

Microbial respiration with chlorine oxyanions: diversity and physiological and biochemical properties of chlorate- and perchlorate-reducing microorganisms

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Chlorine oxyanions are valuable electron acceptors for microorganisms. Recent findings have shed light on the natural formation of chlorine oxyanions in the environment. These suggest a permanent introduction of respective compounds on Earth, long before their anthropogenic manufacture. Microorganisms that are able to grow by the reduction of chlorate and perchlorate are affiliated with phylogenetically diverse lineages, spanning from the Proteobacteria to the Firmicutes and archaeal microorganisms. Microbial reduction of chlorine oxyanions can be found in diverse environments and different environmental conditions (temperature, salinities, pH). It commonly involves the enzymes perchlorate reductase (Pcr) or chlorate reductase (Clr) and chlorite dismutase (Cld). Horizontal gene transfer seems to play an important role for the acquisition of functional genes. Novel and efficient Clds were isolated from microorganisms incapable of growing on chlorine oxyanions. Archaea seem to use a periplasmic Nar-type reductase (pNar) for perchlorate reduction and lack a functional Cld. Chlorite is possibly eliminated by alternative (abiotic) reactions. This was already demonstrated for *Archaeoglobus fulgidus*, which uses reduced sulfur compounds to detoxify chlorite. A broad biochemical diversity of the trait, its environmental dispersal, and the occurrence of relevant enzymes in diverse lineages may indicate early adaptations of life toward chlorine oxyanions on Earth.

Keywords: perchlorate; chlorate; abiotic chlorite elimination; respiration

Introduction

At a time when no oxygen was present on Earth yet, microbes were able to derive energy from anaerobic respiration processes. The microbial reduction of metals and elemental sulfur is considered to stand at the evolutionary beginning of life around 3.5 billion years ago.¹ A long time after, 2.4–2.1 billion years ago, the great oxygenation event on Earth took place.² The evolution of oxygenic photosynthetic microorganisms resulted in the release of large amounts of free dioxygen into the atmosphere.

Anaerobic respiratory processes like CO₂ reduction to methane, sulfur and sulfate reduction, and the reduction of iron(III) and nitrate have been

studied well over the past decades,^{3–6} and their roles in the geochemical cycling of elements have been widely elucidated. However, the diversity of inorganic electron acceptors utilized by microorganisms for energy conservation is much broader, comprising also a range of metals, metalloids, radionuclides,^{7–9} and halogens, such as bromate,¹⁰ iodate,¹¹ and the chlorine oxyanions chlorate and perchlorate.¹²

In this review, we give an overview of the latest discoveries on the microbial reduction of the chlorine oxyanions. Novel findings on the genetics of mesophilic perchlorate- and chlorate-reducing bacteria^{13–17} and recently identified alternative pathways for the reduction of chlorine oxyanions in bacteria and archaea^{18–20} are discussed. In light of growing evidence for natural

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deposition of chlorine oxyanions on Earth²¹ and the large diversity of perchlorate- and chlorate-reducing microorganisms,^{18,22–27} it seems that the geochemical importance of this trait has been underestimated.

The origin and occurrence of chlorine oxyanions

Human-made chlorate has been extensively used as herbicide and also 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, such as chlorite, chlorate, and chloride. The production of sodium chlorate in 2000 was 2.1 million tons in North America and around 3 million tons worldwide.²⁸ However, the chlorine oxyanion most widely dispersed in nature is perchlorate. Both anthropogenic and natural sources of perchlorate are found in the environment.²⁹ Perchlorate has been manufactured for more than 100 years, mainly for its use as an explosive and as rocket propellant in the form of ammonium perchlorate.³⁰ Especially after World War II, an increased demand for perchlorate raised its production to an estimated 18 million kg per year in the United States alone.²⁸ The current production of perchlorate is difficult to determine, since perchlorate is a classified strategic compound in the United States.³⁰ Former disposal practices in the aerospace, military, and chemical industries are the main cause for perchlorate found in groundwater and surface waters. It was reported that 15.9 million kg of perchlorate has been released into the environment since the 1950s,³¹ causing a threat to the environment and human health due to the toxicity of the compound. This raised interests in the biological remediation of perchlorate-polluted sites and drove scientific research on bacteria that reduce chlorine oxyanions. The use of microorganisms for the treatment of contaminated wastewater³² and groundwater³³ was studied. Microbial reduction of chlorine oxyanions was also proposed for the *in situ* bioremediation of soils.³⁴

Research, particularly during the past decade, has led to insight into the natural formation of perchlorate. In contrast to anthropogenic pollution, natural formation and deposition of perchlorate involve much lower concentrations of perchlorate that is

not locally concentrated.²¹ The most significant and best-known natural accumulation of perchlorate on Earth is found in the Atacama Desert in Chile, where it is codeposited with nitrate.³⁵ Perchlorate in the Atacama Desert and depositions found on Mars are both of atmospheric origin. Atmospheric formation and introduction on Earth was also proposed for chlorate. Chlorate was detected in caliches and soils, groundwater, and precipitation samples.³⁶ Several mechanisms have been proposed for the natural formation of perchlorate, such as electrochemical discharge reactions,³⁷ the oxidation of chloride by ozone,³⁸ and photochemically mediated processes in the atmosphere.³⁹ While the most significant mechanism is not yet known, there is consensus about its permanent deposition on Earth, most probably from a stratospheric source. Perchlorate accumulates only in arid environments (e.g., the Atacama Desert, Antarctic dry valleys), which is likely attributable to the inactivity of microorganisms in the absence of water.²¹ Elsewhere on Earth, perchlorate is thought to be biologically reduced to chloride. 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.^{21,40} Taking an average deposition rate of perchlorate on Earth of 3.6 g/km²/year,²¹ around 1.8 million kg of perchlorate is deposited on Earth every year. This rough estimate of natural perchlorate deposition even exceeds the reported anthropogenic release of perchlorate.³¹ 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 enzymes that reduce perchlorate and chlorate.

Microorganisms that reduce chlorine oxyanions

In the early 20th century, the first scientific observations of microbial reduction of chlorate were reported,⁴¹ but it took another 50 years before the first axenic perchlorate-reducing bacterium was isolated and described.³² Research on *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 chlorate and perchlorate reduction.^{42–46}

Almost 100 strains of chlorate- and perchlorate-reducing microorganisms have been obtained over the past 40 years, although the number of publicly deposited organisms is lower. The vast majority of microorganisms that reduce chlorine oxyanions are facultative anaerobes affiliated with the Proteobacteria, predominantly belonging to the class of β -Proteobacteria.⁴⁷ Besides Gram-negative bacteria, members of the Firmicutes and the Euryarchaeota were also reported to grow by the reduction of chlorate and perchlorate.^{22,23,26,27,48} (Table 1). The latter have a perchlorate-reducing metabolism that seems to differ notably from the one found in Proteobacteria.^{18,20,26}

Acetate is a common substrate for chlorate- and perchlorate-reducing bacteria. Other organic electron donors include alcohols,^{22,23} organic acids,^{44,46} aromatic compounds,^{43,49} and aliphatic hydrocarbons.⁵⁰ Inorganic compounds like hydrogen, ferrous iron, zero-valent iron,⁵¹ sulfide, thiosulfate, and elemental sulfur⁵² can also serve as electron donors for the reduction of chlorine oxyanions. Many chlorate- and perchlorate-reducing bacteria have the ability to utilize nitrate or oxygen as electron acceptors besides chlorine oxyanions. Most described chlorate- and perchlorate-degrading microorganisms are mesophiles that grow under neutrophilic low-salinity conditions.⁵³ However, recently several halophilic perchlorate-reducing microorganisms were also described, belonging to the Proteobacteria and the archaeal taxon Halobacteriaceae.^{24,25,27,54} Besides mesophiles, thermophilic strains of Clostridia^{22,48,55} and two hyperthermophilic *Archaeoglobus fulgidus* strains²⁶ were also reported to grow by the reduction of chlorate and perchlorate (Table 1).

Perchlorate- and chlorate-reducing microorganisms have been isolated from a diverse range of environments, comprising pristine and hydrocarbon-polluted soils, aquatic sediments, paper mill waste sludge, farm animal waste lagoons,^{46,53,56,57} sea water and saline lake water samples,⁵⁸ salt ponds and solar salterns,²⁷ marine sediments,^{24,25} activated sludge,^{42,44,52,59} digester sludge,⁴⁵ submarine hot springs,^{26,60} and an underground gas storage site.^{22,23} This shows that the ability to respire using chlorine oxyanions is widespread in nature.

Enzymatic destruction of chlorine oxyanions

The chlorine oxyanions perchlorate (ClO_4^-) and chlorate (ClO_3^-) contain chlorine in an oxidized form (+VII; +V). Sodium perchlorate and sodium chlorate are highly soluble in water; perchlorate is chemically more stable than chlorate and chlorite. The high redox potentials of redox couples involved in the microbial reduction of chlorine oxyanions ($E^0 = \text{ClO}_4^-/\text{ClO}_3^- + 788 \text{ mV}$; $\text{ClO}_3^-/\text{ClO}_2^- + 709 \text{ mV}$; $\text{ClO}_2^-/\text{Cl}^- + 1199 \text{ mV}$) make perchlorate and chlorate ideal electron acceptors for microorganisms, comparable to those of oxygen ($E^0 = \text{O}_2/\text{H}_2\text{O} + 820 \text{ mV}$) or nitrate respiration ($E^0 = \text{NO}_3^-/\text{NO}_2^- + 430 \text{ mV}$; $\text{NO}_2^-/\text{NO} + 350 \text{ mV}$; $2\text{NO}/\text{N}_2\text{O} + 1175 \text{ mV}$; $\text{N}_2\text{O}/\text{N}_2 + 1355 \text{ mV}$; $\text{NO}_2^-/\text{NH}_4^+ + 440 \text{ mV}$).

The metabolism of microbial perchlorate and chlorate reduction in mesophilic bacteria is based on the action of perchlorate reductase (Pcr) or chlorate reductase (Clr), followed by chlorite dismutase. Oxygen formed under anaerobic conditions by Cld is subsequently reduced by a terminal oxidase⁶¹ (Fig. 1). Clr is genetically and structurally different from Pcr.^{13,62,63} Perchlorate reductases and chlorate reductases are more closely related to other enzymes in the DMSO II enzyme family than to each other (Fig. 2). Oxygen formed by chlorite dismutase can also allow the *de facto* aerobic degradation of aromatic and aliphatic compounds under anaerobic conditions, reaching growth rates comparable to the oxidation of respective hydrocarbons with oxygen.^{49,50}

Perchlorate reduction

There is growing evidence that mesophilic perchlorate respiration genes are horizontally transferred. The mesophilic perchlorate-reducing bacteria *Dechloromonas aromatica*, *D. agitata*, *Azospira suillum*, and *Magnetospirillum bellicus* contain genes encoding perchlorate reductase (*pcrABCD*) and chlorite dismutase (*cld*) that were clustered and located on genomic islands on the chromosomes.¹⁶ In *D. aromatica* and *A. suillum*, the genomic islands are very similar and *cld* is located upstream of *pcrD* followed by two genes for cytochromes, a quinol dehydrogenase tetraheme *c*-type and a diheme cytochrome *c*-type protein,

Table 1. Selected isolated strains of chlorate- and perchlorate-reducing microorganisms and their phylogenetic affiliations^a

Strain	Class	e-acceptors	Optimal temperature	Salinity (NaCl)	Optimal pH	Source	Ref.
<i>Azospirillum lipoferum</i> strain VPI Sp 59b	α-Proteobacteria	ClO ₄ ⁻	Mesophilic	0%	–	Grass roots	88, 89
<i>Dechlorospirillum anomalous</i> strain JB116	α-Proteobacteria	ClO ₄ ⁻ , NO ₃ ⁻	Mesophilic	0–0.5%	Neutral, slightly alkaline	Sewage treatment plant	90
<i>Ideonella dechloratans</i> strain CCUG 30898 ^T	β-Proteobacteria	ClO ₃ ⁻ , O ₂ , NO ₃ ⁻	Mesophilic	0%	–	Municipal wastewater treatment plant	42
<i>Azospira oryzae</i> strain GR-1	β-Proteobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , O ₂ , NO ₃ ⁻ , Mn ⁴⁺	Mesophilic	–	Neutral	Activated sludge	44
<i>Azospira</i> sp. strain Percl ace	β-Proteobacteria	ClO ₄ ⁻ , NO ₃ ⁻	Mesophilic	0%	Neutral	Groundwater	91
<i>Dechloromonas agitata</i> strain CKB ^T	β-Proteobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , O ₂	Mesophilic	Opt.: 1%	Neutral	Mix of soil, sediment, and waste sludge	53, 56
<i>Dechloromonas</i> sp. strain JM	β-Proteobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	–	–	–	Activated sludge	92
<i>Dechloromonas aromatica</i> strain RCB	β-Proteobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Mesophilic	–	–	Sediment	43
<i>Dechloromonas aromatica</i> strain JJ	β-Proteobacteria	NO ₃ ⁻ , O ₂	Mesophilic	–	–	Sediment	43
<i>Alicyclophilus denitrificans</i> strain BC	β-Proteobacteria	ClO ₃ ⁻ , O ₂ , NO ₃ ⁻	Mesophilic	–	Neutral	Enrichment culture	49
<i>Dechloromonas hortensis</i> strain MA-1 ^T	β-Proteobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Mesophilic	–	Neutral	Pristine soil	57
<i>Wolinella succinogenes</i> strain HAP-1	ε-Proteobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻	Mesophilic	–	Neutral	Municipal anaerobic reactor	45
<i>Arcobacter</i> sp. strain CAB	ε-Proteobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Mesophilic	Opt.: 3%	Neutral	Marina	25
<i>Sulfurospirillum multivorans</i>	ε-Proteobacteria	ClO ₄ ⁻ , S ₂ O ₃ ²⁻ , S _n ²⁻ , SeO ₄ ²⁻ , AsO ₄ ³⁻ , NO ₃ ⁻ , fumarate, AQDS, PCE, TCE, TMAO, DMSO	Mesophilic	Grown at 0.1%	Neutral	Activated sludge	18, 59, 93
<i>Acinetobacter thermotolerantus</i>	γ-Proteobacteria	ClO ₃ ⁻ , SO ₄ ²⁻	Mesophilic (tolerates up to 47 °C)	–	–	Sewage	94

(Continued)

Table 1. *Continued*

Strain	Class	e-acceptors	Optimal temperature	Salinity (NaCl)	Optimal pH	Source	Ref.
<i>Pseudomonas chloridismutans</i> strain ASK1	γ -Proteobacteria	ClO_3^- , O_2	Mesophilic	–	Neutral	Anaerobic wastewater treatment reactor	57
<i>Shewanella algae</i> strain ACDC	γ -Proteobacteria	ClO_3^- , NO_3^- , SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, O_2 , S^0 , ferric oxyhydroxide, Fe(III)-citrate, fumarate, AQDS, DMSO, TMAO	Mesophilic	Opt.: 2%	Neutral	Marine sediment	13
<i>Sedimenticola selenatireducens</i> strain CUZ	γ -Proteobacteria	ClO_4^- , ClO_3^- , NO_3^- , NO_2^- , O_2	Mesophilic	Opt.: 4%	Neutral	Marina	24
<i>Sedimenticola selenatireducens</i> strain NSS	γ -Proteobacteria	O_2 , ClO_3^- , NO_3^-	Mesophilic	Opt.: 1.5–2.5%	Slightly alkaline	Marine sediment	24
<i>Marinobacter vinifirmus</i> strain P4B1	γ -Proteobacteria	ClO_4^- , NO_3^-	Mesophilic	–	–	–	54
<i>Pseudomonas chloridismutans</i> strain AW1 ^T	γ -Proteobacteria	ClO_3^- , O_2	Mesophilic	Up to 4%	Neutral–basic	Anaerobic chlorate-reducing reactor	95
<i>Vibrio dechloraticans</i> strain Cuznesove B-1168	γ -Proteobacteria	ClO_4^- , ClO_3^- , CO_2 , NO_3	–	–	–	–	32
<i>Sporomusa ovata</i> strain An4	Clostridia	ClO_4^- , ClO_3^- , NO_3^- , CO_2	Mesophilic	–	Neutral	Underground gas storage reservoir	23
<i>Moorella perchloratireducens</i> strain An10	Clostridia	ClO_4^- , ClO_3^- , NO_3^- , $\text{S}_2\text{O}_3^{2-}$, neutralized Fe(III) complexes, AQDS	Thermophilic	Opt.: 1%	Neutral	Underground gas storage reservoir	22
<i>Moorella glycerini</i> strain JW/AS-Y6 ^T	Clostridia	ClO_4^- , $\text{S}_2\text{O}_3^{2-}$	Thermophilic	Range: 0–2%	Slightly acidic	Hot spring	22,96
<i>Moorella mulderi</i> strain TMS	Clostridia	ClO_4^- , $\text{S}_2\text{O}_3^{2-}$	Thermophilic	Opt.: 1%	Neutral	Anaerobic sludge, thermophilic bioreactor	22,97
<i>Moorella stamsii</i> strain E3-O ^T	Clostridia	ClO_4^- , NO_3^- , AQDS	Thermophilic	Grown at 0.03%	Neutral	Anaerobic sludge, solid waste digester	48

(Continued)

Table 1. *Continued*

Strain	Class	e-acceptors	Optimal temperature	Salinity (NaCl)	Optimal pH	Source	Ref.
<i>Moorella humiferrea</i> strain 64-FGQ ^T	Clostridia	ClO ₄ ⁻ , NO ₃ ⁻ , S ₂ O ₃ ²⁻ , humic acid, AQDS	Thermophilic	Opt.: 0%	Neutral	Hydrothermal spring	55
<i>Archaeoglobus fulgidus</i> strain VC-16	Archaeoglobi	ClO ₄ ⁻ , ClO ₃ ⁻ , SO ₄ ²⁻ , S ₂ O ₃ ²⁻ , SO ₃ ²⁻	Hyperthermophilic	–	Neutral	Submarine hot spring	26,60
<i>Haloférx mediterranei</i> strain R-4	Halobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Mesophilic	Range: 7.6–27%	–	Salt pond	27,98
<i>Haloférx denitrificans</i> strain S1	Halobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , NO ₂ ⁻ , O ₂	Mesophilic	Range: 8.8–26.2%	Neutral	Saltern	27,99
<i>Haloférx gibbonsii</i> strain Ma 2.38	Halobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , O ₂	Mesophilic	–	–	Solar saltern	27,100
<i>Haloférx volcanii</i> strain DS2	Halobacteria	ClO ₃ ⁻ , O ₂	Mesophilic	Range: 9.9–14.6%	–	Dead Sea sediment	27,101
<i>Haloarcula vallismortis</i>	Halobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Mesophilic	Opt.: 25%	Neutral	Salt pools	27,102
<i>Haloarcula marismortui</i>	Halobacteria	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Facultative thermophilic	Opt.: 19.8–22.8%	–	Dead Sea	27,103

^aPhysiological and environmental data were added in cases where these were reported.

and *moaA*, encoding an enzyme involved in molybdenum cofactor biosynthesis. Downstream of *pcrA* are genes for a response regulator, a histidine kinase, a PAS-domain sensor peptide, a σ -factor/anti- σ -factor system, and oxidoreductase components. *D. agitata* has a smaller genomic island in which *clD* is located on the opposite side (downstream of *pcrA*), which is followed by genes for a σ -factor/anti- σ -factor system. Like upstream *clD* in *D. aromatica* and *A. suillum*, upstream of *pcrD* in *D. agitata* are genes coding for a quinol dehydrogenase tetraheme *c*-type and diheme *c*-type cytochrome plus *moaA*. In *M. bellicus*, *moaA* is located on the opposite side of *pcrABCD* and downstream of *pcrA*. Upstream of *pcrD*, there is a gene for a quinol dehydrogenase tetraheme *c*-type cytochrome, followed by *clD*, genes for oxidoreductase components, second copies of three *pcr* genes in the order *pcrDBA*, and genes for a response regulator and a histidine kinase. Interestingly, the genomic islands of *D. aromatica*, *D. agitata*, *A. suillum*, and *M. bellicus* have not been found in closely related bacteria (*Dechloromonas* sp. strain JJ and *Magnetospirillum magnetotacticum*).¹⁶ Recently, the genomic island of the newly isolated strain

Sedimenticola selenatireducens CUZ was described, and it contains *pcrABCD* with a gene for quinol dehydrogenase tetraheme *c*-type cytochrome, *clD*, and a transposase upstream of *pcrD*. Downstream of *pcrA* are a gene for cytochrome *b*, a transposase and a response regulator (*pcrR*), a histidine kinase (*pcrS*), and a PAS-domain-containing protein (*pcrP*).²⁴ It would be interesting to find out if and how other genes that are also located on the genomic islands are involved in perchlorate respiration.

Perchlorate reductases are periplasmic heterodimers of PcrA and PcrB ($\alpha\beta$ and $\alpha_3\beta_3$), carrying a Mo-bis (pyranopterin guanine dinucleotide) cofactor and iron-sulfur clusters. Electrons are transferred from a membrane-associated, proton-pumping *c* cytochrome of the NapC/NrfH family to PcrA. PcrC, a soluble multiheme *c* cytochrome, was predicted to participate in electron transport reactions.⁶² Its important role was experimentally confirmed with gene-deletion experiments in *Azospira suillum* PS.¹⁵ However, PcrC is not indispensable for the electron transport chain during perchlorate reduction, as suggested by the absence of the respective gene (*pcrC*) in the

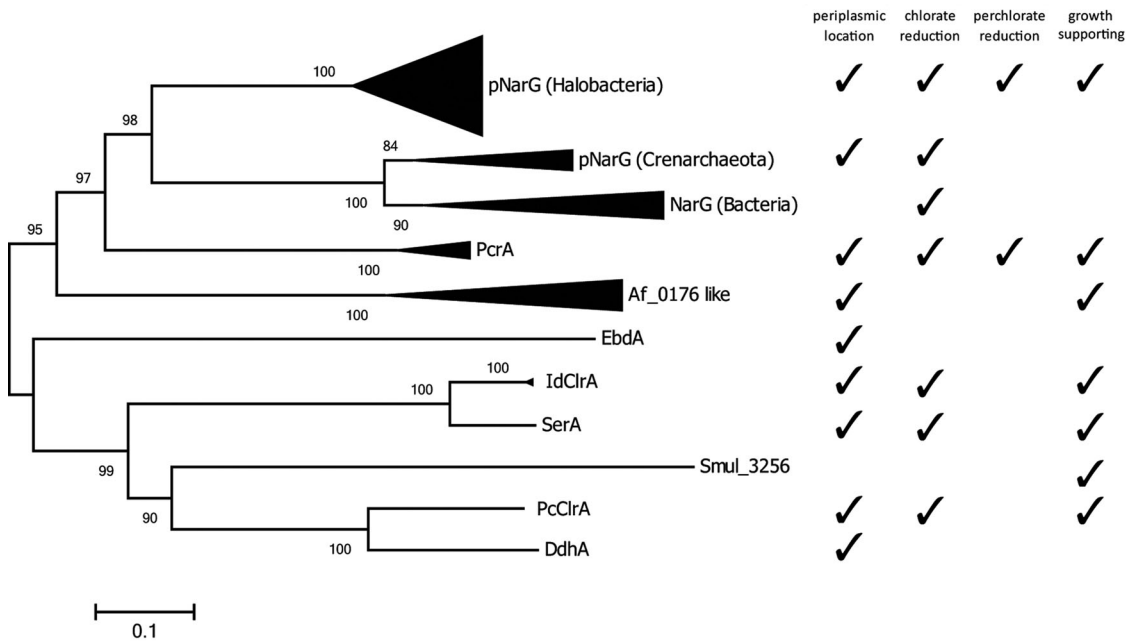


Figure 2. Phylogenetic tree of catalytic subunits of selected DMSO II enzymes (neighbor-joining method). Evolutionary distances of the tree were computed using the Poisson correction method; the scale bar indicates amino acid substitutions per site. Bootstrap values (500 repetitions) are shown. Biochemical characteristics of respective enzyme (groups) are indicated (whenever a certain activity has been demonstrated for at least one representative of the group). The column on the very right shows whether host organisms possessing respective enzymes grow on chlorine oxyanions. We show catalytic subunits of putative and confirmed nitrate reductase (pNarG and NarG), perchlorate reductase (PcrA), ethylbenzene hydroxylase (EbdA), selenate reductase (SerA), and dimethylsulfide dehydrogenase (DdhA). Catalytic subunits of chlorate reductase (ClrA) cluster separately, *I. dechloratans*-like ClrA (IdClrA) cluster with SerA and *P. chloritidismutans*-like ClrA (PcClrA) with DdhA. Af_0176 is the catalytic subunit of the putative perchlorate-reducing enzyme in *Archaeoglobus fulgidus* and Smul_3256 the catalytic subunit of the best enzyme candidate for perchlorate reduction in *Sulfurospirillum multivorans*. For respective enzymes, the subcellular location was predicted using the software PRED-TAT.¹⁰⁴ The phylogenetic tree was constructed with MEGA version 6 (Ref. 105) using sequences listed in the Methods section at the end of manuscript.

D. chlorophilus are very similar and are located on highly similar plasmids.¹³ The genomic islands contain *clD* as well as *clrABDC*. Here *clD* is also located downstream of *clrA* and there is a cytochrome *c553*-encoding gene located between *clD* and *clrA*. Upstream of *clD* is a partial *napC* encoding the γ subunit of a periplasmic nitrate reductase and the insert ISPpu12a/b. This insert contains *merR* regulator-, heavy metal efflux protein-, a lipoprotein signal peptide-, and transposase-encoding open reading frames. Another copy of the insert, ISPpu12b, flanks the other side of the genomic island and is located upstream of *clrC*. Between *clrC* and ISPpu12b, there is an internal insert on the genomic island, ISSal1, that contains three (hypothetical) open reading frames. There are genes for an ATPase and a glycosyl transferase

between *clrC* and ISSal1, and a hypothetical gene and a gene for a methyl-accepting chemotaxis family protein between ISSal1 and ISPpu12b.

Chlorate reductases are heterotrimers ($\alpha\beta\gamma$) located in the periplasm. Similar to Pcr chlorate reductases, they contain a Mo-bis(pyranopterin guanine dinucleotide) cofactor and iron-sulfur clusters.¹² It was proposed that *c*-type cytochromes transfer electrons from a membrane-bound proton-pumping bc_1 complex to Clr.⁶¹ The role of cytochrome *c*-Id1 from *I. dechloratans* as an electron donor for chlorate reduction was demonstrated later.⁶⁶ In perchlorate-reducing bacteria, a consistent set of genes coding for membrane-associated quinol dehydrogenase tetraheme *c*-type cytochromes was reported, and in chlorate reducers other genes were reported coding for

soluble *c*-type cytochromes.^{13,16} This indicates the difference between the two respiratory metabolic pathways on the level of electron transport.⁶⁴

Chlorite disproportionation

The disproportionation of chlorite by heme b oxidoreductases (incorrectly but consistently referred to as “chlorite dismutase”) in the absence of oxygen is one of the few enzymatic reactions (besides photosynthesis and a proposed alternative nitrite reduction pathway⁶⁷) where a covalent O–O bond is formed. Chlorite dismutase activity was demonstrated for perchlorate- and chlorate-reducing bacteria.^{68–70} The genetics and evolution of chlorite dismutase genes, the biochemical/physical properties of chlorite dismutase, and potential biotechnological applications have been reviewed recently.^{12,71–73}

Functional chlorite dismutases can be distinguished into two lineages; lineage I, which contains Cld from microorganisms capable of reduction and growth based on the chlorine oxyanions perchlorate and chlorate; and lineage II, which was defined by the discovery of a Cld in *Nitrobacter winogradskyi*.⁷⁴ While lineage I is characterized by periplasmic enzymes of penta- or hexameric structure, lineage II Cld exhibits a dimeric structure and is predicted to be located inside the cell.⁷⁵ Host microorganisms of lineage II Cld are commonly not capable of growth using the reduction of chlorine oxyanions. Novel lineage II enzymes with efficient chlorite dismutase activity have been characterized from the Cyanobacterium *Cyanotheca* sp.,⁷⁵ the pathogen *Klebsiella pneumoniae*,⁷⁶ and *N. winogradskyi*.⁷⁴

The *in vivo* role of lineage II Cld is not entirely understood. However, authors speculate about the detoxifying role of respective enzymes for endogenously formed chlorite by nitrate reductases upon chlorate exposure.^{74–76} Besides functional Cld, an enormous diversity of Cld-like genes is found throughout the tree of life, comprising bacteria, archaea, and eukaryotic species.^{73,74,77,78} Recent studies demonstrated that some Cld-like gene products carry functions during heme biosynthesis in Gram-positive bacteria (consequently renamed HemQ).^{79–81} Future work is required to identify the exact mechanisms and involvement of HemQ in heme biosynthesis. Horizontal gene transfer seems to be important for the distribution of *cld* and *cld*-like genes.⁷⁸

Alternative pathways for the reduction of chlorine oxyanions

Some microorganisms that lack a functional Cld seem to apply alternative strategies to complete the reduction of chlorine oxyanions.^{18,26,27} As was shown for *A. fulgidus*, the abiotic reactivity of chlorine intermediates with sulfur compounds may play an important role in this aspect and enable continuous and complete reduction of chlorine oxyanions without the involvement of Cld.¹⁹ The initial step of perchlorate reduction in *A. fulgidus* (from perchlorate to chlorite) is likely performed by a periplasmic enzyme that resembles nitrate reductases of the Nar type, a so-called pNar enzyme (Af0174-0176; catalytic subunit: Af0176) (Fig. 2). Related enzymes are predicted based on the genomes of other anaerobic bacteria (e.g., *Carboxydotherrmus ferrireducens*, *C. hydrogenoformans*, *Moorella thermoacetica*, and *M. glycerini*) and archaea (e.g., *Ferroglobus placidus*), of which some have a very similar autecology to *A. fulgidus*.

The ϵ -proteobacterium *Sulfurospirillum multivorans*, an organohalide-respiring microorganism,⁵⁹ was recently described to grow by the reduction of perchlorate. In the genome of *S. multivorans*, only genes remotely related to perchlorate reductases were found.¹⁸ The best candidate enzyme for perchlorate reduction (catalytic subunit: Smul3256) exhibits 29% identity with the PcrA of *D. aromatica* (90% coverage) and 35% identity with ClrA of *I. dechloratans* (92% coverage) (Fig. 2). No genes for chlorite dismutase are encoded in the genome of *S. multivorans*. How the bacterium achieves complete perchlorate reduction without the action of Cld has yet to be investigated, but a similar abiotic detoxification mechanism to the one observed in *A. fulgidus*¹⁹ is possible. Also, *S. multivorans* is routinely grown under anaerobic conditions and in the presence of reducing sulfur compounds. These are chemically very reactive with chlorite and form sulfur compounds of higher redox states. *S. multivorans*, which has the demonstrated ability to use a set of sulfur compounds as electron acceptors,^{18,59} may even be able to use such oxidized sulfur compounds as electron acceptors.

Also, perchlorate-reducing bacteria belonging to the Clostridia are commonly grown in media with reduced sulfur compounds. Although *M. perchloratireducens* was shown to possess Cld activity,²² some

perchlorate-reducing members of the Clostridia lack both a gene encoding Cld and measurable Cld activity, which suggests the existence of alternative mechanisms for chlorite elimination (unpublished results). *Moorella* spp. often have the ability to use sulfur compounds as electron acceptors (Table 1). Theoretically, perchlorate-reducing members of the Clostridia and *S. multivorans* may rely on an abiotic/biotic sulfur-based loop similar to the one found in *A. fulgidus*.²⁶

Members of the Halobacteriaceae also exhibit perchlorate-reducing abilities.²⁷ These halophilic archaea have a periplasmic Nar-type reductase that catalyzes the reduction of perchlorate and chlorate²⁰ (Fig. 2). However, the fate of the generated chlorite is not clear. Although Halobacteriaceae carry a gene that distantly resembles known Clds, no such activity could be determined in cell or cell-free extracts of *Haloferax mediterranei* grown on chlorine oxyanions.²⁰ Additionally, the Cld-like genes in Halobacteriaceae do not share conserved key residues for functional Cld.⁷⁴

Compounds other than reduced sulfur chemicals may possibly serve as equally effective reducing agents (e.g., Fe(II), AHDS, and humic acids) for the abiotic reduction of chlorite and allow for complete perchlorate and chlorate reduction without the involvement of Cld (Fig. 1C). An interesting reaction in this respect is the chlorite–iodide reaction, which follows the stoichiometry $\text{ClO}_2^- + 4 \text{H}^+ + 4 \text{I}^- \rightarrow 2 \text{I}_2 + 2 \text{H}_2\text{O} + \text{Cl}^-$ in aqueous solution. This oxyhalogen–halide reaction is well known for its oscillatory nature.⁸² Knowledge about the biological relevance of this chemical reaction is limited. Iodide (I^-) and iodate (IO_3^-) are the most abundant chemical species of the element iodine in nature. While iodide concentrations in sea water are in the nM to μM range, they are significantly higher in natural gas brines, where they reach up to mM concentrations.⁸³ In such environments, a microbially induced reduction of chlorine oxyanions and the formation of extracellular chlorite would rapidly reduce iodide to iodine and not necessarily rely on a functional Cld. Marine algae such as *Fucus ceranoides* and algae of the Laminariales are known to accumulate iodide.^{84–86} In the Laminariales, the accumulated iodide leads to an apoplastic antioxidant reservoir that can scavenge a variety of

reactive oxygen species, such as peroxide, gaseous ozone, hydroxyl radicals, and superoxide.⁸⁵ Iodide-accumulating bacteria have also been described in marine environments,⁸⁷ but the physiological role of bacterial iodide accumulation is not yet understood.

Until now, only sulfur compounds have been demonstrated to play a role in the reduction of chlorine oxyanions and microbial growth based thereupon. Whether other compounds, and their reactivity with chlorite, are also able to compensate for the lack of Cld needs to be studied further.

Conclusions

From a microbial perspective, given the natural and continuous formation and deposition of chlorine oxyanions on Earth, chlorine oxyanions have always represented an attractive option as electron acceptors, but also a challenging one, because of the intermediate formation of the highly oxidative and toxic chlorite.

Functional genes for microbial chlorate and perchlorate reduction are predominantly found in members of the Proteobacteria, and these genes can be acquired laterally by plasmids or transposons and genomic islands. Highly efficient microbial reduction of chlorine oxyanions is classically associated with genes encoding chlorate- or perchlorate-reducing enzymes and the presence of a chlorite dismutase. However, some perchlorate-reducing microorganisms, particularly in very phylogenetically distant members of the Archaea, seem to lack a functionally efficient Cld, and do not exhibit Cld activity. In at least one case, enzymatically formed chlorite is scavenged by reduced sulfur compounds, which enables energy conservation and continuous reduction of chlorine oxyanions.²⁶ Such an abiotic–biotic interplay may also exist in other chlorate- and perchlorate-reducing microorganisms and could possibly be mediated by compounds other than sulfur (e.g., Fe(II) and iodide).

Enzymes that efficiently reduce chlorate and perchlorate in addition to their canonical substrates (e.g., pNar of the Halobacteria or *A. fulgidus*)²⁰ further expand the biochemical versatility of the trait to respire with chlorine oxyanions^{26,27} (Fig. 2).

The most recent findings on alternative pathways for reduction of chlorine oxyanions and in-depth

studies on the classical mesophilic chlorate and perchlorate reduction pathways^{13,16,17} have expanded the phylogenetic and ecological distribution of the trait and necessitate further research.

Methods

The phylogenetic tree in Figure 2 was constructed with MEGA version 6 (Ref. 105) using the following sequences: Q8GPG4.1, P60068.1 (UniProt), EKA49434.1, AAU40964.1, AHJ14482.1, AAK76387.1, CAB53372.1, AJF27291.1, ACB69917.1, AAO49008.1, ABS59781.1, ADO63825.1 (GenBank), WP_004967656.1, WP_004056332.1, WP_008324614.1, WP_007541437.1, WP_004967656.1, WP_004041371.1, WP_008605318.1, WP_004062055.1, WP_008572697.1, WP_006054602.1, WP_008385261.1, WP_023393128.1, WP_004592418.1, WP_004960754.1, WP_011223493.1, WP_014040599.1, WP_007190097.1, WP_005533997.1, WP_008308778.1, WP_004515484.1, WP_006885135.1, WP_018258505.1, WP_015763420.1, WP_015910069.1, WP_008003395.1, WP_012795090.1, WP_005046213.1, WP_006076868.1, WP_010877688.1, WP_012964659.1, WP_028051983.1, WP_011344974.1, WP_011393408.1, WP_034600133.1, WP_014902929.1, WP_015260881.1, WP_014792875.1, WP_011288314.1, WP_014235273.1, WP_041963832.1, WP_013516315.1, WP_011009509.1, WP_011900001.1, WP_014347351.1, WP_011850581.1, WP_022541560.1, WP_010866283.1, WP_012662440.1, WP_033014854.1, WP_044748717.1, WP_024335701.1, and WP_011750465.1 (NCBI).

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