that couple nitrate reduction to the oxidation of sulfide (6). Mitigation through nitrate injection, however, is considered to be challenging in low temperature reservoirs where it might not be able to prevent the development of a sulfide-generating community deeper in the reservoir (7). Similarly, a zone of sulfide-generating hyperthermophiles might develop in the cold to hot transition zone around the injector well bore of hot oil reservoirs, in case hyperthermophilic nitrate reducers are missing. To our knowledge no nitrate-reducing microorganisms have been isolated from oil fields so far that could grow beyond 80°C, possibly enabling a niche for hyperthermophilic SRP to persist even when nitrate is present. For this reason dosing of nitrite may be an efficient strategy (8), because it directly inhibits SRP on an enzymatic level. However, the high chemical reactivity and potential formation of corrosive elemental sulfur upon sulfide exposure are drawbacks of such a strategy.

Due to the above-mentioned limitations there is a requirement to develop additional strategies for improved souring control in oil reservoirs. The microbial reduction of perchlorate (ClO_4^-) and chlorate (ClO_3^-) [together termed "(per)chlorate"] and in this respect their potential impact on microbial sulfidogenesis is a novel strategy that deserves further investigations.

The diversity of microorganisms that couple (per)chlorate reduction to growth is a rather broad. While most representatives are mesophiles from the phylum Proteobacteria (9), more recent studies identified (per)chlorate reducers also among

the Firmicutes (10, 11) and the archaeal domain of life (12-14). The common mechanism of perchlorate reduction follows a three-step enzymatic cascade employing two enzymes, perchlorate reductase (Pcr) and chlorite dismutase (Cld) (15). Chlorite dismutase is regarded to be the key enzyme for biological (per) chlorate reduction, catalyzing the reaction: $ClO_2^- \rightarrow Cl^- + O_2$, and thereby avoiding the accumulation of highly toxic chlorite (ClO_2^-). Some bacteria lack the ability to reduce perchlorate but are able to reduce chlorate due to the action of a chlorate reductase (Clr). Although Pcr and Clr share some biochemical characteristics they have a different evolutionary history (16).

(Per)chlorate reduction by *Archaeoglobus fulgidus* seems to follow an alternative pathway. In *A. fulgidus* the absence of a chlorite-disproportionating enzyme (Cld) is likely overcome by the highly reduced environment, which the archaeon requires for growth and which is characteristic for subsurface environments like oil reservoirs. Earlier conducted proteome analyses already indicated that sulfur compounds may play an important role in (per)chlorate reduction by *A. fulgidus* (12).

Archaeoglobus spp. are archaeal microorganisms that are ubiquitously found in hot oil reservoirs, where they are considered to be major contributors to reservoir souring by sulfate reduction (17-19). The observation that *A. fulgidus* is able to couple the reduction of (per)chlorate extended this trait to the hyperthermophilic range of life (12). This finding may have implications for the development of novel souring mitigation strategies. The effect of (per)chlorate on the microbial communities of oil fields is unknown. Physiological and molecular studies would be required to evaluate the potential of (per)chlorate for souring mitigation (similar to nitrate). In the current study we describe the interplay of (per)chlorate and sulfur metabolism in *A. fulgidus*. The results are discussed in an integrative way and interpreted in view of a potential future role of (per)chlorate during oil recovery processes.

Materials and methods

Strain, cultivation and medium

The hyperthermophilic archaeon *Archaeoglobus fulgidus* VC-16 (20) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH (DSMZ, Braunschweig, Germany). For growth experiments medium DSM 399, recommended by DSMZ, was used. The amounts of sodium bicarbonate and sodium sulfide in the medium were slightly modified compared to the reference (final concentration of $NaHCO_3$ was 4 g l^{-1} and $Na_2S.9\ H_2O$ was 0.3 g l^{-1}). Additionally, the trace element solution was supplemented with 3 mg l^{-1} Na_2WO_4. Sodium sulfide, sodium bicarbonate and electron donors and acceptors were added from sterile stock solutions to the medium after autoclaving. The pH of the medium was set to 6.5 (at

80°C, which was the incubation temperature for all experimental set-ups). Growth experiments were performed in 120 ml butyl-rubber stoppered serum bottles, containing 50 ml medium and a N_2/CO_2 (80:20; v/v) gas atmosphere of 1.5 bar. Experiments were performed in duplicates *(n=2)* and inoculated with 0.5 ml of fully grown cultures, unless stated otherwise. For standard growth conditions 20 mM sodium lactate and 5 or 10 mM electron acceptor (sodium perchlorate or sodium sulfate) were used. Additionally, sodium nitrate (5 mM), chemical elemental sulfur (4 g l^{-1}), potassium polysulfide (Acros Organics; 0.5 g l^{-1}) and potassium tetrathionate (5 mM) were tested as potential electron acceptors, together with lactate. The last two were tested without any further addition of sodium sulfide to the medium as reducing agent. Reduced conditions in the medium were visualized by resazurin and where indicated also measured by a redox electrode (Qis, Oosterhout, The Netherlands).
The trial monitoring the chemical reactivity of selected chemicals was performed in a water bath, using serum bottles containing sodium chlorite (10 mM) or sodium nitrite (10 mM) in anaerobic sodium phosphate buffer (50 mM, pH 6.5 at 80°C), with a headspace atmosphere of pure N_2. The following chemicals were tested for their reactivity towards one (or both) of the oxyanions, by injecting the respective concentrations twice: FeS (2 mM), $FeCl_2$ (2 mM), $MnCl_2$ (2 mM), $Na_2S_2O_3$ (2 mM), crude oil (1%), humic acid (Na-salt; 0.4%) and Na_2S (2 mM). FeS was produced by adding excessive amounts of $FeCl_2$ (1 M) to a stock of Na_2S (200 mM). After the chemical precipitation was completed, FeS was pelleted, washed (2x) and resuspended in anaerobic water. These steps were performed in an anaerobic tent.

Chromatographic analyses

Oxyanions were measured on a HPLC equipped with an Ion Pac AS22 column (4x250 mm), using an ED 40 electrochemical detector (Dionex, Sunnyvale, CA). The eluent was carbonate-bicarbonate buffer (1.29 g l^{-1} Na_2CO_3.10 H_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 (except in the trial which followed the abiotic reactivity of selected chemicals with chlorite/nitrite).
Lactate and fatty acids were analyzed on a HPLC system using a Varian column (MetaCarb 87H Guard 4.6x50 mm, Middelburg, The Netherlands). Both, a UV and Refractive Index (RI) detector were used. 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}. All chromatographically generated data was later analyzed by the software ChromQuest.

Elemental sulfur and sulfide analyses

Aliquots of 1 ml medium suspension were centrifuged and washed with deionized

water. The final pellet was resuspended in acetone and left for extraction overnight. The analysis of elemental sulfur (S^0) was based on the method described by Sörbo (21); for the calibration concentrations of 25-300 μM chemical S^0 in acetone were used.
Sulfide in the medium was determined with the methylene blue method (22). Aliquots were injected (1:1) into a $ZnCl_2$ solution (5% w/v) in order to precipitate sulfide. After waiting 20 minutes the samples were put at 4°C and analyzed at the end of the experiment. The photometric quantification was performed with the Merck Spectroquant® Multy at 670 nm.

Biomass production

The growth yield was determined by evaluating the protein content in the medium. Cell-free extracts of frozen culture aliquots were produced by disrupting cells with ultra-sonication (5 times 30 sec sonication at 40 kHz followed by 30 sec pause; on ice).
The protein content was determined by a Bradford assay using bovine serum albumin standards (23). Due to potential interferences with elemental sulfur direct OD measurements were abandoned as growth indicator.

Results and Discussion

Chemical reduction of periplasmic chlorite by sulfide

The presence of the enzyme chlorite dismutase is of crucial importance for (per) chlorate-respiring bacteria (9, 24). It was proposed that the lack of this enzyme in the (per)chlorate-reducing hyperthermophile *A. fulgidus* is compensated by the chemical elimination of chlorite with sulfide, allowing continuous (per)chlorate reduction (12). In this study the requirement of sulfide by *A. fulgidus* to perform (per)chlorate reduction was investigated in a medium where excess sulfide (used as reducing agent in the medium) was completely eliminated by precipitation with ferrous iron (Fig. 1A-C). By this treatment the redox potential remained at the same level as compared to a medium with dissolved sulfide (mainly present as HS^-) (both ca. -240 mV at the start), providing optimal circumstances for activity and growth of *A. fulgidus*.

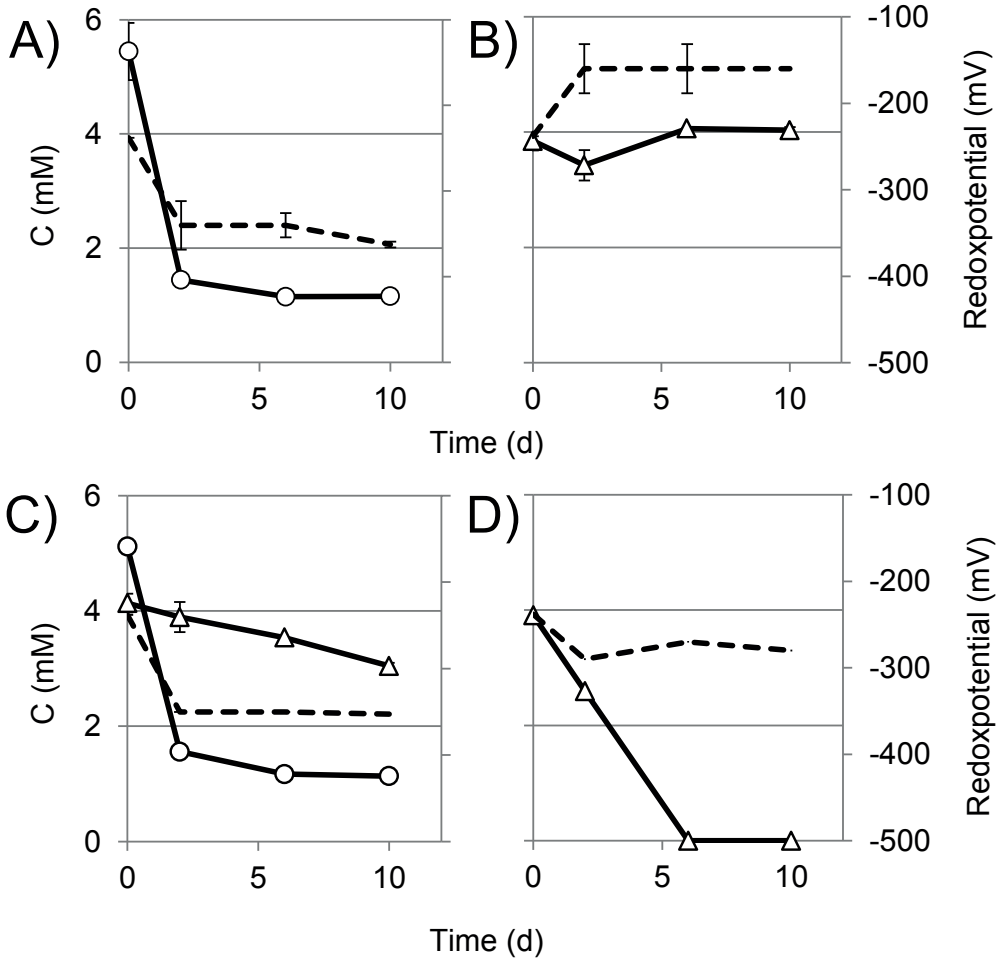

Figure 1: The activity of *A. fulgidus* on **A)** sulfate (circles) **B)** perchlorate (triangles) **C)** sulfate/perchlorate after removal of the excess of sulfide added as reducing agent by precipitation with Fe(II) (added as $FeCl_2$; 10 mM). **D)** Perchlorate reduction in the presence of dissolved sulfide, omitting $FeCl_2$ addition (positive control). Lactate was present in excessive concentrations (20 mM) as electron donor. The redox potential (dashed lines) was followed by a redox electrode. Panels A-C: *n=2*, means ± range (bars); Panel D: *n=1*.

The effect of this treatment on the utilization of sulfate (Fig. 1A) and perchlorate (Fig. 1B) was monitored. The results show that *A. fulgidus* was not able to reduce perchlorate anymore, whenever sulfide was removed by precipitation as FeS (Fig. 1B). Contrarily, the precipitation of sulfide did not result in any negative effect on sulfate reduction by *A. fulgidus* (Fig. 1A). The lowered availability of sulfide as an ubiquitous reagent for the chemical reduction of chlorite inhibited perchlorate reduction right after its start as indicated by an initial increase of the redox potential

(Fig. 1B). Unaltered "fitness" of sulfate reduction with precipitated sulfide points to the important role that sulfide plays (as chlorite scavenger) only during perchlorate reduction. Whenever sulfate and perchlorate were offered together (Fig. 1C) sulfate reduction proceeded unrestrained, while perchlorate was gradually reduced. Most likely due to the biological formation of sulfide in the cytoplasm and its cellular proximity to the (per)chlorate-reducing enzyme in the periplasm, sulfide is partially available to react with chlorite before precipitating with ferrous iron outside the cell. This enables perchlorate reduction to some extent. Under standard conditions where sulfide is in excess in the medium (per)chlorate reduction rates are several times higher than illustrated in Fig. 1C (compare with Fig. 1D).

Biological reduction of S^0 and tetrathionate, closing the "sulfur loop"

In case sulfide is present during (per)chlorate reduction oxidized sulfur compounds are formed by the reduction of chlorite. This was indirectly demonstrated in a previous study by proteome analyses (12), but it is now also illustrated by growth studies comparing sulfate- and perchlorate-reducing conditions where the formation of elemental sulfur (S^0) was followed (Fig. 2).

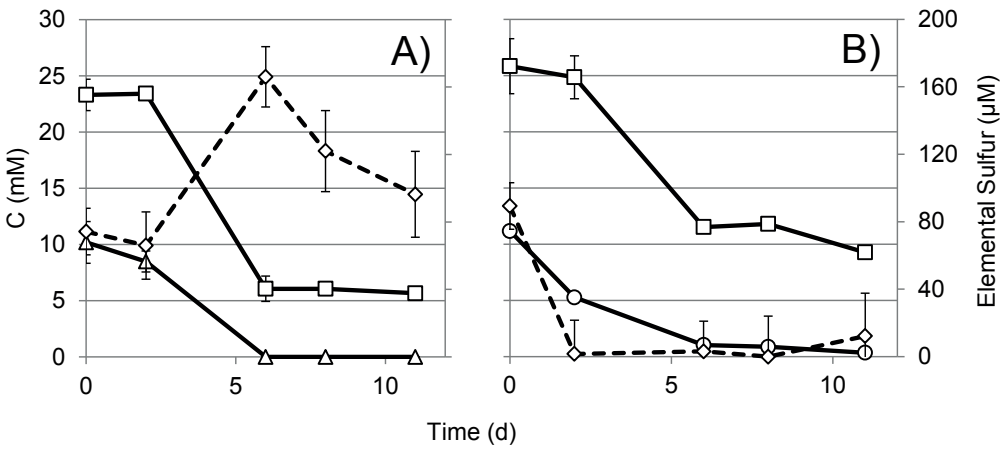

Figure 2: *A. fulgidus* growing on **A)** perchlorate (triangles) or **B)** sulfate (circles), together with lactate (excessive concentrations; squares). Diamonds with dashed lines represent the concentrations of elemental sulfur and are plotted against the secondary y-axis. *n=2*, means ± range (bars).

Elemental sulfur was formed up to 160 µM (equates to ca. 13% of total sulfur) during perchlorate reduction and got reduced again after perchlorate was depleted. The biological reduction of elemental sulfur to sulfide probably occurred already from the beginning of the experiment and prevented an even more pronounced accumulation of elemental sulfur in the medium than observed (compare Fig. 2A and

B). The reduction of elemental sulfur recycles reducing power required to remove the continuously formed chlorite and thereby drives (per)chlorate reduction (Fig. 3).

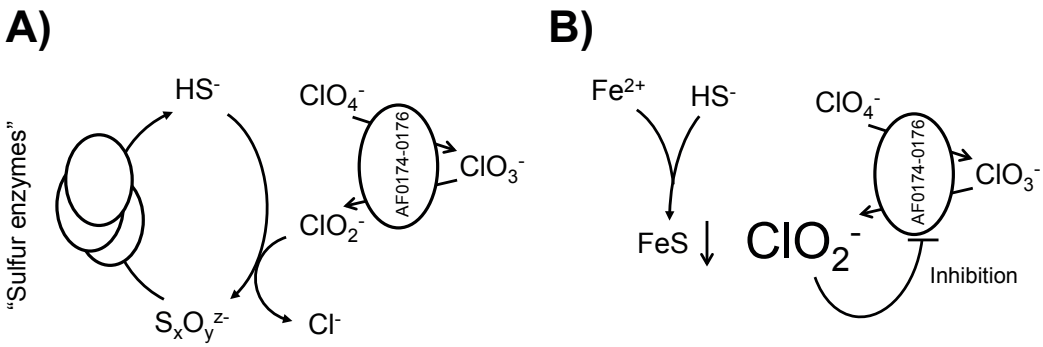

Figure 3: A) Interplay of (per)chlorate reduction and the sulfur metabolism of *A. fulgidus* and **B)** the inhibition of (per)chlorate reduction in the absence of sulfide (precipitated by ferrous iron).

However, while the reduction of elemental sulfur was observed, growth of *A. fulgidus* with S^0 or polysulfide as electron acceptor did not occur, confirming earlier observations (20). The chemical nature and bioavailability of externally added sulfur in aqueous solutions depends on several factors and may thus have restricted its utilization (25). Tetrathionate on the other hand was used as terminal electron acceptor for growth by *A. fulgidus* promoting growth (Table 1).

Table 1: The reduction of tetrathionate (5 mM) by *A. fulgidus* coupled to growth, followed by the formation of sulfide, oxidation of lactate and growth (represented by the protein content). The biological control (where tetrathionate was omitted) did not show any activity or growth. -, not detected; the inoculum (0.25%) derived from a culture grown on tetrathionate and lactate; *n=2*, means ± range.

		Time		
Condition	**Parameters**	**0 days**	**2 days**	**4 days**
Tetrathionate added (5mM)	Sulfide (mM)	-	4.8 ± 2.6	8.5 ± 0.8
	Lactate (mM)	19.7 ± 0.4	13. 1 ± 3.0	6.8 ± 0.4
	Protein content (mg l^{-1})	-	43.6 ± 34.9	66.6 ± 1.3
Tetrathionate omitted	Sulfide (mM)	-	-	-
	Lactate (mM)	19.7 ± 0.4	18.5 ± 0.3	18.7 ± 0.2
	Protein content (mg l^{-1})	-	-	-

The ability of *A. fulgidus* to utilize sulfur and polythionates (or more specifically tetrathionate) was suggested earlier by proteomic data (Chapter 3), but it was not proven experimentally yet. The several fold higher expression of complexes related to tetrathionate reductase (AF0157-0159) and S^0/polysulfide reductase (AF2384-2386) of *A. fulgidus* grown on (per)chlorate was indicative for the involvement of sulfur compounds in (per)chlorate reduction (12). The reduction of oxidized sulfur compounds may even be the primary source of energy generation by *A. fulgidus* growing on (per)chlorate.

Taken together the chemical oxidation and biological reduction of sulfur during (per) chlorate reduction a closed intraspecies "biotic/abiotic sulfur loop" is established. Next to the sulfur fractions monitored in this study, other sulfur compounds may be formed (26) during (per)chlorate reduction that are relevant for this loop (Fig. 3).

Alternative chlorite scavengers

The presence of sulfide is a common characteristic in the natural environments of *A. fulgidus* and is also required for growth in the culture medium. The chemical reactivity of chlorite with reduced sulfur compounds is known (26, 27). However, in the environment compounds other than sulfide may also act as efficient scavengers for the reduction and detoxification of chlorite. Potential candidates may be ferrous iron or manganese(II) or organic compounds that are present in the environment of *A. fulgidus*, such as hydrocarbons (28, 29).

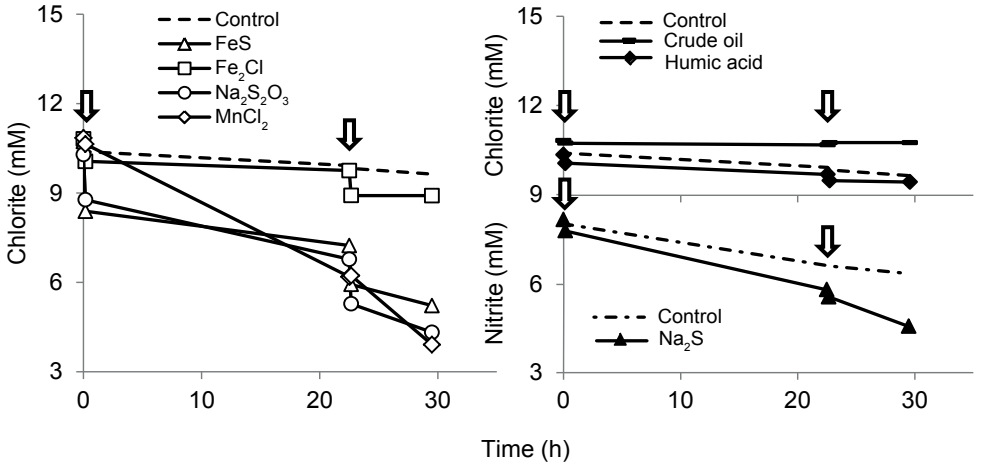

Figure 4: Chemical reactivity of chlorite (left and upper right panel) or nitrite (lower right panel) with selected chemicals (in anaerobic aqueous solution, 50 mM phosphate; pH = 6.5; 80°C); (no microorganisms were added). Arrows indicate the injection of the respective chemicals (each time 2 mM). means, *n=2*.

A chemical trial was performed to identify substances that have a high reactivity with chlorite and might thus enable (per)chlorate reduction in *A. fulgidus* via scavenging chlorite (Fig. 4, left and upper right panel). Ferrous iron ($FeCl_2$) showed among other chemicals an immediate reaction upon chlorite exposure. This observation was also made in other studies where ferrous iron and chlorite reacted to ferric iron hydroxides and chloride in a stoichiometry of 4:1, Fe^{2+}:ClO_2^- (30). However, the chemical reactivity of ferrous iron could not support growth in experiments with perchlorate (Fig. 1B). The concentration of dissolved ferrous iron in the medium (after sulfide precipitation) would have been sufficient for the conversion of at least 2 mM of chlorite (taking the above given stoichiometry into account). Probably ferrous iron, (in case present in dissolved form as ferrous chloride) reacts with chlorite, but causes a locally restricted (periplasmic) increase of redox potential (Fig. 1B) that leads to a stop of the (per)chlorate-reducing metabolism and growth. This is prevented by the archaeon, in case sulfide is present (Fig. 1D) or generated (Fig. 1C). Then, the emerging oxidized sulfur compounds ($S_xO_y^{z-}$) may get formed in relative proximity to periplasmic molybdo-enzymes that utilize them promptly and thus regenerate sulfide (12). Another reason may be that the energy production during (per)chlorate reduction is mainly based on the reduction of therefrom (chemically) generated $S_xO_y^{z-}$ compounds.

The chemical trial would furthermore suggest ferrous sulfide (FeS) as good candidate for a quick elimination of chlorite. FeS gets quickly oxidized whenever chlorite is provided in excess (Fig. 4, left panel), however biologically formed chlorite is present at low concentrations in the periplasm where it is hardly accessible for precipitated FeS. Also thiosulfate was tested and showed a quick reactivity with chlorite. The hereupon tested substitution of sulfide with thiosulfate in the medium (10 mM; omitting any other reducing agent) was also able to initiate (per)chlorate reduction with *A. fulgidus*.

Manganese(II)chloride ($MnCl_2$) seemed to react with chlorite, but at lower rates, whereas crude oil and humic acid were not reactive at all with chlorite (Fig. 4, left and upper right panel).

Nitrate reduction and accumulation of nitrite

Growth of *A. fulgidus* with nitrate as electron acceptor has until now not been reported (31, 32). However, in the current study it was observed that when sulfide was completely omitted from the growth medium inoculated with a perchlorate pre-grown culture, nitrate was reduced, partially accumulating nitrite (Fig. 5B) and forming acetate and formate from lactate.

In incubations with sulfide-amended medium reduction of nitrate did not occur, neither was it observed in earlier conducted methylviologen-based enzyme assays

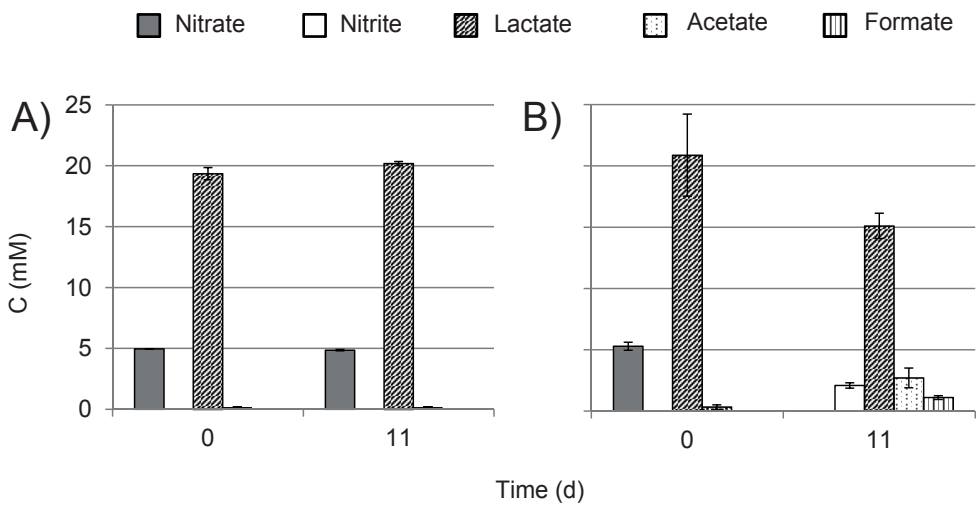

Figure 5: *Archaeoglobus fulgidus* exposed to nitrate as potential electron acceptor (together with excessive concentrations of lactate) under **A)** standard growth conditions, reducing the medium with sulfide (1.25 mM) and **B)** anaerobic conditions omitting sulfide. The inoculum derived from a culture grown on perchlorate and lactate. *n=2*, means ± range (bars).

using dithionite as reducing agent (12). It is possible that nitrate reduction is catalyzed by the same enzyme that reduces (per)chlorate in *A. fulgidus* (AF0174-0176). This enzyme was studied because of its resemblance with respiratory Nar-type reductases (33, 34). The inability of *A. fulgidus* to reduce nitrate in the presence of sulfide (Fig. 5A) might be related to the low and thus unfavorable redox conditions. Nitrate reduction is a process that takes place at a high redox potential, which might not be compatible with the metabolism of a strictly anaerobic microorganism like *A. fulgidus* and consequently not result in growth (compare protein concentrations in Fig. 5 with Table 1). However, (per)chlorate reduction, a metabolism with comparable redox chemistry as nitrate reduction (35, 36) is coupled to growth. This seems to be possible due to the "sulfur-based loop", a mechanism that may theoretically be coupled to nitrate reduction as well, but has not been observed. Potentially formed nitrite (by nitrate reduction) could then react chemically with sulfide, forming elemental sulfur (or polysulfide) and ammonium (37). However, this reaction seems to be slower than the one between chlorite with sulfide resulting in more long-lasting unfavorable high redox conditions due to the accumulation of nitrite when exposed to nitrate (compare Fig. 4, lower right panel with Fig. S3 in Chapter 3). In the genome of *A. fulgidus* a putative assimilatory nitrite reductase (AF0164) is encoded, but its biochemical functionality has never been demonstrated (38). In the experimental set-up exposing *A. fulgidus* cells to nitrate, a substantial amount of nitrite accumulated in the medium (Fig. 5B).

Nitrate has been successfully used for the mitigation of souring in oil fields (2). In a former study with thermophilic isolates from produced water of a petroleum field, strains related to *Thermodesulforhabdus norvegicus* and *A. fulgidus* did not show any inhibition in terms of sulfide formation upon nitrate injection. However, nitrite strongly inhibited the microorganisms (8).
The finding that *A. fulgidus* is able to reduce nitrate to nitrite, in addition to (per) chlorate, is particularly interesting in view of souring mitigation.

Changing electron acceptor conditions

The activity of perchlorate-reducing *A. fulgidus* cultures shown in this study was obtained after several subsequent transfers to perchlorate. The simultaneous exposure of (per)chlorate-adapted cultures to (per)chlorate and sulfate indicated a change in the microorganism's sulfate reduction capabilities (12). When perchlorate-adapted cultures of *A. fulgidus* were transferred back solely to sulfate as electron acceptor, a clear lag-phase was observed (Fig. 6, lower row); this was in comparison to transfers to medium containing perchlorate only (Fig. 6, upper row).

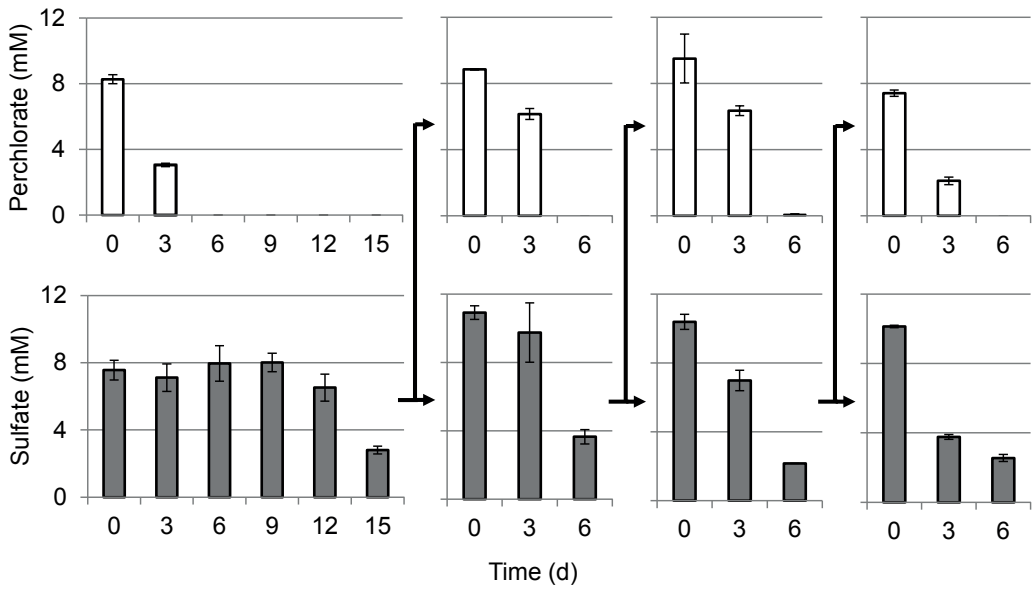

Figure 6: Electron acceptor utilization of *A. fulgidus* under sulfate- (lower panel) and perchlorate-reducing (upper panel) conditions (coupled to lactate; offered in excess). The arrows indicate where inocula (1%) for next generations of cultures derived from. The culture that was used as source for starting the experiment had been transferred for multiple times to fresh medium containing perchlorate only (ca. 10 generations). *n=2*, means ± range (bars).

It took three to four subsequent transfers on sulfate (each taking at least a week) before the sulfate-reducing capacity of *A. fulgidus* was restored to a level that was common for sulfate-adapted cultures (Fig. 6, lower row). Interestingly, the degree of perchlorate reduction after these repeated transfers on sulfate did not decline (Fig. 6, upper row). Such observations (though not made in a continuous system) are important for souring mitigation. It may prevent an immediate re-activation of sulfate reduction after an (unintended) stop of a (per)chlorate treatment (due to e.g. lack of injection chemicals, or failing injection pumps), while keeping the microorganisms adjusted to (per)chlorate for a subsequent treatment (with shorter conditioning time).

Conclusions

It is shown that *A. fulgidus* requires a "sulfur-based" loop for continuous (per) chlorate reduction to overcome the lack of a chlorite-disproportionating enzyme. The interplay of (per)chlorate reduction and the sulfur metabolism in *A. fulgidus* comprises the chemical formation of oxidized sulfur fractions from the reaction of sulfide with chlorite and the ability of biologically reducing (per)chlorate, elemental sulfur and tetrathionate. Depending on the availability of larger or "catalytic" amounts of sulfide, the preferential reduction of (per)chlorate (as demonstrated for *A. fulgidus*; after adaptation) may not only drain the pool of electron donors (volatile acids, hydrocarbons, etc.) and thereby limit sulfide generation, but could also oxidize existing sulfide to more oxidized sulfur compounds (elemental sulfur, tetrathionate, etc.). As a consequence, a significant fraction of sulfur (in case of S^0) may be retained in the reservoir resulting in produced fluids and gas containing less sulfide. In situations where sulfide is completely absent, traces of accumulating chlorite would cause cell damage and inhibit the sulfide-producing microbial community. This study also demonstrated that under specific conditions, *A. fulgidus* accumulates nitrite upon exposure to nitrate. Nitrite is known as an inhibitor of the sulfite reductase of SRP. Due to their similar structure a comparable effect of nitrite and chlorite would not be surprising. Besides, both compounds have a high reactivity and general toxic effect when they accumulate in microorganisms. The finding that long term exposure to perchlorate resulted in prolonged times required for re-adaptation to sulfate, implies that temporary downtime of a (per)chlorate injection unit, will not immediately allow souring to resume. The long time needed for the prokaryote's sulfate-reducing capability to recover might also provide opportunities to use pulsed-wise dosing strategies with (per)chlorate rather than continuous to save chemicals and reduce costs. The potential advantage of (per)chlorate-based souring mitigation over nitrate, might be that given its direct impact on sulfate reduction (of e.g. *A. fulgidus*), it would probably not rely on the development of a separate (per)chlorate-reducing community that would need to compete for exactly the same electron donors with

the sulfate-reducing community as it is often the case for nitrate. Costs for use of (per)chlorate are expected to be comparable to those of nitrate, and significantly lower than costs for sulfate removal. Summarizing, the use of (per)chlorate might thus access groups of microorganisms and conditions where nitrate treatments might be less efficient. In hot oil reservoirs, where also *A. fulgidus* is commonly found, nitrate-based mitigation of souring may be challenging in case hyperthermophilic nitrate reducers are missing. The combined or alternating use of (per)chlorate and nitrate could (for instance in presence of *A. fulgidus*) lead to an *in-situ* formation and release of nitrite or chlorite to the oil field, affecting adjacent microorganisms as well. As such, strategies combining effects of nitrate, (per)chlorate and possibly also biocides may increase the effectiveness of souring mitigation treatments in future. This also seems a promising strategy for breaking microbial zonations after continuous nitrate dosing in reservoirs of lower temperatures and would promote the dispersal and effectiveness of the treatment beyond the near well-bore zone (7).

Acknowledgements

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Chapter 5

Microbial perchlorate reduction by a thermophilic consortium with acetate as carbon and energy source

adapted from:
Martin G. Liebensteiner, Irene Sanchéz-Andrea, Alfons J.M. Stams, Bart P. Lomans. Microbial perchlorate reduction by a thermophilic consortium with acetate as carbon and energy source, *in preparation.*

Abstract

Classical microbial perchlorate reduction is based on the enzymes, perchlorate reductase, reducing perchlorate to chlorite and chlorite dismutase, disproportionating chlorite to chloride and oxygen. Acetate is a common substrate for perchlorate-reducing bacteria, but thus far no thermophilic microorganisms have been described that grow with acetate and perchlorate or chlorate. We enriched a thermophilic microbial consortium from a hydrocarbon-polluted soil that grows with acetate and perchlorate. Perchlorate was completely reduced to chloride by the thermophilic consortium. The presence of sulfide was a critical prerequisite for growth of the culture, in contrast to known mesophilic perchlorate-reducing bacteria (such as *Dechloromonas agitata*). Attempts to obtain an axenic perchlorate-reducing culture with acetate failed until now indicating a possible syntrophy of the microbes involved. A similar mechanism like the one described for *Archaeoglobus fuldigus* might occur, where chlorite reacts chemically with sulfide, producing chlorite and oxidized species of sulfur. This reaction seems to provide electron acceptors for other microorganisms reducing sulfur compounds. Sulfide would consequently be regenerated by latter microorganisms and thus be available for chlorite elimination and the continuous reduction of perchlorate based on an interspecies "sulfur loop". This hypothesis was supported by 16S rRNA gene clone library analysis where potential perchlorate-, sulfate- and sulfur-reducing microorganisms were identified. By its dominant abundance a microorganism that is affiliated with the genus *Thermanaeromonas*, seems to be the best candidate for the reduction of perchlorate.

Keywords: syntrophic perchlorate reduction, high temperature, interspecies "sulfur loop", *Thermanaeromonas*

Introduction

Perchlorate and chlorate [together abbreviated (per)chlorate] have been discussed as new agents for biologically based processes in the oil upstream industry, like microbial enhanced oil recovery (MEOR) and souring mitigation control (Chapter 2).

A large number of facultative anaerobic Gram-negative mesophilic bacteria have been described that can use (per)chlorate as terminal acceptor for growth such as *Dechloromonas agitata* and *Azospira oryzae* strain GR-1 (1, 2). Classical microbial perchlorate reduction is based on the enzymes perchlorate reductase, reducing perchlorate (via chlorate) to chlorite; and chlorite dismutase, disproportionating chlorite to chloride and oxygen (2). Some microorganisms cannot reduce perchlorate but only chlorate to chlorite (followed by disproportionation of chlorite) which is catalyzed by so-called chlorate reductases (3). The reduction of (per)chlorate is coupled to a broad set of inorganic and organic electron donors, where acetate represents the most commonly utilized electron donor by (per)chlorate-reducers (4). Microbial perchlorate reduction at elevated temperatures has up until now only been investigated in a few studies. For the respective (hyper)thermophiles it was demonstrated that the utilization of substrates like lactate, methanol, fructose and formate is coupled to (per)chlorate reduction (5, 6). However, except *Archaeoglobus fulgidus* (also coupling (per)chlorate reduction to butyrate oxidation; Chapter 3) no other microorganism has yet been reported to use volatile fatty acids (VFA) such as acetate, propionate and butyrate for this metabolism.

Acetate, propionate and butyrate are compounds that accumulate in deep subsurface environments such as oil fields at elevated temperatures (7, 8). Particularly acetate accumulation rises up to concentrations in the mM range; even up to 50 mM in produced fluids from a Venezuelan offshore well at 78°C (9). In contrast to hot oil reservoirs, higher biological turnover rates in low and moderately hot oil fields prevent the accumulation of acetate, propionate and butyrate. These compounds (and especially acetate) are ubiquitous in oil reservoirs and represent attractive substrates for microbial respiration processes. For the aforementioned applications in the oil recovery business addressing MEOR and souring control, processes that rely on externally added substrates as little as possible are preferred.

The aim of this work was to explore the potential of acetate as electron donor for (per)chlorate reduction at elevated temperature. Enrichments were performed using a hydrocarbon-polluted deep soil layer as source of microorganisms. The successfully enriched thermophilic perchlorate-reducing consortium was physiologically characterized and its microbial diversity determined.

Materials and Methods

Cultivation and media

The sample used for enrichments was a deep anoxic soil layer (6-10 m depth), taken from below the ground water table, of a site polluted with hydrocarbons (BTEX and mineral oil) and MtBE (Methyl tert-butyl ether). For initial enrichment cultures, a sample 0.5 g (1%; w/v) were transferred to anaerobic medium, contained in rubber stoppered 120 ml serum bottles. The headspace of the bottles was filled with a N_2/CO_2 (80/20; v/v; 1.5 bar) gas atmosphere. The medium used throughout the study was bicarbonate/CO_2 and phosphate buffered at neutral pH containing yeast extract (0.2 g l^{-1}) (10). The only sulfur source in the medium was sulfide (which was used as reducing agent). The medium was routinely supplemented with soil extract, deriving from the original substrate (5% v/v), in order to maintain perchlorate-reducing activity.

The soil extract was prepared by mixing soil and demi water (5%, w/v) in anoxic conditions followed by 2 hours shaking at 200 rpm. After settling by gravitational force (1h) the supernatant was autoclaved twice and stored under anoxic conditions (with pure N_2 gas in the headspace). Due to the partial loss of perchlorate-reducing activity in subsequent transfers, sodium sulfide (1.25 mM) was added to the medium as a reducing agent, which could fully recover the initial activity.

Initial enrichments were set up using chlorate or perchlorate as sole electron acceptor (10 mM), together with acetate (10 mM). Electron acceptors and donors were added to the medium from sterile anaerobic stocks after autoclaving.

Experimental set-ups were incubated at 60°C and 30°C. Latter represents ideal conditions for most known (per)chlorate-reducing microorganisms (11).

Analysis of metabolites

Oxyanions were analyzed using a HPLC with an Ion Pac AS22 column (4 x 250 mm), using an ED 40 electrochemical detector (Dionex, Sunnyvale, CA). Carbonate/bicarbonate buffer (1.29 g l^{-1} Na_2CO_3.10 H_2O and 0.12 g l^{-1} $NaHCO_3$) was used as eluent with a flow of 1.2 ml min^{-1} and the analysis was performed at 35°C. Pure standards (analytical grade) of chloride, nitrate, chlorate and perchlorate were used at 2.5; 5; 10 and 20 mM concentrations. Sodium iodide (NaI) was used as internal standard in every sample with a final concentration of 2 mM.

Acetate and potentially formed products were analyzed at a HPLC system using a Varian column (MetaCarb 87H Guard 4.6x50 mm, Middelburg, The Netherlands). Sulfuric acid was used as eluent at a concentration of 10 mM with a flow rate of 0.8 ml min^{-1} and the analysis was performed at 30°C. The internal standard was L-arabinose at 10 mM. Chromatographic data was analyzed using the software

package ChromQuest.
The turbidity in the medium caused by the soil extract did not allow proper monitoring of growth by optical density. Thus growth was determined by the protein content of cell lysates. Aliquots of 1 ml of culture were disrupted by ultra-sonication (5 times; 30 sec sonication at 20 kHz followed by 30 sec pause) and centrifuged (10 min, 16100 g).
The protein content in these supernatants was determined by a commercially available Bradford assay using bovine serum albumin as standard (12); concentrations of 0, 25, 50, 75 and 100 μg ml^{-1} were used.

Clone library

Cloning of the 16S rRNA gene was performed for the determination of the microbial diversity in the enrichment. The total genomic DNA was extracted using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). The yielded DNA was quantified on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA).
Bacterial 16S rRNA genes were amplified in triplicate with the primer set 27F-1492R (Ta = 57°C) and cloned in Escherichia coli DH5α competent cells using the pGEM-T vector (Promega, Madison, WI). The 16S amplification for archaeal 16S rRNA genes was performed using the primers 25F-1492R (Ta = 52°C). Next to some unspecific amplification of short bacterial fragments no archaeal 16S rRNA clones were obtained. Positive control PCR reactions were performed with the addition of amplifiable reference DNA (e.g. for archaeal PCR DNA of *A. fulgidus* VC-16) and negative controls were done without addition of template. Furthermore control PCR reactions were performed on the added soil extract (see "cultivation and media"), and consistently yielded no product. Purified PCR products were cloned in *Escherichia coli* DH5α competent cells by using the pGEM-T vector (Promega, Madison, WI) according to the manufacturer's instructions. Plasmid DNA was sequenced using a Big-Dye sequencing kit (Applied Biosystems) following the manufacturer´s instructions with Sanger sequencing method by GATC (Konstanz, Germany).
DNABaser software 3.5.3 was used to assemble the sequences. Vector sequences flanking the 16S rRNA inserts were identified using VecScreen tool (NCBI) (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) and manually removed. Afterwards the sequences were checked for chimeras (http://decipher.cee.wisc.edu/FindChimeras.html).
The sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.0) (13, 14). Identical reads were identified, the unique reads were clustered (OTUs), and the reference read of each OTU was classified phylogenetically.

The selected representative sequences of each OTU were added to a database of over 230000 homologous prokaryotic 16S rRNA genes using the merging tool of the ARB program package (15). Phylogenetic reconstruction was performed in the ARB package using three different algorithms and the Neighbor-Joining tree constructed using bootstrap analysis.
The 16S rRNA gene of the mesophilic pure culture was directly amplified from the total genomic DNA, followed by PCR using primers 27F and 1492R.
16S rRNA genes obtained in this study were deposited in the European Nucleotide Archive (ENA) under accession numbers LM643753-LM643767.

Results and Discussion

Initial enrichments were set up using both chlorate and perchlorate as sole electron acceptor and acetate as electron donor, incubated at 30 and 60°C. From these four conditions only the mesophilic condition with chlorate and the thermophilic condition with perchlorate resulted in microbial activity as indicated by the complete reduction of the respective electron acceptor monitored over one month of incubation.

Mesophilic chlorate reduction

The mesophilic culture (30°C), representing the ideal conditions of classical (per) chlorate-reducing bacteria (16), showed higher activity and led in subsequent transfers and dilution series to the isolation of a microorganism (strain ClrAce30) affiliated with the genus *Pseudomonas* (*P. chloritidismutans* AW-1; coverage 99%, identity 99%). Several *Pseudomonas* strains were demonstrated to grow by chlorate reduction before. Strain AW-1 was isolated from an anaerobic bioreactor treating chlorate- and bromate-polluted waste streams (17). It was reported to grow by the reduction of chlorate or oxygen together with acetate, but lacked the ability to reduce perchlorate, nitrate or bromate. Strain AW-1 largely constitutes an archetype of mesophilic chlorate-reducing bacteria, which have been investigated over several decades (4, 11, 16). Due to the phylogenetic and physiological resemblance with these microorganisms no further in-depth investigations were conducted with strain ClrAce30.

Thermophilic perchlorate reduction

Initial enrichments of perchlorate reduction coupled to acetate oxidation at 60°C were made without the addition of sulfide to the medium, but its addition in the subsequent transfers was crucial to maintain perchlorate-reducing activity. Sulfide addition as reducing agent was not necessary for the mesophilic culture enriched in this study; neither is it normally added for growth of other classical (per)chlorate-reducing

microorganisms due to their facultative anaerobic autecology.
Over the course of time and multiple transfers, the activity of the perchlorate-reducing enrichment culture increased considerably, being fully-grown after ca. 1 week (Fig. 1). Under the chosen conditions (60°C, 1.25 mM of sulfide, 5% soil extract), acetate was completely mineralized to CO_2 by the microbial consortium, while perchlorate was reduced all the way to chloride (Fig. 1). The reduction of perchlorate led to the accumulation of stoichiometric amounts of chloride in the medium. Methane or other products were not detected in the medium at any time.

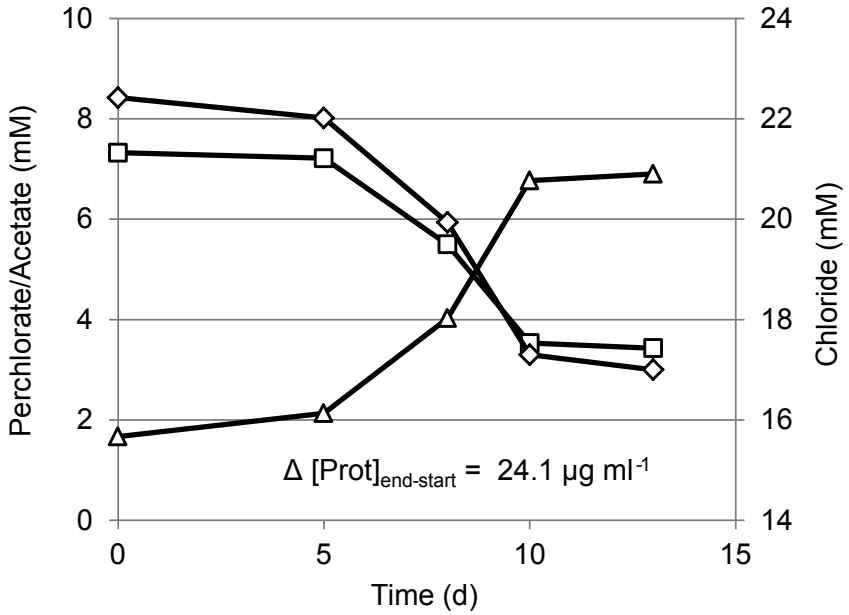

Figure 1: The reduction of perchlorate (diamonds) coupled to acetate oxidation (squares) and growth (expressed in the protein yield) by a microbial consortium at 60°C. The reaction forms chloride (triangles) and carbon dioxide (not measured). The experiment was performed in reduced medium (1.25 mM sodium sulfide) and in the presence of 5% soil extract; *n=2*.

The observed stoichiometry of the reaction required approximately equimolar amounts of acetate and perchlorate which roughly meets the expected stoichiometry.

$$ClO_4^- + CH_3CO_2^- \rightarrow Cl^- + 2HCO_3^- + H^+ \qquad \Delta G^{0'} = -966.8 \text{ kJ}$$

Transferring the culture to 30°C instead of 60°C did not result in any growth, which indicated the thermophilic nature of the perchlorate-reducing culture rather than activity of mesophilic microorganisms at suboptimal conditions. In addition, known

acetate-oxidizing (per)chlorate reducers have their upper limit around 37 to 40°C (17-19).

Perchlorate reduction was not observed in the control incubations, where acetate or the inoculum was omitted. Surprisingly, the culture was not able to reduce chlorate efficiently; chlorate reduction stopped after ca. 1-2 mM of chlorate was reduced. This might be explained by the enzyme kinetics of perchlorate reductases and related enzymes, which have a higher specific activity with chlorate than perchlorate (20, 21). In the absence of a functional Cld, the use of chlorate as electron acceptor may (in contrast to perchlorate) exceed the abiotic reduction rates of chlorite with sulfide, which accumulates toxic levels of chlorite and terminates chlorate reduction.

Substituting perchlorate with nitrate resulted in the reduction of nitrate (results not shown). Unlike perchlorate reduction, nitrate reduction was faster whenever sulfide was omitted from the medium. Perchlorate was also reduced when acetate was replaced by H_2/CO_2. Additionally, perchlorate reduction occurred with lactate, as electron donor and C-source.

Attempts to obtain an axenic perchlorate-reducing culture growing on acetate failed, although several isolation methods were applied (such as dilution series and roll tubes).

The microbial diversity of the thermophilic acetate-degrading perchlorate-reducing culture was analyzed by cloning and sequencing of the 16S rRNA gene. While no archaeal sequences were obtained, the bacterial clone library resulted in 85 good quality sequences of which 2 turned out to be chimera. The remaining 83 valid sequences clustered in 14 OTUs grouped by 98% threshold. Some of the sequences were classifiable to the genus level identifying microorganisms that belonged to the genera *Desulfotomaculum*, *Thermanaeromonas*, *Thermacetogenium* and *Thermosediminibacter* (Fig. 2).

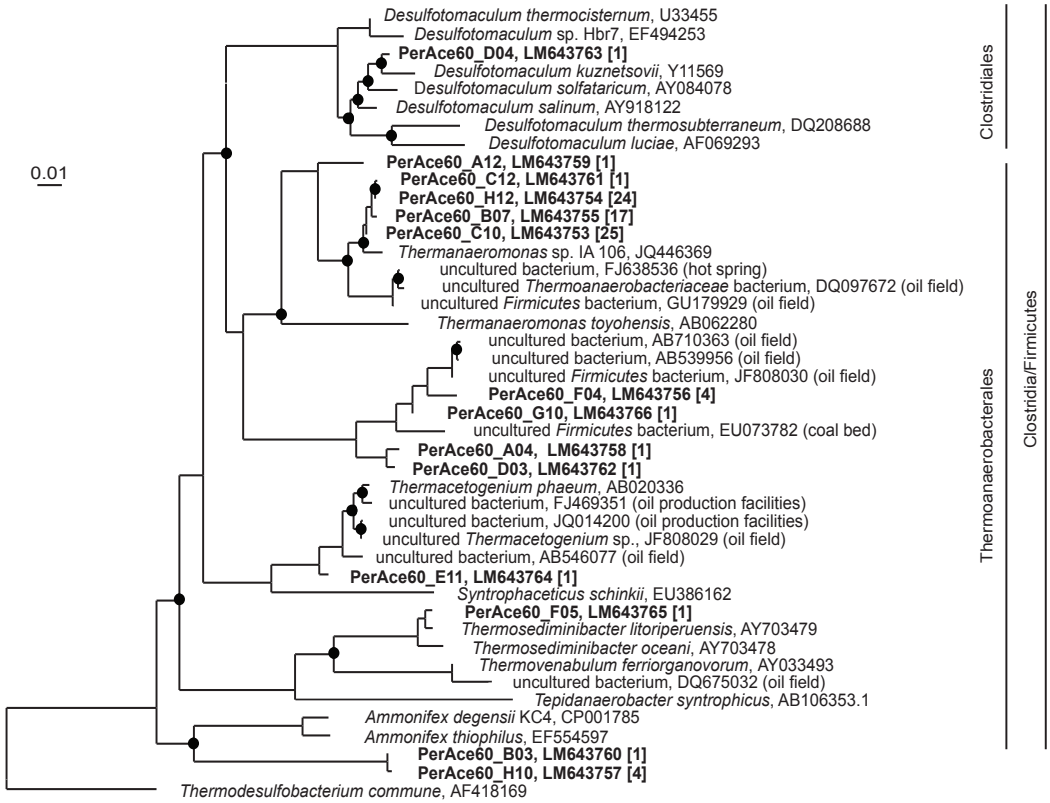

Figure 2: Phylogenetic affiliation of sequences obtained (in bold) by bacterial 16S rRNA gene cloning of the perchlorate-reducing culture, grown at 60°C using acetate. Absolute abundances of clones are given after the respective names of the clones (in squared brackets). Accession numbers and relevant environmental sources are given after the sequence names. Neighbor-Joining method with Jukes-Cantor correction was chosen after applying the three algorithms as implemented in the ARB package. Based on 1000 replications, significant values of each branch (above 70%) are indicated at the nodes by circles. The scale bar illustrates substitutions per site.

The high abundance (82%) of clones related to *Thermanaeromonas* (OTUs represented by PerAce60_C10, H12, B07, A12, C12) is likely an indication of the functional importance of members of that genus for the reduction of perchlorate. The most closely related isolate to these sequences is *Thermanaeromonas toyohensis* (identity 92%, coverage 99%) (gb: AB062280). This strictly anaerobic strain was isolated from geothermal waters of a Japanese metal mine (22). It can utilize thiosulfate, nitrate and nitrite as electron acceptors and has an optimum growth temperature of 70°C. Substrates used for the reduction of thiosulfate were reported to be arabinose, cellobiose, fructose, glucose, inositol, maltose, mannose, sucrose, trehalose xylose, yeast extract, formate, lactate and pyruvate; acetate and H_2/CO_2 were not utilized.

The ability of *T. toyohensis* to reduce nitrate and nitrite was successfully tested with lactate. More closely related *Thermanaeromonas*-like sequences (ranging from 93 to 99% identity to PerAce60_C10, H12, B07, A12, C12) derive from uncultured microorganisms of a hot spring environment (gb: FJ638536.1), subsurface circulation water (gb: JQ446369.1) and low (gb: GU179929.1) and high (gb: DQ097672.1) temperature oil reservoirs (23) (Fig. 2).

Thermoacetogenium phaeum, a microorganism isolated from a methanogenic reactor, is the most closely related isolate to sequence PerAce60_E11 (24) (coverage 92%, identity 98%). Sequences of uncultured microorganisms with high resemblance to the PerAce60_E11 clone (ranging from 98 to 99% identity) were also found in pipelines (gb: JQ014200.1) (25), oil facilities (gb: FJ469351.1) (26) and oil reservoirs (gb: JF808029.1 and AB546077.1) (27, 28).

PerAce60_F05 (*Thermosediminibacter* sp.) had highest resemblance with sequences deriving from *Thermosediminibacter litoriperuensis* (93% coverage and 99% identity) and *Thermosediminibacter oceani* (100% coverage, 99% identity), which were isolated from a deep sea sediment core (29). Environmental sequences more distantly related to PerAce60_F05 originated also from oil reservoirs (gb: DQ675032.1) (30) (92% coverage, 90% identity).

PerAce60_D04 is affiliated with the genus *Desulfotomaculum*. Members of this genus are commonly found in subsurface environments and more specifically in oil reservoirs, like e.g. the isolates *Desulfotomaculum salinum* (gb: AY918122.1) (31) and *Desulfotomaculum thermocisternum* (gb: U33455.1) (32).

The presence of several microorganisms in the perchlorate-reducing consortium that are related to microorganisms with a sulfur metabolism was surprising considering that sulfur was only added to the medium in form of sulfide (1.25 mM). Closest related isolates to the here obtained OTUs such as *Desulfotomaculum kuznetsovii* (PerAce60_D04), *T. oceanii*/*T. litoriperuensis* (PerAce60_F05) and *T. phaeum* (PerAce60_E11) have the capabilities to reduce sulfate (*D. kuznetsovii*, *T. phaeum*), sulfite (*D. kuznetsovii*), thiosulfate (*D. kuznetsovii*, *T. oceanii*/*T. litoriperuensis*, *T. phaeum*) and elemental sulfur (*T. oceanii*/*T. litoriperuensis*) (24, 29, 33, 34). Measurements of sulfate in the medium confirmed that its concentration was below 60 μM at all times of the experiment; thiosulfate concentrations were below detection levels.

The presence of microorganisms with a sulfur metabolism may indicate a similar mechanism for perchlorate reduction as was reported for *A. fulgidus* (Chapter 3 and 4). *A. fulgidus* reduces perchlorate and chlorate to chlorite, followed by a chemical scavenging of chlorite by sulfide. The formed oxidized sulfur compounds are in turn biologically reduced again. This constitutes an intraspecies "sulfur loop" that involves biotic and abiotic reactions driving the biological reduction of perchlorate. Other than *A. fulgidus* complete (per)chlorate reduction in the here characterized

consortium may be spread over functionally diverse microorganisms and form an interspecies "sulfur loop".

We propose that *Thermanaeromonas* sp. is the most probable candidate to reduce perchlorate to chlorite, followed by the chemical reduction of chlorite with sulfide, forming oxidized sulfur compounds. This would explain the coexistence of sulfate- and sulfur-reducing microorganisms in the consortium, that are reducing oxidized sulfur compounds to sulfide and also why it was not possible to obtain an axenic culture of an acetate-utilizing perchlorate reducer.

The low similarity of OTUs represented by PerAce60_F04, A04, D03, G10, B03 and H10 to known isolates (Fig. 2) resulted in a low taxonomic resolution using two independent taxonomic identification tools, SILVA (35) and the Ribosomal Database Project (RDP) (36). Although OTUs represented by PerAce60_ A04 and G10 are remotely associated with members of the genus *Desulfotomaculum* and F04 and D03 with *Thermananaeromonas* (for all identities < 90% using BLAST) they could not be classified beyond the level of Clostridia/Firmicutes. More closely related clones to the above-mentioned sequences can also be found in samples from oil reservoirs (gb: AB710363.1, JF808030.1 and AB539956.1) (28, 37) and a coal bed sample (gb: EU073782.2).

OTUs PerAce60_B03 and H10 were taxonomically unclassifiable and only distantly related to the genus *Ammonifex* (sequence identities of 86% with *A. degensii* using BLAST). Both described isolates within the genus *Ammonifex* were isolated from hot springs (38, 39).

From both a phylogenetic and physiological perspective, it would be desirable to shed more light on the respective microorganisms to understand their exact role in the perchlorate-reducing consortium.

Conclusions and Outlook

The results of this study demonstrated the feasibility of coupling a readily and abundantly available substrate in anaerobic environments like acetate to perchlorate reduction at high temperature. The presence of related environmental sequences found in the subsurface and petroleum reservoirs suggests that the injection of perchlorate would likely result in perchlorate reduction in respective environments.

Intensified effort is currently spent on the isolation of the *Thermanaeromonas*-like bacterium, which seems the most probable perchlorate-reducing candidate. So far, efforts for obtaining an axenic perchlorate-reducing culture did not succeed. An explanation therefore may be the dependence of perchlorate-reducing microorganisms on microbial partners for the co-degradation of perchlorate.

Contrarily to the mesophilic (per)chlorate-reducing enrichment where *P. stutzeri*, a facultative anaerobe, dominated; 16S rRNA sequencing of the thermophilic

perchlorate-reducing enrichment showed the exclusive presence of strictly anaerobic microorganisms. These results combined with the necessity of sulfide for commencing perchlorate reduction in the thermophilic culture suggest a similar mechanism as described for perchlorate reduction by *A. fulgidus* (Chapter 3 and 4). In contrast to *A. fulgidus* the here presented consortium may enable complete perchlorate reduction by an interspecies "sulfur loop"; a model which is strengthened by the presence of a diverse group of microorganisms affiliated with known sulfur- and sulfate-reducing microorganisms. Future experiments are required to gain more evidence for the model of an interspecies "sulfur loop" for complete perchlorate reduction.

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Chapter 6

Extreme temperatures and (per)chlorate reduction: Consistent traits and differences to the "classical" mesophilic metabolism

adapted from:
Martin G. Liebensteiner, Martijn W.H. Pinkse, Bart Nijsse, Peter D.E.M. Verhaert, Alfons J.M. Stams, Bart P. Lomans. Extreme temperatures and (per)chlorate reduction: Consistent traits and differences to the "classical" mesophilic metabolism. *in preparation.*

Abstract

The knowledge on hyperthermophilic chlorate and perchlorate [together termed (per)chlorate] reducers is thus far only based on the recently described metabolism in *Archaeoglobus fulgidus*; a trait that seems to differ considerably from the physiology of classical (per)chlorate-reducing mesophiles. In this study, we describe the ability of other thermo- and hyperthermophilic microorganisms to use perchlorate and chlorate as electron acceptors. *Aeropyrum pernix*, previously described as a strictly aerobic Crenarchaeon, is able to grow anaerobically by the reduction of (per)chlorate. Physiological, genomic and proteome analyses suggest that *A. pernix* performs (per)chlorate reduction by employing a periplasmic enzyme related to nitrate reductases (pNar) for reducing perchlorate to chlorite (via chlorate), but lacks a functional chlorite-disproportionating enzyme (Cld) to complete the pathway. Similar to *A. fulgidus*, chemical reactivity of reduced sulfur compounds and chlorite seems to play a crucial role in (per)chlorate reduction of *A. pernix*. The chemical oxidation of thiosulfate (in excessive amounts present in the medium) to sulfate and the concomitant release of chloride anions from the reduction of chlorite are the products of a biotic-abiotic (per)chlorate reduction pathway.

The lack of Cld in two (per)chlorate-reducing Firmicutes (*Carboxydothermus hydrogenoformans* and *Moorella glycerini* strain NMP) and the strict necessity of sulfide for (per)chlorate reduction in these bacteria is consistent to the observations made on *A. fulgidus* and *A. pernix*. All microorganisms employ similar strategies for the reduction of (per)chlorate at high temperatures and differ notably from the classical (per)chlorate-reducing mesophiles. This paper further strengthens the hypothesis that (hyper)thermophilic (per)chlorate reduction commonly occurs based on a biotic-abiotic pathway, missing Cld.

Keywords: *Aeropyrum pernix*, (per)chlorate reduction, pNar, hot environments, Cld deficiency, Firmicutes

Introduction

Dissimilatory reduction of perchlorate (ClO_4^-) and chlorate (ClO_3^-) [jointly termed (per)chlorate] is a microbial metabolism under oxygen free conditions that is known for almost 100 years (1). (Per)chlorate-reducing microorganisms are predominantly represented by members of the Proteobacteria and characterized by a mesophilic and facultative anaerobic lifestyle (2). The respective microorganisms employ either a perchlorate (reducing perchlorate to chlorate and chlorite) or a chlorate reductase (reducing chlorate to chlorite), followed by an enzyme disproportionating chlorite to dioxygen and chloride, called chlorite dismutase (Cld) (3-5).

Due to the environmental release of man-made perchlorate sediment- and soil-inhabiting (per)chlorate-reducing microorganisms have received attention for their potential role in biological remediation of polluted sites. However, the presence of (per)chlorate on Earth and the development of enzymes reducing these compounds may date back already to pre-anthropogenic eras (6-8). Geophysical studies of the last years have demonstrated several (photo)chemical mechanisms of natural perchlorate formation that introduce perchlorate on Earth and Mars (9). Ancient (per)chlorate deposition on Earth has possibly already influenced the evolution of early prokaryotic enzymes used for energy conservation. The degree of homology of perchlorate reductases with nitrate reductases suggests an evolutionary relatedness of these enzymes (10, 11). The promiscuity of numerous enzymes in the DMSO enzyme family using chlorate (and possibly perchlorate) as substrate may have evolved in ancestral enzymes already. Previous reports of archaea growing by the reduction of (per)chlorate might support arguments for an early evolution of enzymes that reduce (per)chlorate in anaerobic environments (12, 13). However, in classical perchlorate- and chlorate-reducing mesophiles horizontal gene transfer seems to play a major role for the acquisition of this trait as well (10, 14). The related nitrate reductase enzymes have a wide dispersal over diverse groups of prokaryotes. A complex evolution of these enzymes involving several mechanisms, amongst which horizontal gene transfer, has been described (15).

New discoveries in the field of microbial (per)chlorate reduction are not only interesting in terms of phylogenetic diversity or the biochemical background of the metabolism. Findings of recent years also discovered (per)chlorate-reducing capabilities in ecologically diverse microorganisms and expanded this trait beyond moderate temperatures to thermophilic (16) and hyperthermophilic life forms (13).

In the current study we examined (hyper)thermophiles for the ability to grow by the reduction of (per)chlorate. 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 (per)chlorate. It extends microbial (per)chlorate reduction up to 100°C. This novel physiological characteristic

of *A. pernix* was investigated in more detail by genomic and proteomic analyses. Similarly, two thermophilic bacteria, *Carboxydothermus hydrogenoformans* and a strain related to *Moorella glycerini* showed the capabilities to reduce perchlorate and chlorate. The presented work enlarges the current knowledge on (per)chlorate-reducing microorganisms at high temperatures and confirms the important role of sulfur compounds for this trait.

Materials and Methods

Strains, media and cultivation

Aeropyrum pernix strain K1 (17) and *Carboxydothermus hydrogenoformans* strain Z-2901 (18) were purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. Strain NMP, which turned out to be affiliated with *Moorella glycerini* (19) (99% sequence identity, 96% coverage) was isolated in our laboratory from a culture previously obtained from an underground gas storage site (16). In contrast to the type strain of *M. glycerini*, strain NMP was not able to use glycerol as substrate. The cultivations of the two first mentioned microorganisms were done according to the recommendations of the supplier, using DSM media nr. 820 *(A. pernix)* and nr. 507 *(C. hydrogenoformans)*. The substrates for growing *A. pernix* were yeast extract (1 g l^{-1}) and peptone (5 g l^{-1}), whereas pyruvate (20 mM) was used to grow *C. hydrogenoformans*. The medium used for strain NMP was described earlier (20); 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 were used as substrates for the growth of strain NMP.

Physiological trials were conducted in 120 ml bottles containing 50 ml medium. The headspace of the serum bottles was filled with a N_2/CO_2 (80/20; v/v; 1.5 bar) gas mixture (for strain NMP and *C. hydrogenoformans*) or pure N_2 (for anaerobic incubation of *A. pernix*). Aerobic cultures of *A. pernix* were also grown in serum bottles. Prior to autoclaving the headspace was completely saturated with atmospheric air, stoppered and sealed.

Stocks of electron acceptors (sodium chlorate, sodium perchlorate), medium additives (sodium sulfate, sodium thiosulfate, sodium bicarbonate, sodium sulfide) and electron donors (sodium formate, methanol) were separately autoclaved and added to the media afterwards. Vitamin solutions and sodium pyruvate were filter-sterilized and added to the medium after autoclaving.

In the trial determining sulfur fluxes (Fig. 2), sulfate in the medium was lowered to 4 mM in order to increase the analytical accuracy for detecting additionally formed sulfate. The standard medium used for growing *A. pernix* (DSM nr. 820, see above) contained 4 mM sodium thiosulfate and 23 mM sodium sulfate.

Strain NMP was cultivated at 60°C, whereas *C. hydrogenoformans* and *A. pernix* were grown at 70 and 90°C, respectively. All experiments were performed with biological duplicates.
Next to the here mentioned (per)chlorate-reducing microorganisms other pure cultures were tested for this trait, using anaerobic medium. The following thermophilic strains were selected based on the presence of a gene encoded in their genomes that resembles chlorite dismutase, however none of the respective microorganisms was able to reduce (per)chlorate: *Geobacillus debilis* Tf, DSM 16016 (DSM medium nr. 220), *Thermoplasma volcanium* GSS1 DSM 4299, (in DSM medium nr. 398), *Thermus scotoductus* SE-1, DSM 8553 (DSM 878), *Marinithermus hydrothermalis* T1, DSM 14884 (DSM medium nr. 973) and *Sulfolobus solfataricus* P2, DSM 1617 (DSM medium 182). Chlorate and perchlorate were tested at concentrations of 5 mM in recommended media using cultures pregrown under standard conditions.

Chromatographic analyses and Cld activity

Oxyanions were measured on a HPLC equipped with an Ion Pac AS22 column (4x250 mm), using an ED 40 electrochemical detector (Dionex, Sunnyvale, CA). The eluent was carbonate-bicarbonate buffer (1.29 g l^{-1} Na_2CO_3.10 H_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 analyzed on a HPLC system using a Varian column (MetaCarb 87H Guard 4.6x50 mm, Middelburg, The Netherlands) equipped with a UV and Refractive Index (RI) 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 analyzing the chromatograms later.
The potential activity of a chlorite-disproportionating enzyme in crude extracts and cell suspensions was determined by using a Clark electrode detecting the formation of oxygen. The preparation of cell free extract was performed as described earlier (13). The assay was performed in anaerobic phosphate buffer and under oxygen free conditions at 60°C.

Genome Sequencing and Proteome analysis

Genomic DNA of strain NMP was sent to Baseclear (Leiden, NL), where paired-end sequencing of the genome was performed. A *de novo* assembly was done using the *de novo* assembler Ray (21). Pilon (http://www.broadinstitute.org/software/pilon/) was used for assembly improvement afterwards. The assembled scaffolds were annotated using an in-house annotation pipeline (VAAP). This annotation includes (amongst others); predicted rRNA genes, predicted proteins and a blast search against the

SwissProt database, in an RDF database format. PRED-TAT was used to predict the subcellular location of proteins of interest (22).
The proteome analyses of *A. pernix* cells grown with oxygen, chlorate or perchlorate were performed using LC-MS/MS (23). For all 3 conditions independent duplicates of 500 ml 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 minutes 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, U.S.) for 90 min at a constant voltage of 120 mV using Tris-SDS as running buffer. Label free quantitative proteomics type experiments were carried out to find differentially expressed proteins under all different growth conditions studied. 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 200V 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 dithiotreitol 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% DMSO and subjected to nano LC-MS/MS using an EasyLC 1000 and an Orbitrap Q-Exactive Plus instrument (Thermofisher Scientific). Each peptide sample was auto-sampled and separated over a 25 cm analytical column (75 µm inner diameter) in-house packed with 5 µm C18 column material (Reprosil Pur-AQ, Dr. Maisch) 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 MS1 spectra were acquired in the positive ion mode from m/z 300-1200 at a resolution of 70000 after accumulation of 3 x 10^6 ions within a maximal injection time of 250 ms. A top20 method was used to acquire MS2 spectra at a resolution of 17500 after accumulation of 1 x 10^5 ions within a maximal injection time of 50ms. Parent ions were isolated with a 2.5 m/z window and fragmented with a HCD energy of 28. Only multiply charged ions were selected and the dynamic exclusion time was set to 30 seconds. Raw data were analyzed using Proteome Discoverer 1.4 (ThermoFischer Scientific) and Mascot 2.2 (matrixscience) was used as search engine. A database containing all protein entries of *A. pernix* listed in Uniprot was used to search the data. Search settings used were; 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 (ProteomeSoftware) 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 *A. pernix* is publicly available and encodes 1752 genes, from which 1700 genes are predicted to be protein-coding (24, 25).
The raw proteome analysis resulted in the identification of 993 different proteins (with at least 2 unique peptides identified). A likelihood ratio G-test for independence (26) 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 5-times more abundant in one condition compared to another are listed in the appendix (Table S1). Proteins that possibly play an important role under different growth conditions are discussed in more detail in the text (Table 1).

Results and Discussion

In this study we examined the ability of (hyper)thermophilic microorganisms to reduce the chlorine oxyanions perchlorate and chlorate. Next to the here successfully tested and described (per)chlorate reducers other microorganisms were tested but could not grow by the reduction of (per)chlorate, such as *G. debilis*, *T. volcanium*, *T. scotoductus*, *M. hydrothermalis*, and *S. solfataricus* (see Materials and Methods). Contrarily to the earlier described hyperthermophilic (per)chlorate-reducing microorganism *A. fulgidus*, which is strictly anaerobic, *A. pernix* is known as a strict aerobe (17). However, we showed that *A. pernix* was able to grow efficiently under anaerobic conditions using perchlorate and chlorate as electron acceptors (Fig. 1). The microorganism was not able to grow by nitrate reduction or fermentation of proteinaceous substrates (yeast extract, peptone), which confirms earlier observations (17). The newly discovered metabolism of *A. pernix* was investigated in more detail to get better insight in the mechanism of (per)chlorate reduction at extremely high temperatures.

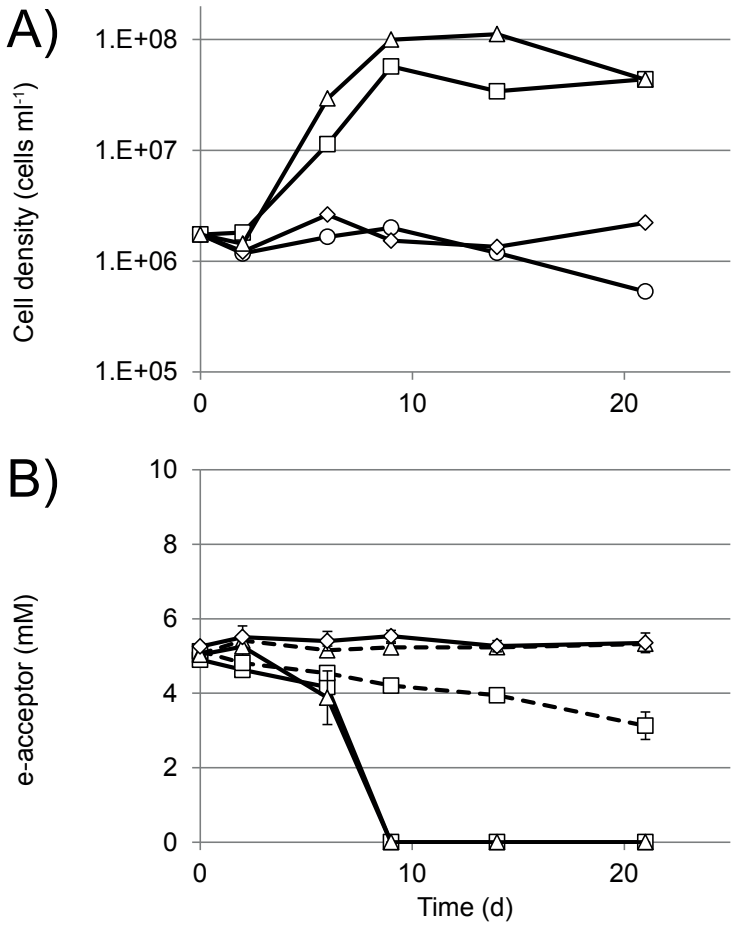


Figure 1: Growth (A) and electron acceptor utilization (B) of *Aeropyrum pernix* in anaerobic medium (DSM medium 820) at 90°C containing either perchlorate (triangles), chlorate (squares), nitrate (diamonds) or no additional e-acceptor (circles); solid lines indicate biological experiments (2% inoculum)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

In the genome of *A. pernix*, no genes are encoded that closely resemble known perchlorate or chlorate reductases. 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, *A. camini* (100% coverage, 95% identity). APE_1288.1 has also 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 TAT sequence motif, which is indicative for a

periplasmic location of the catalytic subunit. The pNar of *P. aerophilum* was reported earlier to use chlorate, besides nitrate as substrate; the V_{max} with chlorate was slightly higher compared to nitrate (27); perchlorate was not tested as substrate.

Besides the identification of a probable enzyme for reducing perchlorate and chlorate in *A. pernix*, the remaining question was how the archaeon eliminates chlorite; an intermediate that is highly toxic for prokaryotes. Chlorite is normally disproportionated in mesophilic (per)chlorate reducers by the action of a chlorite dismutase. Although a protein (APE_0237.1; NP_147071.2) was found in the genome of *A. pernix* (24) that belongs to the same protein family as functional chlorite dismutases (Cld) (Pfam06778) its overall similarity with respective enzymes is very low. Additionally, there was no Cld activity observed in *A. pernix* cell extracts and whole cells (grown with perchlorate) upon chlorite injection.

A closer examination of this Cld-like protein in *A. pernix* revealed the absence of earlier defined signature residues that are present in functional Cld (Ile88, Trp97, Leu122, Arg127, Glu167 - position refers to *Nitrobacter winogradskyi*) (28). From the broad diversity of assigned Cld-like proteins in bacterial and archaeal microbes only a low number is carrying the respective signature residues and for an even smaller number the chlorite-disproportionating activity could be proven (28, 29).

To obtain more understanding of the (per)chlorate-reducing metabolism of *A. pernix*, a proteome analysis was conducted. The presence and abundance of proteins in cells grown with oxygen and grown with chlorate and perchlorate were compared.

The proteome analysis of *A. pernix* cells grown with oxygen, chlorate and perchlorate resulted in coverage of ca. 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).

Table 1: The expression of selected proteins/enzymes of *A. pernix* grown with oxygen, or anaerobically with chlorate or perchlorate as electron acceptors. An extended table listing differentially abundant proteins (5-times more) can be found in the Supplemental Material (Table S1).

Protein	Locus tag	Spectral counts					
		oxygen		chlorate		perchlorate	
Bipolar DNA helicase	APE_0107	38	43	3	4	11	8
5' to 3' nuclease repair protein	APE_0109.1	12	9	0	0	3	1
Putative exonuclease	APE_0181	20	18	5	1	10	4
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
DNA repair photolyase	APE_1326.1	4	4	0	0	0	0
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

Nitrate reductases of the Nar-type as well as perchlorate and chlorate reductases are normally negatively regulated by oxygen (11, 30, 31). However, already former proteomic studies of *A. pernix* grown with oxygen resulted in high expression levels of the Nar-type complex (32). Transcriptomic analyses of *P. aerophilum* demonstrated that its pNar is also expressed independently of the presence or absence of oxygen and nitrate (33). Considering the close relatedness of pNar of *P. aerophilum* with the putative nitrate reductase in *A. pernix* the similarity in the regulation of the two enzymes seems plausible. Based on the observation made by Palmieri et al. (32), the authors debated whether *A. pernix* was a real strict aerobe. The microorganism's ability to grow without oxygen shown in this study proves their earlier thoughts now experimentally right.

APE_0237.1, the chlorite dismutase family protein of *A. pernix*, had a low number of total spectral counts, which remained in the same range under all different growth conditions (Table 1). As was experimentally shown in other studies the fate of chlorite may not only be dependent on the presence of a chlorite-disproportionating enzyme, but is due to its high chemical reactivity also determined by the availability of reducing chemicals in the medium (Chapter 3 and 4). Following the routinely added sulfur components (thiosulfate, sulfate) in the medium (DSM 820) it was demonstrated that, similar to *A. fulgidus* (and sulfide), (per)chlorate reduction by *A. pernix* resulted in a concomitant oxidation of thiosulfate (compare Fig. 2A and B).

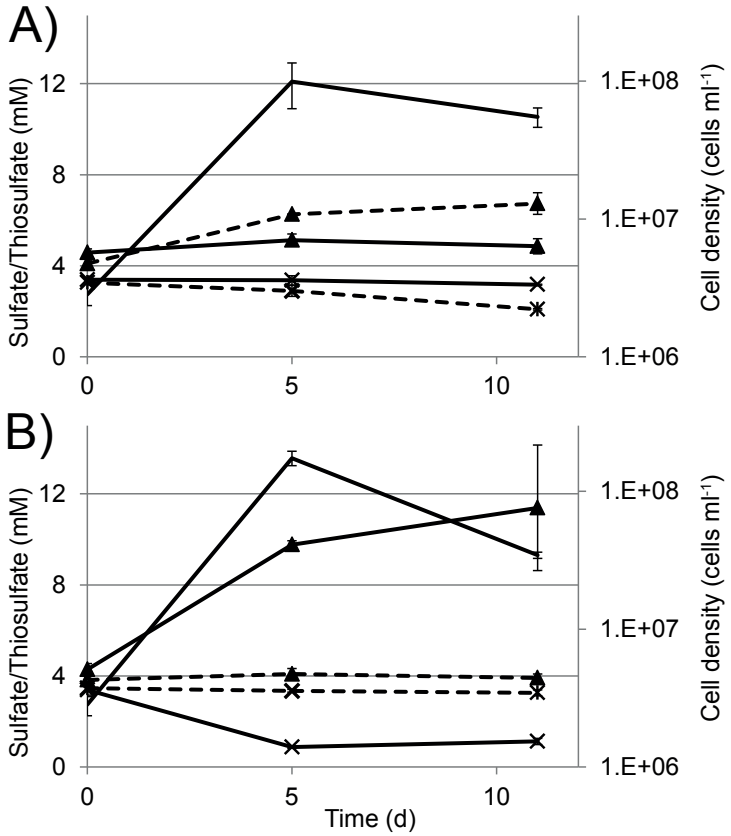
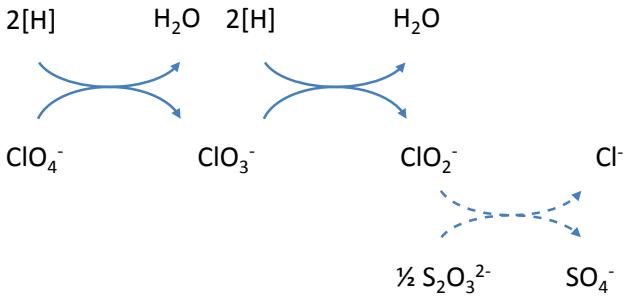


Figure 2: Oxidation of thiosulfate during growth of *Aeropyrum pernix* with either oxygen **(A)** or perchlorate **(B)**. The primary y-axis shows thiosulfate (crosses) and sulfate concentrations (triangles); cell densities (solid lines/no symbols) are plotted against the secondary y-axis. Dashed lines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

The chemical reactivity of reduced sulfur compounds (including thiosulfate) with chlorite has been investigated thoroughly in the past [Mao et al. (34) and Chapter 3 and 4]. Their role as chemical scavengers in the biological reduction of (per)chlorate in microorganisms that lack a chlorite-disproportionating enzyme was reported recently (13) and seems to be crucial for (per)chlorate reduction in *A. pernix* as well (Fig. 3).



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

2) $ClO_2^- + ½S_2O_3^{2-} + ½H_2O \rightarrow Cl^- + SO_4^{2-} + H^+$ $\Delta G^{0'} = -617.1$ kJ chemical

Figure 3: The proposed mechanism of (per)chlorate reduction in *A. pernix* and the thermodynamics of the biologically (equ. 1; solid lines) and chemically mediated branches (equ. 2; dashed lines) of this metabolism under standard conditions (pH 7, 25°C).

Enzymes related to the sulfur metabolism of the archaeon showed differential expression comparing chlorate-, perchlorate- and oxygen-grown cells (Table 1). An enzyme significantly more abundant in aerobically grown cells, a putative ATP sulfurylase (APE_1197.1), is probably assimilating sulfate; which seems less required during growth with perchlorate (Fig. 2), where it accumulates to higher concentrations. APS kinase (APE_1195.1), the subsequent enzyme in the assimilatory sulfate pathway could not be detected in the proteome analysis.

The ability of heterotrophic microorganisms to gain energy from the oxidation of thiosulfate in presence of oxygen has been reported for several microorganisms (35). Also for *A. pernix* cultures grown with oxygen growth-stimulating effects of thiosulfate and its biological oxidation were demonstrated before (17). The strongly increased abundance of a molybdopterin oxidoreducase related to tetrathionate reductase enzymes (APE_2605.1, APE_2607, APE_2610) in oxygen- compared to perchlorate-grown cells (Table 1) seems to relate the results of this study with the above-mentioned observation. In previous studies it was already shown that the oxidation of thiosulfate to tetrathionate, as well as the reversed reaction (reduction from tetrathionate to thiosulfate) can be catalyzed by the same enzyme (36) or the same microorganisms (35), depending on the availability of oxygen. In the current study, the biological oxidation of thiosulfate (represented by the abundance of tetrathionate reductase) seems to be slightly or completely repressed when cells were grown with chlorate or perchlorate, respectively (Table 1). This might be attributed to an inability of transferring electrons deriving from thiosulfate oxidation to electron acceptors other than oxygen. Also a potentially faster chemical reactivity

between chlorite and thiosulfate than between oxygen and thiosulfate (Fig. 2 and Chapter 4) may compete with the biological oxidation of thiosulfate and combined with the above-mentioned aspect be the cause of lower abundances of the enzyme (APE_2605.1, APE_2607, APE_2610).
Contrary to the proteome analysis performed on *A. fulgidus* (13), growth with perchlorate and chlorate did not result in a strong redox and oxygen stress response in *A. pernix*. There was rather increased abundances of potentially redox-sensing proteins in the presence of oxygen (e.g.: APE_0107, APE_0109.1, APE_0181, APE_1326.1), than under anaerobic conditions with (per)chlorate (Table 1). This is comprehensible considering the strictly anaerobic lifestyle of *A. fulgidus* compared to the aerobic metabolism of *A. pernix*.
Besides (per)chlorate reduction there are indications for an even broader metabolic flexibility in *A. pernix*. The increased abundance of a putative arsenite oxidase in presence of oxygen (but absence of arsenite) (Table S1) may indicate a pre-adapted metabolic preference.
Additionally the putative tetrathionate reductase encoded in the genome (which was discussed above) may also get expressed under anaerobic conditions in case tetrathionate is present. This would possibly enable another anaerobic respiration pathway next to (per)chlorate reduction in *A. pernix*.

(Per)chorate-reducing members of the Firmicutes

Two Gram-positive bacteria of the Firmicutes, namely the strict anaerobe *Carboxydothermus hydrogenoformans* (strain Z-2901) and strain NMP, affiliated with the type strain of *Moorella glycerini* (strain JW/AS-Y6) (18, 19) were also able to reduce perchlorate (Fig. 4A and 5) and chlorate (Fig. 4B) in the presence of sulfide (see Material and Methods). (Per)chlorate reduction in the absence of sulfide was tested with strain NMP and did not result in any activity. Other members of the genus *Moorella* have also been reported earlier to grow by the reduction of (per) chlorate (16, 37). The genomes of both, *C. hydrogenoformans* and *M. glycerini* NMP harbor 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 *C. hydrogenoformans* (Chy_2082, YP_360901.1) is predicted to be located outside of the cell using PRED-TAT (22). This characteristic has also been found for the presumed (per)chlorate-reducing enzymes of *A. pernix* and *A. fulgidus* (Chapter 3). The best enzyme candidate for the reduction of (per)chlorate in strain NMP (also predicted to have an extracellular catalytic subunit) has a high similarity with the nitrate reductase of *C. hydrogenoformans* (alpha-subunits: 99% coverage, 73% identity) and with a molydopterin oxidoreductase in *Moorella thermoacetica* ATTC 39073 (alpha-subunits: 99% coverage, 93% identity).

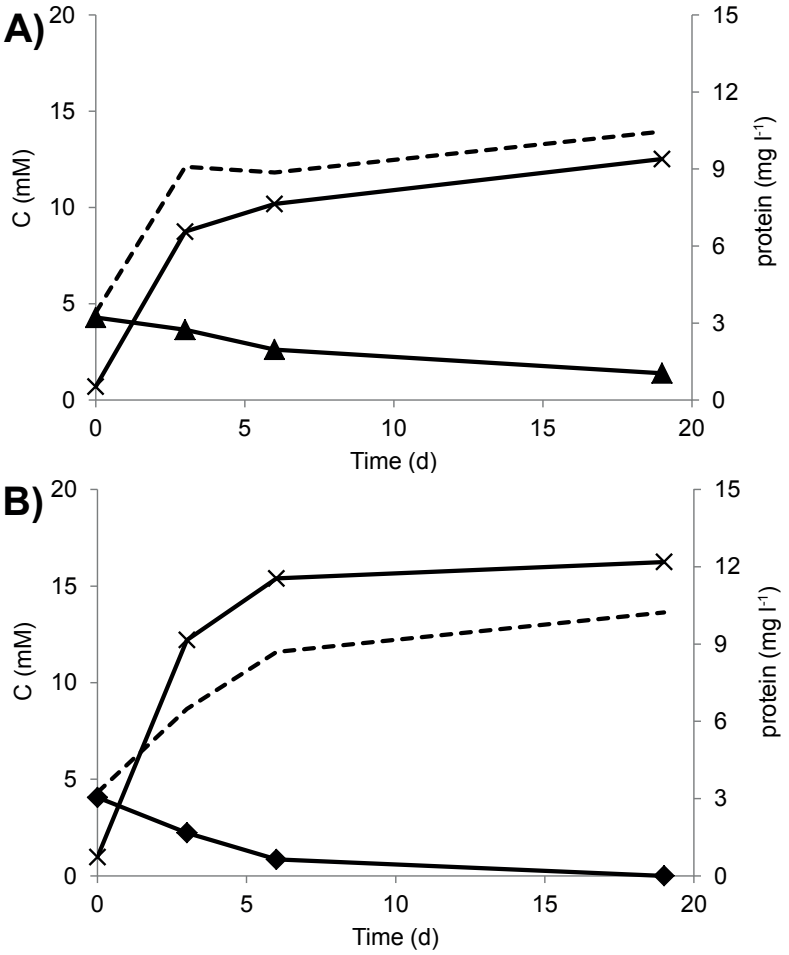


Figure 4: The reduction of **A)** perchlorate (triangles) and **B)** chlorate (diamonds) by *Carboxydothermus hydrogenoformans* Z-2901 in sulfide-reduced medium at 70°C. Pyruvate was used as electron donor (not measured) and acetate accumulated as product (crosses). The increase of biomass is indicated by the protein concentration (dashed line) over time. Data points are means of *n=2*.

Both, the draft genome of strain NMP and the genome of *C. hydrogenoformans* do not encode any enzyme resembling chlorite dismutases. In addition, no chlorite dismutase activity was detected with whole cells and cell free extracts of strain NMP; *C. hydrogenoformans* was not tested in this respect. Although earlier studies reported chlorite-disproportionating activities in (per)chlorate-grown members of the Firmicutes (16, 38), the mechanism of perchlorate reduction observed in this study seems to differ.

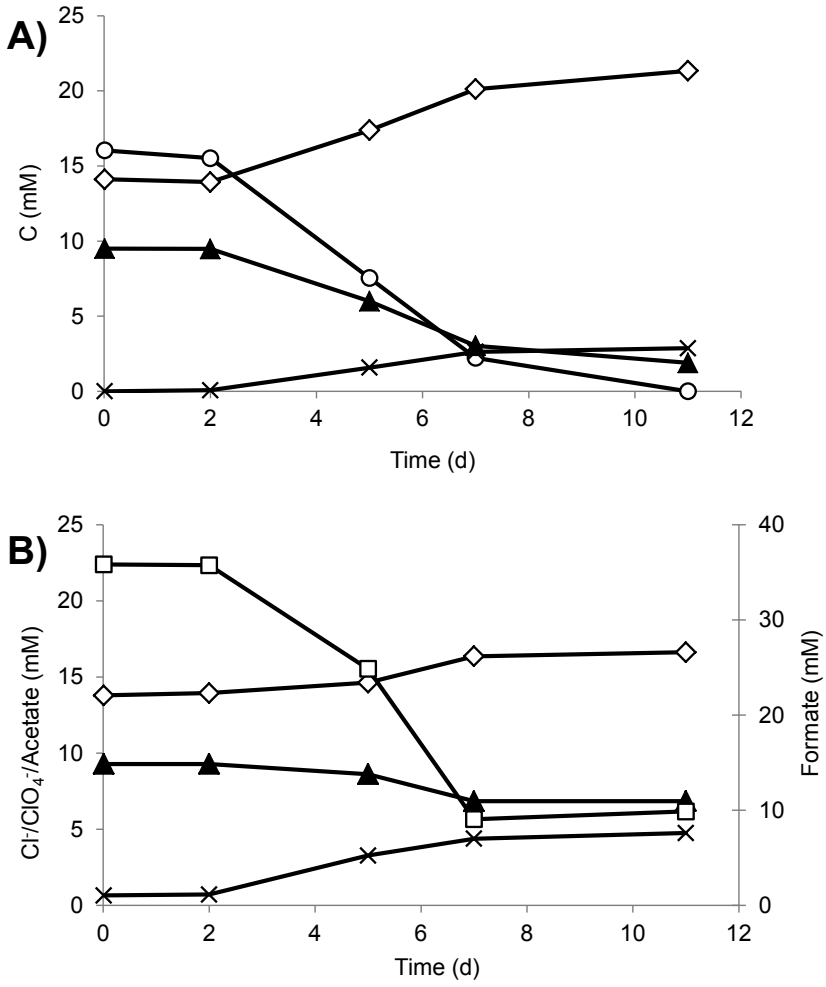


Figure 5: The reduction of perchlorate (ClO_4^-, triangles) by *Moorella glycerini* strain NMP at 60°C. **A)** Methanol (circles) or **B)** formate (squares) were used as substrates and acetate (crosses) accumulated over time. The complete reduction resulted in the formation of chloride anions (Cl^-, diamonds). Plotted data are means of *n=2*.

C. hydrogenoformans has been reported to grow by the reduction of sulfite, thiosulfate, sulfur, nitrate and fumarate, but not sulfate (39). Similarly also strain NMP was able to grow by the reduction of thiosulfate and sulfite, but not sulfate. Strain NMP had difficulties to grow with chlorate (which is the intermediate of perchlorate reduction), though perchlorate served as excellent electron acceptor (Fig. S1). This may be related to the kinetics of the perchlorate-reducing enzyme combined with the lack of a functional Cld. Higher specific activities of Pcr with chlorate compared

to perchlorate were shown earlier (3, 40). Thus, chlorate reduction might exceed the abiotic reduction rates of chlorite with sulfide (in case chlorate is offered as electron acceptor instead of perchlorate), which accumulates toxic levels of chlorite and terminates chlorate reduction.
For the two members of the Firmicutes tested in this study it seems probable that complete (per)chlorate reduction proceeds in a similar manner as described for *A. fulgidus* and *A. pernix*, where the absence of Cld is compensated by chemical chlorite reduction, involving reduced sulfur compounds. Further studies are required to obtain additional support for the involvement of an intracellular "sulfur loop" in (per)chlorate reduction, similar to the one in *A. fulgidus* (Chapter 3 and 4).

Conclusions

This study broadened the knowledge on high temperature (per)chlorate reduction and strengthened the idea that classical microbial (per)chlorate reduction is restricted to mesophilic microorganisms. There is consistency in the observation that all up to now described (hyper)thermophiles [with one exception, *Moorella perchloratireducens* (16)] lack a functional chlorite-disproportionating enzyme. Similar to *A. fulgidus* also *A. pernix* coupled the complete reduction of (per)chlorate to sulfur compounds. By the chemical oxidation of available thiosulfate to sulfate, chlorite is reduced to chloride anions. In line with the two above-mentioned archaea also the, here tested, Firmicutes [*Carboxydothermus hydrogenoformans* (strain Z-2901) and strain NMP] seem to lack a chlorite dismutase. Furthermore, sulfide in the medium was essential for the initiation of (per)chlorate reduction. Another common characteristic of (hyper)thermophilic (per)chlorate-reducing microorganisms known thus far, is the periplasmic location of the enzyme reducing (per)chlorate. In the respective microorganisms (per)chlorate reduction seems to be catalyzed by enzymes resembling Nar-type nitrate reductases. Nar-type nitrate reductase can reduce chlorate (41) (and possibly perchlorate, although this has hardly been investigated on the biochemical level), 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 (42) (see also Fig. 1 in Chapter 7), which may enable microorganisms to grow with (per) chlorate even in the absence of a chlorite dismutase (the key enzyme of classical (per)chlorate-reducing bacteria).
Substrate promiscuity of evolutionary related enzymes, like nitrate reductases and perchlorate reductases seems to enable (per)chlorate reduction beyond the classical mesophilic metabolism (employing chlorite dismutase). A broadened substrate range of these 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 this consideration seems intriguing. This study expands the diversity of (per)chlorate-reducing microorganisms growing at high temperatures and reports the first Crenarchaeon growing by this metabolism. Results so far suggest that microbial (per)chlorate reduction at extremely elevated temperatures is characterized by the lack of chlorite dismutase and enabled by a combination of biotic and abiotic reactions.

Acknowledgements

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Supplemental Material

Table S1: Proteins of *Aeropyrum pernix* that were found to be at least 5-times more abundant under one growth condition compared with another (">>>"). Different levels of significance of are indicated ($p \leq 0.05$, ** and $p \leq 0.01$, ***).

Oxygen >>> Chlorate			
Proteins	Locus tag	UniProt	
Bipolar DNA helicase	APE_0107	Q9YFZ4_AERPE	***
5' to 3' nuclease repair protein	APE_0109.1	Q9YFZ2_AERPE	***
ORC1-type DNA replication protein	APE_0152.1	CDC62_AERPE	***
Uncharacterized protein	APE_0187	Q9YFR2_AERPE	***
MRP/NBP35 family protein	APE_0230.1	Q9YFL8_AERPE	***
30S ribosomal protein S4e	APE_0356.1	RS4E_AERPE	***
30S ribosomal protein S19	APE_0367.1	RS19_AERPE	**
Medium-chain-fatty-acid--CoA ligase	APE_0396.1	Q9YF45_AERPE	***
Uncharacterized protein	APE_0413	Q9YF27_AERPE	***
Uncharacterized protein	APE_0416.1	Q9YF24_AERPE	***
Glycerol-1-phosphate dehydrogenase [$NAD(P)^+$]	APE_0519.1	G1PDH_AERPE	***
Putative dehydrogenase	APE_0595.1	Q9YEI1_AERPE	***
Aldehyde dehydrogenase	APE_0708.1	Q9YE62_AERPE	***
Uncharacterized protein	APE_0763.1	Q9YE06_AERPE	***
tRNA (guanine(26)-N(2))-dimethyltransferase	APE_0782.1	TRM1_AERPE	***
Uncharacterized protein	APE_0784.1	Q9YDY5_AERPE	***
Heme-copper oxidase subunit II	APE_0792.1	AOX2_AERPE	***
Heme-copper oxidase subunit I+III	APE_0793.1	AOX1_AERPE	***
Branched-chain amino acid ABC transporter	APE_0919	Q9YDJ4_AERPE	***
Protease HtpX homolog	APE_1045.1	HTPX_AERPE	**
Uncharacterized protein	APE_1059	Q9YD53_AERPE	**
tRNA N6-adenosine threonylcarbamoyltransferase	APE_1135	KAE1_AERPE	**
Uncharacterized protein	APE_1164	Q9YCU8_AERPE	***
dTDP-glucose 4,6-dehydratase	APE_1180	Q9YCT1_AERPE	***
Sulfate adenylyltransferase	APE_1197.1	SAT_AERPE	***
Uncharacterized protein	APE_1326.1	Q9YCD0_AERPE	**

7-cyano-7-deazaguanine synthase	APE_1470.1	QUEC_AERPE	***
Uncharacterized protein	APE_1495.1	Q9YBV5_AERPE	***
Threonine dehydratase	APE_1498.1	Q9YBV1_AERPE	***
Uncharacterized protein	APE_1510.1	Q9YBT9_AERPE	***
Putative tRNA(Met) cytidine acetyltransferase	APE_1543	TMCA_AERPE	***
ABC transporter	APE_1548.1	Q9YBQ1_AERPE	***
Putative ATP-dependent adenyltransferase	APE_1594	Q9YBK4_AERPE	***
Signal recognition particle	APE_1735	SRP54_AERPE	***
Transcription factor E	APE_2004.1	TFE_AERPE	***
Probable cysteine desulfurase	APE_2023	CSD_AERPE	***
UPF0284 protein	APE_2029.1	Y2029_AERPE	***
Glycosyl transferase, group 1	APE_2066.1	Q9YA73_AERPE	**
Uncharacterized protein	APE_2178.1	Q9Y9W1_AERPE	***
Protein kinase	APE_2208.1	Q9Y9T0_AERPE	***
Aldehyde dehydrogenase, large subunit	APE_2216.1	Q9Y9S2_AERPE	***
Aldehyde dehydrogenase, middle subunit	APE_2219	Q9Y9R9_AERPE	***
Long-chain-fatty-acid--CoA ligase	APE_2284.1	Q9Y9K4_AERPE	***
Uncharacterized protein	APE_2360.1	Q9Y9C7_AERPE	**
Electron transfer flavoprotein beta-subunit	APE_2418.1	Q9Y967_AERPE	***
Uncharacterized protein	APE_2425	Q9Y960_AERPE	***
RNA (Cytosine-C(5)-)-methyltransferase	APE_2465.1	Q9Y919_AERPE	***
Uncharacterized protein	APE_2481.1	Q9Y903_AERPE	***
Branched-chain amino acid ABC transporter	APE_2526	Q9Y8V8_AERPE	**
Probable arsenite oxidase large subunit	APE_2556.1	Q9Y8S7_AERPE	***

O**xygen >>> Perchlorate**			
Proteins	Locus tag	UniProt	
Uncharacterized protein	APE_0258.1	Q9YFI8_AERPE	***
Uncharacterized protein	APE_0416.1	Q9YF24_AERPE	***
Xaa-Pro dipeptidase	APE_0526.1	Q9YEQ3_AERPE	***
Phospho-2-dehydro-3-deoxyheptonate aldolase	APE_0581.1	Q9YEJ7_AERPE	**
Uncharacterized protein	APE_0605.1	Q9YEH1_AERPE	***

Uncharacterized protein	APE_0784.1	Q9YDY5_AERPE	***
Heme-copper oxidase subunit II	APE_0792.1	AOX2_AERPE	***
Heme-copper oxidase subunit I+III	APE_0793.1	AOX1_AERPE	***
UPF0130 protein	APE_0816	Y816_AERPE	***
Uncharacterized protein	APE_1059	Q9YD53_AERPE	**
tRNA N6-adenosine threonylcarbamoyltransferase	APE_1135	KAE1_AERPE	**
Uncharacterized protein	APE_1164	Q9YCU8_AERPE	***
Sulfate adenylyltransferase	APE_1197.1	SAT_AERPE	***
Uncharacterized protein	APE_1326.1	Q9YCD0_AERPE	**
7-cyano-7-deazaguanine synthase	APE_1470.1	QUEC_AERPE	***
Oligopeptide ABC transporter, ATP binding protei	APE_1578	Q9YBM0_AERPE	***
Alcohol dehydrogenase	APE_1963.1	Q9YAH6_AERPE	**
Uncharacterized protein	APE_1966	Q9YAH3_AERPE	**
Transcription factor E	APE_2004.1	TFE_AERPE	***
Glycosyl transferase, group 1	APE_2066.1	Q9YA73_AERPE	**
Aldehyde dehydrogenase, large subunit	APE_2216.1	Q9Y9S2_AERPE	***
Dipeptide ABC transporter	APE_2263.1	Q9Y9M5_AERPE	***
Long-chain-fatty-acid--CoA ligase	APE_2284.1	Q9Y9K4_AERPE	***
Uncharacterized protein	APE_2311.1	Q9Y9H7_AERPE	***
Uncharacterized protein	APE_2352.1	Q9Y9D5_AERPE	***
Uncharacterized protein	APE_2458a	Q05DW9_AERPE	**
Tryptophan--tRNA ligase	APE_2461.1	SYW_AERPE	***
RNA (Cytosine-C(5)-)-methyltransferase	APE_2465.1	Q9Y919_AERPE	***
Branched-chain amino acid ABC transporter	APE_2526	Q9Y8V8_AERPE	**
Probable arsenite oxidase large subunit	APE_2556.1	Q9Y8S7_AERPE	***
Molybdopterin oxidoreductase, iron-sulfur binding subunit	APE_2605.1	Q9Y8M7_AERPE	***
Molybdopterin oxidoreductase, membrane subunit	APE_2607	Q9Y8M5_AERPE	***
Molybdopterin oxidoreductase, molybdopterin binding subunit	APE_2610	Q9Y8M2_AERPE	***
Chlorate >>> Oxygen			

Proteins	Locus tag	UniProt	
Uncharacterized protein	APE_0725.1	Q9YE45_AERPE	***
Chlorate >>> Perchlorate			
Proteins	Locus tag	UniProt	
Uncharacterized protein	APE_0258.1	Q9YFI8_AERPE	**
Uncharacterized protein	APE_1966	Q9YAH3_AERPE	**
Thymidylate synthase ThyX	APE_2064.1	THYX_AERPE	***
Dipeptide ABC transporter	APE_2263.1	Q9Y9M5_AERPE	***
Uncharacterized protein	APE_2352.1	Q9Y9D5_AERPE	***
Tryptophan--tRNA ligase	APE_2461.1	SYW_AERPE	***
Molybdopterin oxidoreductase, membrane subunit	APE_2607	Q9Y8M5_AERPE	***
Molybdopterin oxidoreductase, molybdopterin binding subunit	APE_2610	Q9Y8M2_AERPE	***
Perchlorate >>> Oxygen			
Proteins	Locus tag	UniProt	
Uncharacterized protein	APE_0725.1	Q9YE45_AERPE	***
Uncharacterized protein	APE_1971.1	Q9YAG8_AERPE	***
Perchlorate >>> Chlorate			
Proteins	Locus tag	UniProt	
30S ribosomal protein S4e	APE_0356.1	RS4E_AERPE	***
30S ribosomal protein S19	APE_0367.1	RS19_AERPE	***
Probable tRNA sulfurtransferase	APE_0465.1	THII_AERPE	***
tRNA (guanine(26)-N(2))-dimethyltransferase	APE_0782.1	TRM1_AERPE	***
Branched-chain amino acid ABC transporter	APE_0919	Q9YDJ4_AERPE	***
Uncharacterized protein	APE_1510.1	Q9YBT9_AERPE	**
Transport system kinase	APE_1683.1	Q9YBB4_AERPE	***
Probable cysteine desulfurase	APE_2023	CSD_AERPE	***
Protein kinase	APE_2208.1	Q9Y9T0_AERPE	***
Glutamyl-tRNA reductase	APE_2296	HEM1_AERPE	***
Hydantoin utilization protein	APE_2530.1	Q9Y8V4_AERPE	***

Chapter 7
General Discussion

General Discussion

Microbial (per)chlorate reduction was reported for the first time in 1928 (1). Especially research conducted in the fourth quarter of the 20th century unraveled this metabolism of facultative anaerobic mesophilic bacteria (2). The isolation of the first axenic cultures of (per)chlorate-reducing bacteria, the characterization of key enzymes and the exploration of the genetic fundament has described an "exotic" anaerobic metabolism, that forms dioxygen under oxygen-free conditions (3). Microbial (per)chlorate reduction at high temperature has barely been reported (4, 5), although it is a thermodynamically favorable process (6). In the current work, research on microbial (per)chlorate reduction at elevated temperatures is described. Results of this study did not only expand microbial (per) chlorate reduction up to 100°C, but also discovered a metabolism that differs from the classical (per)chlorate reduction at low temperatures. Consistent observations were made regarding the interactions of biotic and abiotic reactions involving sulfur compounds that enable complete (per)chlorate reduction in (hyper)thermophiles.
In addition to the scientific value of this work the potential benefit of (per)chlorate and (per)chlorate-reducing microorganisms for industrial applications is discussed from the angle of petroleum recovery.

Classical (per)chlorate reduction

Almost all (per)chlorate-reducing microorganisms that have been described so far are mesophilic bacteria (7). They are mostly facultative anaerobes affiliated with the phylum Proteobacteria (two others are belonging to the Firmicutes) and they can be found in a broad range of environments, including pristine soils. (Per)chlorate reducers couple the reduction of (per)chlorate to the oxidation of organic and inorganic electron donors, while acetate is the most commonly utilized substrate by the microorganisms (2).
The classical pathway of complete biological perchlorate reduction involves the action of two enzymes, perchlorate (Pcr) or chlorate reductase (Clr) and chlorite dismutase (Cld) (8, 9). Perchlorate reductase catalyzes the first two reduction steps, from perchlorate to chlorate and from chlorate to chlorite, while chlorite is disproportioned by chlorite dismutase to chloride and molecular oxygen. Microorganisms that cannot use perchlorate but reduce chlorate employ genetically distinct chlorate reductases (10). Pcr and Clr are enzymes that belong to the DMSO II type reductases and have their alpha-subunits located in the periplasm. Pcr and Clr resemble nitrate reductases and other DMSO II oxidoreductases that use molybdopterin as cofactor (11).
Chlorite dismutase is an enzyme that forms oxygen and chloride by the disproportionation of chlorite under oxygen-free conditions (3). The detoxification

of chlorite, a highly toxic compound for the cell is the crucial step in (per)chlorate reduction and assigns a key function to chlorite dismutase in this metabolism.
In addition to the functionally characterized Cld from known (per)chlorate-reducing bacteria, there is a huge number of microorganisms with Cld-like proteins encoded in their genomes (12, 13). In the Integrated Microbial Genomes (IMG) database (14), around 500 completed genomes contain genes that encode chlorite-dismutase family proteins (pfam06778) (Chapter 2). It is unknown how many of these proteins can actually dispoportionate chlorite and whether this may enable respective microorganisms to grow by (per)chlorate reduction. Recently a highly efficient Cld of *Nitrobacter winogradskyi* was described; this enzyme is different in size and structure from previously characterized Cld (13). This finding indicates the yet unknown diversity of functionally efficient enzymes and microorganisms involved in the reduction of (per)chlorate. Based on the presence of genes which remotely resemble the ones of Cld in genome-sequenced microorganisms, we selected and tested (hyper)thermophilic bacteria and archaea for their capability to grow by (per) chlorate reduction (Chapter 6).
The investigations described in this thesis discovered unexpected mechanisms during microbial (per)chlorate reduction that do not employ the action of Cld; normally regarded as prerequisite for (per)chlorate reduction. These alternative strategies for complete (per)chlorate reduction coupled to growth are described hereafter and are compared with the classical metabolism relying on Cld.

(PER)CHLORATE REDUCTION *SENSU LATO*

This thesis presented new insights in microbial (per)chlorate reduction at high temperature, a rarely explored field of research until now. Findings of this study extended this anaerobic metabolism to hyperthermophilic archaea and additional thermophilic Gram-positive bacteria (Chapter 3, Chapter 6) and described an alternative pathway to classical mesophilic (per)chlorate reduction. It was shown that the complete microbial reduction of (per)chlorate to chloride does not necessarily rely on the presence of a functional chlorite dismutase (Chapter 3). The presence and regeneration of reduced sulfur compounds enables an alternative pathway of (per)chlorate reduction in *Archaeoglobus fulgidus* (lacking Cld), where continuously formed chlorite is eliminated by sulfide, forming sulfur compounds of higher oxidation states ($S_xO_y^{z-}$).

Biological (per)chlorate reduction

The initial step of (per)chlorate reduction in *A. fulgidus* (from perchlorate to chlorite) is mediated by a DMSO II oxidoreductase (Af_0174-0176; Af_0173 is a chaperone)

that is only moderately related to known Pcr (Chapter 3). This enzyme has been associated with Nar-type enzymes, but unlike the bacterial Nar, Af_0173-0176 seems to have its catalytic subunit located in the periplasm (15).
Such periplasmic Nar-type nitrate reductases were biochemically characterized from three archaeal species (16-18). Similar to respiratory nitrate reductases of bacteria, the pNar from *Haloferax meditteranei* and *Pyrobaculum aerophilum* utilize chlorate (17, 19). The activity toward perchlorate has unfortunately not been assessed.
Similary, *Aeropyrum pernix* was shown to grow by the reduction of (per)chlorate in the absence of a functional chlorite dismutase (Chapter 6). In contrast to *A. fulgidus* this crenarchaeal microorganism was formerly described as a strict aerobe. The newly discovered trait in *A. pernix* showed, next to the absence of a functional Cld, other consistencies with the (per)chlorate-reducing metabolism of *A. fulgidus*. A putative nitrate reductase was identified as the most probable enzyme for the reduction of perchlorate and chlorate to chlorite. Also this enzyme is other than bacterial Nar-type enzymes positioned with its catalytic subunit on the outside of the cell. The role of sulfur compounds for the abiotic oxidation of chlorite during (per)chlorate reduction was demonstrated. In contrast to *A. fulgidus* (per)chlorate reduction in *A. pernix* is not based on sulfide, but the reactivity of thiosulfate (present in the medium) with chlorite, accumulating sulfate and chloride. In Chapter 6 also two Firmicutes are described, *C. hydrogenoformans* and a strain belonging to *Moorella glycerini* (strain NMP), that were able to couple growth to (per)chlorate reduction, while lacking chlorite dismutase. The similarity to the above-mentioned archaeal (per)chlorate-reducing metabolism is obvious. As for another *Moorella* strain chlorite dismutase activity was reported (4) further research is needed to elucidate the perchlorate-reducing pathway in this genus.

Chemical chlorite elimination

The periplasmic location of (per)chlorate-reducing enzymes may be the key for complete (per)chlorate reduction in the absence of a Cld, exposing chlorite to potential chemical scavengers in the environment (e.g. sulfide, thiosulfate). The fate of chlorite during (per)chlorate reduction and the hereupon-based formation of oxidized sulfur compounds was investigated in more detail in Chapter 4. Additional evidence was obtained that *A. fulgidus* depends on abiotic reactions (reducing chlorite) based on sulfur compounds to drive (per)chlorate reduction. An intraspecies “sulfur loop” in *A. fulgidus* is established, consisting of abiotic oxidation and biotic reduction processes, that recycles reducing agents for further chlorite scavenging. This was also indicated by the increased presence of enzymes involved in the reduction of elemental sulfur/polysulfide and tetrathionate during growth on (per)chlorate (Chapter 3). The (periodic) accumulation of elemental sulfur

and the biological reduction of elemental sulfur during (per)chlorate reduction in *A. fulgidus* was shown (Chapter 4), which strengthened the model of a "sulfur loop". Another potentially important intermediate in the "sulfur-based" loop, tetrathionate was shown to be reduced as sole electron acceptor by *A. fulgidus*. It is therefore apparent that *A. fulgidus* is able to grow on (per)chlorate because it couples (per) chlorate reduction to its sulfur metabolism.

The above discussed intraspecies "sulfur loop" during (per)chlorate reduction was most detailed investigated with *A. fulgidus*, but also the other, here described thermophilic (per)chlorate-reducing species are likely employing such strategies. Particularly *C. hydrogenoformans* and *M. glycerini* strain NMP, which grew by (per)chlorate reduction, have a wide set of "sulfur enzymes" (20) (Chapter 6) that possibly regenerate sulfide during (per)chlorate reduction, needed for the chemical elimination of chlorite.

The high chemical reactivity of other potentially relevant chemical scavengers for chlorite (e.g. ferrous iron) was confirmed in Chapter 4; however (per)chlorate reduction of *A. fulgidus* was only observed in presence of sulfide or thiosulfate (as reducing agents). Although ferrous chloride was added in excess to the medium (with parts of it precipitated as FeS), *A. fulgidus* was not able to grow by perchlorate reduction. Possibly reaction rates of ferrous iron with chlorite are lower than compared with the ones of sulfide with chlorite (which could not be assessed accurately enough in the trials of Chapter 4) and may consequently lead to the inhibition of (per)chlorate reduction.

Extracellular chlorite formation

Apart from the dependence on efficient chlorite scavengers, the subcellular location of the (per)chlorate-reducing enzyme seems of major importance. The ability of bacterial nitrate reductases (Nar-type) to reduce chlorate has been known for a long time (21). However, due to their cytoplasmic location this reduction accumulates toxic chlorite in the cell, which does not enable growth or the continuous reduction of chlorate. Perchlorate and chlorate reductases have their catalytic subunits located outside of the cell, where chlorite is rapidly disproportionated by the periplasmic enzyme chlorite dismutase during classical (per)chlorate reduction.

In the absence of chlorite dismutase, the periplasmic location of (per)chlorate-reducing enzymes seems to matter particularly, since chlorite has to be accessible for chemical reducing agents that promptly eliminate the toxic intermediate (Chapter 3 and 4).

Indirect proof for this assumption was shown within this thesis by the observation that all here identified (per)chlorate-reducing (hyper)thermophiles employ enzymes with periplasmic alpha-subunits for (per)chlorate reduction (Chapter 3 and 6).

Substrate ambiguity in Mo-enzymes

The ambiguity in the substrate range of enzymes within the DMSO II family allows speculation on the diversity of enzymes (next to classical chlorate and perchlorate reductases) that can reduce perchlorate or chlorate; forming chlorite. Particularly interesting are the above-mentioned (p)Nar-type nitrate reductases and selenate reductases that are closely related to perchlorate and chlorate reductases within the DMSO II enzyme family. Both enzymes in fact reduce chlorate in addition to nitrate or selenate (19, 22). Other molybdenum-enzymes like DMSO reductase (Dor) and trimethylamine N-oxide reductase (Tor) have also been reported to reduce chlorate, whereas the periplasmic Nap-type nitrate reductase lacks this trait (23-25). The activity of these enzymes toward perchlorate has hardly been tested. One of the few exceptions is the respiratory nitrate reductase of *Marinobacter hydrocarbonoclasticus* strain 617 (22). For this enzyme, a significantly lower efficiency for perchlorate reduction compared to chlorate and nitrate reduction was measured.

In summary it seems that a considerable range of molybdenum enzymes in general, and DMSO II enzymes in particular, have the potential to reduce chlorate (and possibly perchlorate) (Fig. 1), although in most cases with lower efficiency. In this context also the pNar-like enzyme of *A. fulgidus* and its relatedness to other enzymes suggests promising candidates for (per)chlorate reduction.

Proteins that have a high similarity with the pNarG-like subunit (Af_0176) of *A. fulgidus* (Chapter 3) can be found in other archaea (*Ferroglobus placidus*, Ferp_0124), mesophilic (*Desulfosporosinus meridiei*, Desmer_2075; *Desulfitobacterium dehalogenans*, Desde_0947; *Desulfitobacterium dichloroeliminans*, Desdi_0326) and thermophilic bacteria [*Carboxydothermus hydrogenoformans*, Cyh_2082; *Moorella thermoacetica*, Moth_1908; *Moorella glycerini*, strain NMP, Mo-oxidoreductase (Chapter 6)]. For some of these enzymes, the potential function as a pNar was discussed earlier (19). Here, their potential as (per)chlorate-reducing enzymes is proposed.

The sequence similarity and probable periplasmic location of these enzymes combined with circumstantial analogies of the respective microbes with *A. fulgidus* [(hyper)thermophily and (strict) anaerobicity] may be indicators for (per)chlorate reduction. Taking into account that all the respective microbes lack Cld, it would require chlorite elimination strategies similar to the ones in *A. fulgidus* to enable complete (per)chlorate reduction. The observation that *C. hydrogenoformans* (and *M. glycerini* strain NMP) were found to reduce (per)chlorate (Chapter 6) strengthens these assumptions.

C. hydrogenoformans has two putative nitrate reductases encoded in its genome (27), one is a putative pNar-type reductase (pNarG: Cyh_2082) and resembles Af_0173-0176 of *A. fulgidus* and the other one a nitrate reductase related to the Nap-type (NapA:

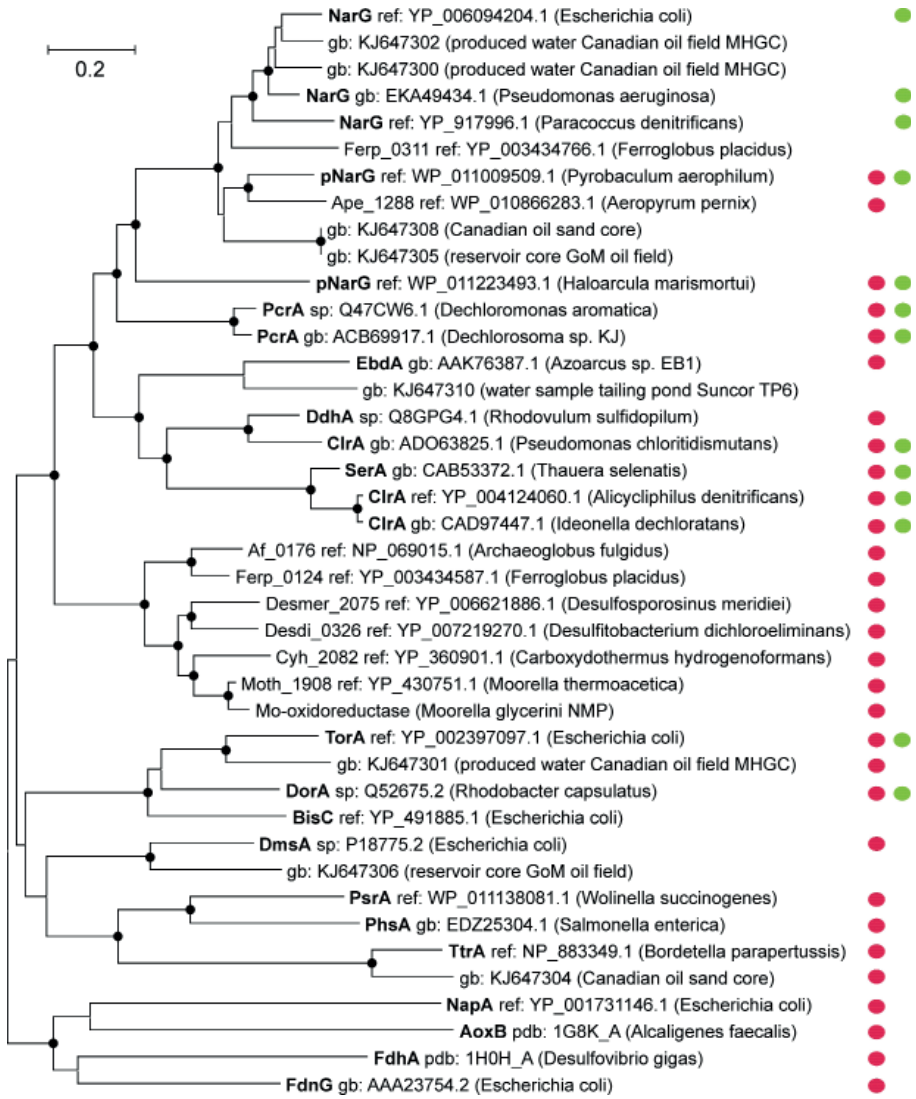

Figure 1: Diversity of catalytic subunits of selected DMSO Mo-enzymes. Protein sequences of characterized enzymes (bold) and uncharacterized molybdopterin oxidoreductases. (Partial) sequences retrieved from metagenomic datasets of oil reservoir environments are also displayed; red dots mark the periplasmic location of the catalytic subunit for characterized enzymes [otherwise predicted by PRED-TAT, Bagos et al. (26)], whereas green dots indicate activity with chlorate (next to the canonical enzyme function). For most of the enzymes no data are available regarding their activity toward perchlorate. The phylogenetic tree was constructed using the Neighbor-Joining method. Bootstrap values above 70% (based on 500 replicates) are indicated by nodes at the respective branches. Evolutionary distances of the tree were computed using the Poisson correction method; the scale bar indicates amino acid substitutions per site.

Chy_0601) (although not predicted to be translocated via the membrane). Given the similarity with Af_0173-0176 it seems likely that (per)chlorate reduction by *C. hydrogenoformans* (Chapter 6) is based on the first of the two mentioned enzymes, whereas it is not clear which of the two enzymes is reducing nitrate; a trait earlier reported for *C. hydrogenoformans* (20). Interestingly, the nitrate reducer *Ferroglobus placidus* possesses in addition to the above-mentioned enzyme resembling Af_0173-0176 (Ferp_0121-0124), also a cytoplasmic Nar-type enzyme in its genome (alpha-subunit: Ferp_0311, whole enzyme: Ferp_0311-0314) (28). The above-mentioned Ferp_0121-0124 is a molybdopterin oxidoreductase with unknown function and has its catalytic subunit predicted to be outside of the cell. The high similarity between Af_0176 and Ferp_0124 suggests that *F. placidus* has a system for the reduction of (per)chlorate that is distinct from the one for the reduction of nitrate. Further investigations are required to confirm this hypothesis.
Overall, the above-mentioned group of enzymes related to Af_0173-0176 seem to be a deep-rooting branch in the phylogeny of DMSO II enzymes (Fig. 2), which may indicate the ancient character of these (probably chlorate- and perchlorate-reducing) enzymes.

Syntrophic perchlorate reduction

Next to classical (per)chlorate reduction based on Cld (Fig. 2A) and the complete (per)chlorate reduction, involving an intraspecies "sulfur loop" (like in *A. fulgidus*) (Fig. 2B), it was speculated whether (per)chlorate reduction could also be established based on an interspecies "sulfur loop". This would require a close interaction between two (or more) microorganisms, in which one partner reduces perchlorate to chlorite and the other one reduces oxidized sulfur compounds, deriving from the chemical reduction of chlorite to regenerate sulfide for continuous (per)chlorate reduction (Fig. 2C). Such syntrophic interactions during (per)chlorate reduction may theoretically also be based on elements other than sulfur such as iron, involving an iron-reducing and a (per)chlorate-reducing microorganism. The most critical factor determining the feasibility of this syntrophy will probably be once more the availability and reactivity of the reduced scavenging compound with chlorite.
Some indications for syntrophic perchlorate reduction were obtained for a consortium that grew with perchlorate and acetate (Chapter 5). Despite the efforts, no pure culture could be obtained, indicating the necessity for biological complexity under these conditions. Supporting arguments for syntrophic perchlorate reduction derived from a community analysis, confirming mostly species related to known sulfur/sulfate reducers in the culture. In addition to that a microorganism related to a Gram-positive nitrate-reducing thermophile was identified; the most promising candidate for (per)chlorate reduction. Given the fact that sulfur in the medium was only added

as sulfide it is likely that similar reactions as described for *A. fulgidus* occur, but mediated by an interspecies "sulfur loop" involving several microorganisms (Fig. 2C). To get more insight in the exact dimension of this interspecies "sulfur loop" and its participants, future experiments are required.

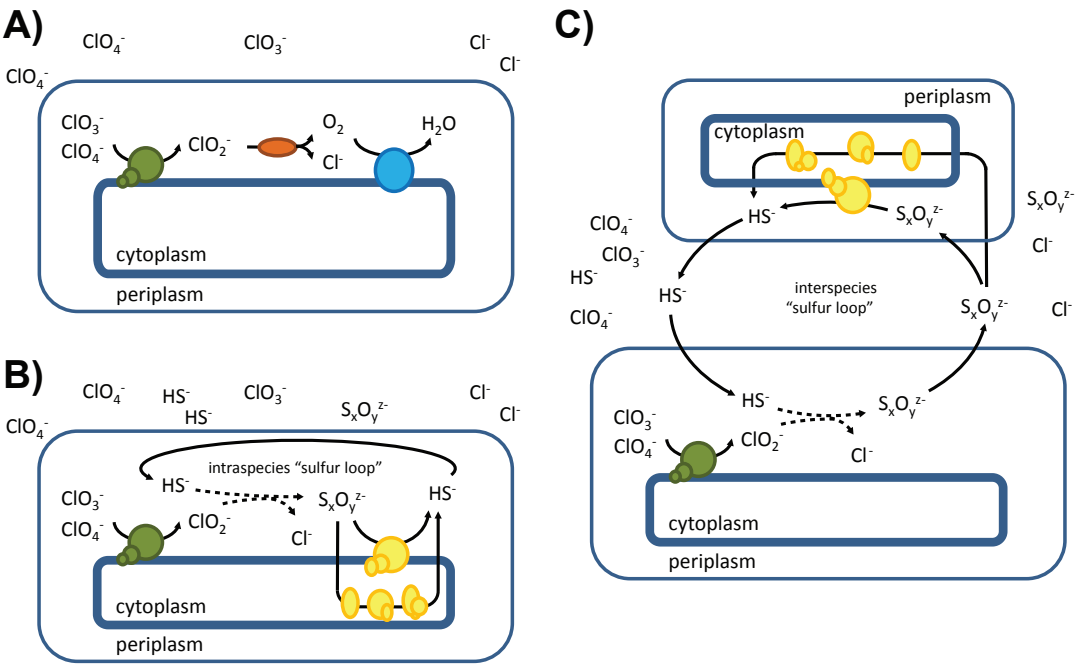

Figure 2: Schematic overview of microbial (per)chlorate reduction. **A)** "classical" (per)chlorate reduction involving a functional chlorite dismutase (Cld) (mainly found in mesophilic Proteobacteria), **B)** (per)chlorate reduction in the absence of Cld, employing an intraspecies "sulfur loop" (as found in *Archaeoglobus fulgidus*), **C)** syntrophic (per)chlorate reduction, involving interspecies sulfur transfer (as Chapter 5 indicates). (Per)chlorate-reducing enzymes are shown in green, chlorite dismutase and terminal oxidase in red and blue respectively. Enzymes involved in the reduction of oxidized sulfur compounds (e.g. S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, etc.) are shown in yellow. (This figure was also used in a recently submitted manuscript).

7

(Per)chlorate reduction and ancient life

Estimates of how long ago natural perchlorate formation and deposition on Earth began range up to many millions of years (29). This has given rise to speculations on the early evolution of enzymes adapted to (per)chlorate (5). The broad range of stereochemically distinct substrates (nitrate, chlorate and possibly perchlorate) (21) used by some enzymes within the DMSO enzyme family could have been such an

early adaptation.
The growth-coupled reduction of (per)chlorate by ancient microorganisms linked to sulfur metabolism, like it was found in *A. fulgidus*, represents a "living on the edge" situation where highly oxidative intermediates are formed, causing notable redox stress response in the metabolism of strict anaerobes (Chapter 3). This might have occurred already a long time ago in sulfur-/sulfate-utilising microorganisms under the highly reduced atmosphere of early Earth (Fig. 2B) or in consortia of microorganisms that metabolized (per)chlorate syntrophically (as indicated in Chapter 5) (Fig. 2C). Such reactions may have influenced the development of enzymes and life forms adapted to an oxidized atmosphere.

(Per)chlorate in the oil business

There is also industrial interest for studying microorganisms that perform (per) chlorate reduction at elevated temperatures. As extensively covered in Chapter 2 there is a broad diversity of microorganisms indigenous to oil reservoirs, of which some are associated with unwanted activities for petroleum recovery (like sulfate-reducing microorganisms causing reservoir souring).
Searching metagenomic datasets for the existence of enzymes involved in the microbial reduction of (per)chlorate in oil reservoirs resulted in the identification of sequences related to characterized DMSO enzymes with known chlorate-reducing capability, like Nar, pNar and Tor (Fig. 1) (17, 21, 23). This may indicate the presence of enzymes that are able to reduce chlorate (and probably perchlorate) to chlorite. Most of the respective "oil reservoir sequences" are incomplete. Nevertheless, some of the sequences carry motifs, indicative for a periplasmic location of the protein, using PRED-TAT (26). Although neither classical perchlorate and chlorate reductases (Fig. 1), nor chlorite dismutases were identified (Fig. 1 in Chapter 2) it is likely that (per)chlorate reduction *sensu lato* occurs in subsurface environments (containing sulfide or other reduced compounds that can scavenge chlorite) upon the introduction of (per)chlorate.

Souring control

During the secondary recovery phase of petroleum production water injection is performed to increase the pressure and oil sweep efficiency in the reservoir. Injected water introduces high concentrations of sulfate (contained in sea water) into offshore oil fields. Sulfate-reducing prokaryotes (SRP) thriving in oil fields, such as *A. fulgidus*, reduce sulfate to hydrogen sulfide coupled to organic carbon oxidation causing reservoir souring. Sulfide formation is associated with corrosion, metal precipitations (plugging) and high toxicities (30). These effects are commonly

mitigated by the addition of nitrate during water injection in oil reservoirs, acting on several levels: the competitive exclusion of SRP by more efficient nitrate-reducing bacteria competing for same electron donors (31); the enzymatic inhibition of the dissimilatory sulfite reductase by nitrite (an intermediate during the reduction of nitrate) (32); and the oxidation of formed sulfide by nitrate-reducing bacteria (33).
However, in both low and hot temperature oil reservoirs nitrate often proves to have insufficient impact on the sulfate-reducing community. For both scenarios this is associated with the zonation of different functional groups of microorganisms (SRP, NRP, etc.) throughout the reservoir (34) (Chapter 2).
In order to find improved strategies for souring mitigation the potential of (per) chlorate was evaluated in this study. Although mesophilic (per)chlorate reducers may be present in or introduced to oil reservoirs (Chapter 2) their viability throughout (mostly) hot oil fields is doubtful.
Archaeoglobus fulgidus on the other hand is a microorganism widely found in hot subsurface environments and it is regarded as one of the major contributors to reservoir souring in hot oil fields (35, 36). Observations that the archaeon had difficulties to resume with the reduction of sulfate after the exposure to chlorate (Chapter 3), were particularly interesting in the context of souring mitigation. In follow-up experiments (Chapter 4), it was shown that *A. fulgidus* had prolonged lag phases on sulfate after long-term exposure to perchlorate. This was only overcome after several subsequent transfers of the cultures to sulfate as sole electron acceptor. The inability to readapt quickly to sulfate is a desired property from an operational view in souring control since the dosing of (per)chlorate to the oil reservoir may not be required continuously, and thus will save costs.
The molecular mechanisms of this inhibitory effect are not identified yet, but may be associated with increased redox stress caused during (per)chlorate reduction in *A. fulgidus* (Chapter 3) and possibly with an inhibiting interaction of perchlorate (a chemical analogue of molybdate) with ATP sulfurylase.
The formation of nitrite by *A. fulgidus* in unreduced medium pointed to another valuable property of the microorganism for souring mitigation, the accumulation of nitrite by nitrate reduction (Chapter 4). The incomplete reduction of nitrate was not coupled to growth and occurred only in perchlorate pre-grown cultures, whenever sulfide was left out from anaerobic medium (causing a raise in redox potential). Nitrite is an efficient inhibitor of the sulfite reductase of SRP and may be particularly effective against thermophiles, due to the absence of nitrite reductase (37). In field operations it might be feasible to trigger the *in-situ* formation of nitrite by alternating dosing of (per)chlorate and nitrate for souring control. In contrast to the direct dosage of nitrite, this *in-situ* formation would become effective deep in the reservoir and directly affect SRP (in case of *A. fulgidus*) or SRP close-by. Additionally the biologically in-field release of nitrite would, contrary to the massive dosing of nitrite,

not have detrimental consequences in terms of corrosion.
Due to the apparent low abundance (or absence) of hyperthermophilic nitrate reducers [based on meta-analyses of metagenome datasets (Chapter 2) and indirectly confirmed by the inexistence of respective isolates], nitrate may be less effective for souring control in hot oil reservoirs than (per)chlorate. It seems as if the use of (per)chlorate has complementary effects to the use of nitrate, which suggests the additional benefit of its application for souring control in (hot) oil reservoirs.

Microbial enhanced oil recovery (MEOR)

The ability of *A. fulgidus* to couple (per)chlorate reduction to butyrate oxidation (Chapter 3), one of the volatile fatty acids that is commonly found in oil reservoirs (besides hydrocarbons) (38, 39), may result in growth without the additional dosing of further substrates [besides (per)chlorate] to oil reservoirs. This is from an economical point a valuable factor for the feasibility of a new application. Acetate which is the most abundant volatile fatty acid in oil reservoir could also be coupled to high temperature perchlorate reduction (Chapter 5).
The recently demonstrated anaerobic oxidation of long-chain alkanes and alkenes by *A. fulgidus* coupled to sulfate reduction (40, 41) may also be linked to (per)chlorate reduction and in case that occurs in oil fields, drain substrates that are normally used for sulfide generation. This would not only result in lowered sulfide formation (and thus reduced reservoir souring), but also stimulate growth based on easily available substrates; a beneficial effect for higher sweeping efficiencies during water flooding (through conformance control based on *in-situ* biomass formation). The use of (per) chlorate would consequently result in microbial enhanced oil recovery (MEOR) in addition to souring mitigation.

Concluding remarks and outlook

This thesis gained insight in microbial (per)chlorate reduction at high temperatures and expanded this trait to hyperthermophilic microorganisms in the phylum Crenarchaeota and Euryarcheaota (Fig. 3).
Ample evidence was collected for a metabolism where the complete reduction of (per) chlorate is tightly bound to (a)biotic redox reactions involving sulfur compounds. In this sense the described physiology of (hyper)thermophilic (per)chlorate reducers differs notably from the classical metabolism known from mesophilic bacteria. Considering the number of enzymes reducing chlorate (and possibly perchlorate) it seems apparent that this trait is present in a yet underestimated diversity of microorganisms, particularly in reduced environments (and in presence of sulfur). This could enable axenic and syntrophic cultures to grow by the reduction of (per)

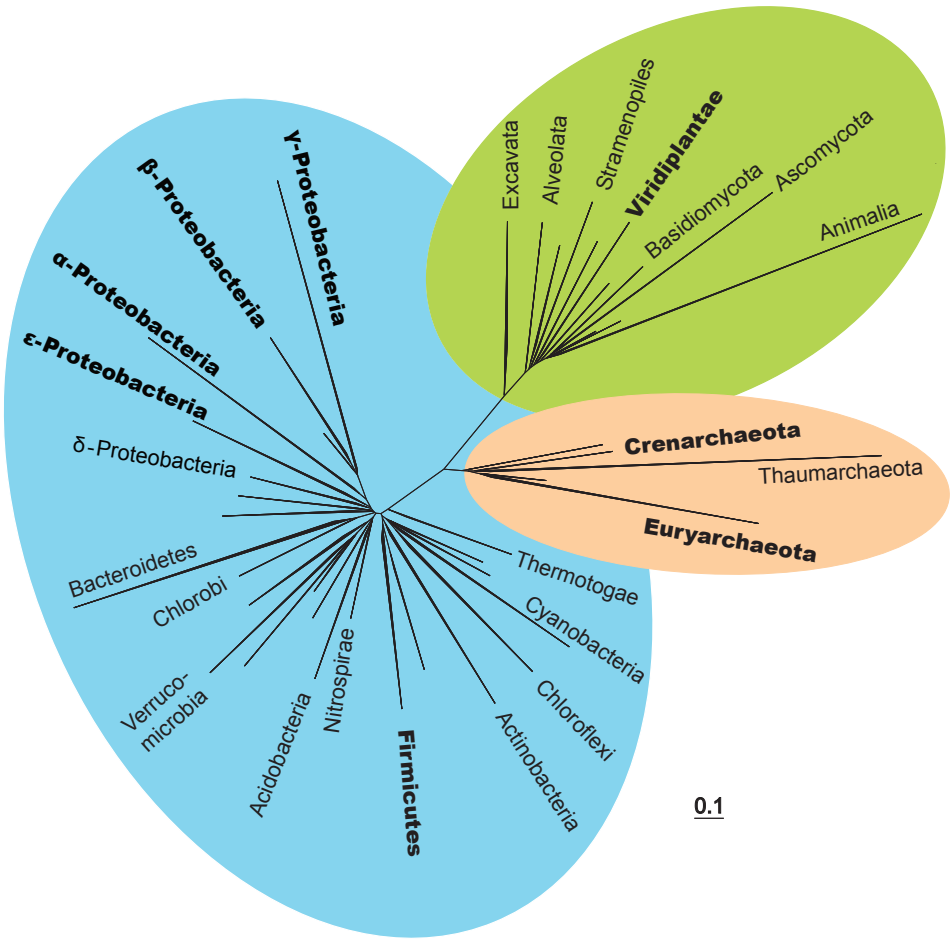

Figure 3: The tree of life, displaying the three different domains: Bacteria (blue), Archaea (orange) and Eukarya (green). Phyla containing (per)chlorate-reducing members are written in bold. The current study expanded the knowledge on microbial (per)chlorate reduction to hyperthermophilic members of the Euryarcheaota and to the Crenarchaeota.

chlorate in the absence of a chlorite-disproportionating enzyme. It is tempting to speculate on the utilization of naturally formed perchlorate by ancient anaerobes on early Earth and how that influenced the evolution of redox- and oxygen stress response mechanisms. Particularly interesting would also be to shed further light on the functions of uncharacterized molybdopterin oxidoreductases of hyperthermophiles, which harbor a rich potential of unknown biotransformations, such as (per)chlorate reduction.

The obtained results show that it is worthy to further assess the use of (per)chlorate for industrial applications, such as souring control in oil reservoirs. It was demonstrated that the sulfate reduction capabilities of *Archaeoglobus fulgidus* were only slowly resuming after exposure to perchlorate. These results are of great industrial interest and it will be the objective of applied research efforts in future to confirm these findings in continuous systems (preferably core-flooding experiments with changing electron acceptor conditions) to simulate more closely in-field situations and to develop a sustainable process.

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Appendices

Summary

Samenvatting

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List of publications

Overview of completed training activities

Summary

The microbial reduction of chlorate and perchlorate has been known for long as a respiratory process of mesophilic bacteria that thrive in diverse environments such as soils, marine and freshwater sediments. Chlorate and perchlorate are found in nature deriving from anthropogenic and natural sources and can, in the absence of oxygen, be reduced by respective microorganisms to chloride coupled to energy conservation and growth. These classical chlorate- and perchlorate-reducing microorganisms employ enzymes that reduce perchlorate (or chlorate) to the intermediate chlorite, followed by the disproportionation of chlorite to chloride and dioxygen. The latter has been regarded as key reaction for complete (per)chlorate reduction, catalyzed by the enzyme chlorite dismutase, which forms oxygen under anaerobic conditions. This *de novo* produced oxygen is reduced by terminal oxidases in the metabolism of facultative anaerobic (per) chlorate-reducing microorganisms and can be used by oxygenases for the activation of recalcitrant substrates, as was shown earlier for hydrocarbons. The potentially stimulating effect of chlorate and perchlorate on microorganisms indigenous to petroleum reservoirs was discussed, seeking new strategies for microbial enhanced oil recovery (based on subsurface growth stimulation and partial hydrocarbon degradation) and reservoir souring control (by inhibiting sulfate-reducing prokaryotes and diminishing sulfide formation).

This thesis reports the capability of hyperthermophilic and thermophilic prokaryotes that originate from subsurface environments to grow by the reduction of chlorate and/or perchlorate. In contrast to the classical metabolism of mesophilic (per)chlorate-reducing microorganisms this study demonstrated that a chlorite-disproportionating enzyme is commonly absent in (hyper)thermophilic (per)chlorate reducers. The absence of this enzyme that was previously defined as prerequisite for (per)chlorate reduction is overcome by the chemical reactivity of reduced sulfur compounds with chlorite generated. In the here more closely investigated hyperthermophilic archaea (*Archaeoglobus fulgidus* and *Aeropyrum pernix*) and thermophilic Firmicutes (*Carboxydothermus hydrogenoformans* and *Moorella glycerini* strain NMP) chlorite is formed by the activity of molybdopterin oxidoreductases. The respective enzymes are remotely related to perchlorate reductases of mesophilic bacteria and nitrate reductases of the bacterial Nar-type. In contrast to classical bacterial Nar-type enzymes, above-mentioned enzymes seem to have their catalytic subunits outside of the cell. As a consequence the reduction of (per)chlorate forms chlorite extracellularly where it reacts with reduced sulfur species present in the medium/environment (e.g. sulfide), forming chloride anions and oxidized sulfur species ($S_xO_y^{z-}$).

The hyperthermophilic archaeon *Archaeoglobus fulgidus* reduces these chemically formed sulfur species concomitantly to (per)chlorate reduction, which regenerates

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sulfide for the continuous reduction of (per)chlorate. This interaction of biotic and abiotic reactions during (per)chlorate reduction establishes an intraspecies "sulfur loop" that enables complete reduction of perchlorate to chloride.
Whereas *A. pernix* also relies on the chemical reactivity of chlorite with thiosulfate, this archaeon does not employ systems for regenerating the reducing agents biologically; which is reflected by the accumulation of sulfate during perchlorate reduction. The Crenarchaeon *A. pernix*, formerly known as a strictly aerobic microorganism, expands the trait of microbial (per)chlorate reduction up to 100°C.
In addition to the intraspecies "sulfur loop" of *A. fulgidus*, there were indications that the reduction of perchlorate may also proceed syntrophically, as indicated by a thermophilic bacterial consortium. In the respective culture, it seems that one microorganism reduces perchlorate, forming chlorite, which is chemically reduced by sulfide to chloride anions and oxidized sulfur compounds. Another group of microorganisms uses the respective sulfur compounds as electron acceptors and regenerates sulfide. Sulfur (of different redox states) depicts the mediating agent in this interspecies "sulfur loop", but may possibly be substituted in nature by other compounds such as ferrous/ferric iron.
Here presented (per)chlorate reduction *sensu lato*, which lacks the action of a chlorite-disproportionating enzyme may be widely spread among prokaryotes. For example enzymes closely resembling the designated (per)chlorate-reducing enzyme in *Archaeoglobus fulgidus* are also found in other strictly anaerobic thermophiles, of which *C. hydrogenoformans* and *M. glycerini* NMP were already confirmed to grow by the reduction of (per)chlorate as well.
The substrate ambiguity of particular periplasmic DMSO enzymes may enable a broader group of microorganisms of (per)chlorate reduction *sensu lato*, in case sulfide is present in the environment. A broadened substrate spectrum of respective enzymes (beyond their canonical function) may possibly have had evolutionary advantages. Chlorine oxyanions are naturally formed and have been introduced on Earth for ages already. The reduction of (per)chlorate and formation of chlorite in ancient anaerobic microorganisms may even have contributed to the evolution of proteins adapted to oxidizing conditions on early Earth and preceded the evolution of oxygenic photosynthesis.
It is shown that subsurface-inhabiting (hyper)thermophiles are able to grow by the reduction of (per)chlorate, which is also of interest for applications in the field of oil recovery. The finding that (per)chlorate reduction is interfering with the sulfur metabolism of a major contributor to reservoir souring in hot oil fields, *A. fulgidus*, draws promising scenarios for future attempts in developing novel souring control strategies.
(Per)chlorate reduction by *A. fulgidus* was also coupled to the oxidation of butyrate, a volatile fatty acid commonly present in petroleum reservoirs. For sustainable

applications in the oil recovery business, it is desirable to rely, as little as possible, on external substrates. In this respect the fact that *A. fulgidus* couples (per)chlorate reduction to the oxidation of butyrate is advantageous. Possibly the microorganism can also degrade long-chain alkanes and alkenes coupled to (per)chlorate reduction, a feature that was shown earlier coupled to sulfate reduction.
All together a shift of *A. fulgidus* from sulfate reduction to (per)chlorate reduction in oil fields would not only diminish souring, but maintain/stimulate *in-situ* growth of the microorganism (based on intrinsic carbon sources) which has additionally advantageous effects for improved sweeping efficiencies during water flooding.

Samenvatting

De microbiële reductie van chloraat en perchloraat is sedert lange tijd bekend als een respiratoir proces uitgevoerd door mesofiele bacteriën die gedijen in diverse ecosystemen, zoals de aardbodem en zee- en zoetwatersedimenten. Het in de natuur aanwezige chloraat en perchloraat, afkomstig van zowel antropogene als natuurlijke bronnen, kan door de betreffende mirco-organismen in afwezigheid van zuurstof worden gereduceerd tot chloride ten behoeve van energie productie en groei. Deze klassieke chlorate- en perchloraat-reducerende micro-organismen gebruiken enzymen die perchloraat (of chloraat) omzetten in het intermediair chloriet, dat vervolgens middels disproportionering leidt tot de vorming van chloride en moleculair zuurstof. De laatstgenoemde wordt beschouwd als de belangrijkste reactie voor volledige (per)chloraat reductie die wordt gekatalyseerd door het enzym chlorietdismutase dat daarmee zuurstof vormt onder anaërobe omstandigheden. Dit *de novo* geproduceerde zuurstof wordt gereduceerd door terminale oxidases in het metabolisme van facultatief anaërobe (per)chloraat-reducerende micro-organismen en kan middels oxygenases worden gebruikt voor de activering van recalcitrante substraten, zoals eerder werd aangetoond voor koolwaterstoffen. Het potentieel stimulerend effect van chloraat en perchloraat op de van nature in petroleum reservoirs aanwezige micro-organismen wordt besproken in relatie tot het ontwikkelen van nieuwe strategieën voor het verhogen van de olie productie (gebaseerd op het stimuleren van groei van deze micro-organismen in de ondergrond en de daarvoor benodigde gedeeltelijke afbraak van koolwaterstoffen) en het voorkomen van de verzuring van olie reservoirs (door het remmen van sulfaat-reducerende prokaryoten en daarmee verminderde sulfide vorming).
Dit proefschrift beschrijft het vermogen van thermofiele en hyperthermofiele prokaryoten, die afkomstig zijn uit de ondergrond, om te groeien door middel van de reductie van chloraat en/of perchloraat. In tegenstelling tot het klassieke metabolisme van mesofiele (per)chloraat-reducerende micro-organismen toonde deze studie aan dat een chloriet disproportionerend enzym gewoonlijk afwezig is in (hyper)thermofiele (per)chloraat-reduceerders. De afwezigheid van dit enzym, dat eerder werd gezien als een strikte voorwaarde voor (per)chloraat reductie, wordt gecompenseerd door de chemische reactiviteit van gereduceerde zwavelverbindingen met het geproduceerde chloriet. In de in deze studie nader onderzochte hyperthermofiele archaea *(Archaeoglobus fulgidus en Aeropyrum pernix)* en thermofiele Firmicutes (*Carboxydothermus hydrogenoformans* en *Moorella glycerini* stam NMP) wordt chloriet gevormd door de activiteit van molybdopterin oxidoreductases. Deze enzymen zijn ver verwant aan perchloraat reductases van mesofiele bacteriën en nitraat reductases van het bacteriële Nar-type. In tegenstelling tot de klassieke bacteriële Nar-verwante enzymen, lijken de bovengenoemde enzymen hun

katalytische subeenheden buiten de cel te hebben. Als gevolg van de reductie van (per)chloraat wordt chloriet daardoor extracellulair gevormd, waarna het reageert met gereduceerde zwavelverbindingen aanwezig in het medium of natuurlijk milieu (bijvoorbeeld sulfide). Dit resulteert in de vorming van chloride-anionen en geoxideerde zwavelverbindingen ($S_xO_y^{z-}$).
De hyperthermofiele archaeon *Archaeoglobus fulgidus* reduceert deze chemisch gevormde geoxideerde zwavelverbindingen en regenereert daarmee sulfide nodig voor de reductie van chloriet en drijft daarmee de chloraat reductie aan. Deze interactie van biotische en abiotische reacties tijdens (per)chloraat reductie creëert een intraspecies "zwavel cyclus" dat de volledige reductie van perchloraat tot chloride mogelijk maakt.
Alhoewel de (per)chloraat reductie in *A. pernix* ook van de chemische reactiviteit van chloriet met in dit geval thiosulfaat afhankelijk is, heeft deze archaeon geen systemen voor het regenereren van thiosulfaat, wat daarom leidt tot de accumulatie van sulfaat tijdens perchloraat reductie. Met de acidofiele crenarchaeon *A. pernix*, voorheen bekend als een strikt aeroob micro-organisme, breidt de eigenschap van microbiële (per)chloraat reductie zich uit tot 100°C.
Naast de intraspecies "zwavel cyclus" van *A. fulgidus*, blijkt uit een thermofiel bacterieel consortium dat de reductie van perchloraat ook syntroof kan verlopen. In de desbetreffende cultuur lijkt één groep micro-organismen perchloraat te reduceren en daarbij chloriet te produceren, dat vervolgens gereduceerd wordt tot chloride waarbij tevens geoxideerde zwavelverbindingen worden gevormd. Deze zwavelverbindingen worden op hun beurt weer gereduceerd tot sulfide door een andere groep micro-organismen. Zwavel (in verschillende redox staten) fungeert als de intermediaire verbinding in deze interspecies "zwavel cyclus", maar kan eventueel in de natuur door andere stoffen worden vervangen, zoals ferro/ferri-ijzerionen.
De hier voorgestelde (per)chloraat reductie *sensu lato*, dat de werking van een chloriet disproportionerend enzym mist, is mogelijk zeer veel voorkomend onder prokaryoten. Enzymen die gelijkenis vertonen met het (per)chloraat-reducerende enzym van *Archaeoglobus fulgidus* zijn ook in andere strikt anaërobe thermofielen gevonden, waarvan voor *C. hydrogenoformans* en *M. glycerini* NMP reeds werd bevestigd dat die kunnen groeien door reductie van (per)chloraat.
Door de brede substraat specificiteit van periplasmatische DMSO enzymen is waarschijnlijk een grotere groep van micro-organismen in staat tot (per)chloraat reductie *sensu lato*, mits er sulfide aanwezig is. Een breed substraat spectrum van deze enzymen (buiten hun canonieke functie) kan mogelijk evolutionaire voordelen hebben gehad. Chloor-bevattende anionen worden al reeds miljoenen jaren natuurlijk gevormd en daarmee geïntroduceerd op Aarde. Tijdens de ontwikkeling van leven op Aarde heeft de reductie van (per)chloraat en vorming van chloriet in oude anaerobe microorganismen mogelijk bijgedragen tot de evolutie van aan oxiderende

omstandigheden aangepaste eiwitten die daarbij voorafging aan de ontwikkeling van oxygene fotosynthese.
De bevinding dat (hyper)thermofiele micro-organismen uit de ondergrond kunnen groeien door reductie van (per)chloraat is ook van belang voor de ontwikkeling van toepassingen op het gebied van aardoliewinning. Het feit dat (per)chloraat reductie interfereert met het zwavel metabolisme van *A. fulgidus* (waarvan bewezen is dat deze bijdragen aan de verzuring van olie reservoirs) biedt kansen voor het ontwikkelen van strategieën om deze verzuring tegen te gaan.
Daarnaast is het voor een succesvolle microbieel verhoogde olie productie strategie noodzakelijk om zo weinig mogelijk afhankelijk te zijn van substraten die geinjecteerd dienen te worden. In dat verband is het zeer gunstig dat (per)chloraat reductie door *A. fulgidus* tevens aan de oxidatie van butyraat gekoppeld kan zijn, aangezien butyraat een vluchtig vetzuur is dat veel voorkomt in aardolie reservoirs. Naast butyraat kunnen mogelijk ook alkanen en alkenen dienst doen als substraten voor (per)chloraat reductie, een functie die eerder werd aangetoond voor sulfaat.
Samenvattend zou de verschuiving van *A. fulgidus* van sulfaat- naar (per)chloraat-reductie in aardolie reservoirs dus niet alleen kunnen leiden tot een vermindering van de verzuring van de desbetreffende reservoirs, maar zou tevens middels groei op basis van intrinsieke koolstof bronnen kunnen leiden tot een verhoogde aardolie productie.

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About the author

Martin (Gerald) Liebensteiner was born on 22nd of February, 1981 in Linz, Austria. He started the study of Biology at the University of Innsbruck (Austria) in 2000 and obtained the degree of Master of Science in Microbiology and Zoology (in 2006 and 2007 respectively) by completing his studies with an interdisciplinary diploma thesis investigating the interactions between earthworms and microorganisms. In October 2008 he joined the group of Prof. Dr. Fons Stams at the Laboratory of Microbiology (Wageningen University) with a Leonardo da Vinci scholarship of the European Union. For half a year Martin investigated methanogenic and sulfidogenic communities of sea sediments under the supervision of Dr. Caroline Plugge. Right after that, in 2009, Martin was appointed as a PhD student in the same work group. From then on he investigated microbial (per)chlorate reduction and particularly the occurence of this metabolism in thermophilic microorganisms. This PhD study was carried out under the supervision of Dr. Bart Lomans and Prof. Dr. Fons Stams.

List of publications

Martin G. Liebensteiner, Nicolas Tsesmetzis, Alfons J.M. Stams, Bart P. Lomans (2014) Microbial redox processes in deep subsurface environments and the potential application of (per)chlorate in oil reservoirs. Frontiers in Microbiology 5:428.

Martin G. Liebensteiner, Alfons J.M. Stams, Bart P. Lomans (2014) (Per)chlorate reduction at high temperature: Physiological study of *Archaeoglobus fulgidus* and potential implications for novel souring mitigation strategies, International Biodeterioration and Biodegradation. *in press*.

Martin G. Liebensteiner, Martijn W.H. Pinkse, Peter J. Schaap, Alfons J.M. Stams, Bart P. Lomans (2013) Archaeal (per)chlorate reduction at high temperature; an interplay of biotic and abiotic reactions. Science 340:85-87.

Martin G. Liebensteiner, Siavash Atashgahi, Hauke Smidt, Alfons J.M. Stams, Detmer Sipkema. How microorganisms drive the global chlorine cycle. *in revision.*

Martin G. Liebensteiner, Irene Sanchéz-Andrea, Alfons J.M. Stams, Bart P. Lomans. Microbial perchlorate reduction by a thermophilic consortium with acetate as carbon and energy source. *in preparation.*

Martin G. Liebensteiner, Martijn W.H. Pinkse, Bart Nijsse, Peter D.E.M. Verhaert, Alfons J.M. Stams, Bart P. Lomans. Extreme temperatures and (per)chlorate reduction: Consistent traits and differences to the “classical” mesophilic metabolism. *in preparation.*

Sidnei Cerqueira dos Santos, Martin G. Liebensteiner, Leila C. Silva das Virgens de Souza, Catia L. Santos Ramos, Luiz L. Franco Batista, Josilene Borges Torres Lima Matos, Cristina M. Quintella, Paulo F. de Almeida (2014) Crude glycerol as substrate for sulfate-reducing bacteria from a mature oil field and its potential impact on souring. Journal of Petroleum Science and Technology. *accepted.*

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- Principles of Ecological Genomics (2011)

Other PhD and Advanced MSc Courses

- Project and Time Management (2011)
- Advanced Environmental Biotechnology (2011)
- Biorefinery for Biomolecules (2012)

Management and Didactic Skills Training

- Teaching at MSc courses Research Methods Microbiology and Microbial Physiology (2009-2013)
- Supervising BSc thesis 'Gram-positive (per)chlorate-reducing bacteria' (2010)

Oral Presentations

- *Insight in the physiology of Moorella perchloratireducens, a gram-positive perchlorate reducing bacterium*. PhD trip (Peking University), 18 April 2011, Beijing, China
- *Biological (per)chlorate reduction – a novel concept for stimulating oil reservoir microbiota*. Reservoir Microbiology Forum (RMF), 22-23 November 2011, London, United Kingdom
- *Biological (per)chlorate reduction, a tool for stimulating oil reservoir microbiota*. SENSE Symposium 'Microbes for Sustainability', 4-5 April 2012, Wageningen, The Netherlands
- *Potential of (per)chlorate for souring mitigation?!* Reservoir Microbiology Forum (RMF), 27-28 November 2012 London, United Kingdom
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