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Microbial metabolism of oxochlorates: A bioenergetic perspective ☆

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ABSTRACT

The microbial metabolism of oxochlorates is part of the biogeochemical cycle of chlorine. Organisms capable of growth using perchlorate or chlorate as respiratory electron acceptors are also interesting for applications in biotreatment of oxochlorate-containing effluents or bioremediation of contaminated areas. In this review, we discuss the reactions of oxochlorate respiration, the corresponding enzymes, and the relation to respiratory electron transport that can contribute to a proton gradient across the cell membrane. Enzymes specific for oxochlorate respiration are oxochlorate reductases and chlorite dismutase. The former belong to DMSO reductase family of molybdenum-containing enzymes. The heme protein chlorite dismutase, which decomposes chlorite into chloride and molecular oxygen, is only distantly related to other proteins with known functions. Pathways for electron transport may be different in perchlorate and chlorate reducers, but appear in both cases to be similar to pathways found in other respiratory systems. This article is part of a Special Issue entitled: Evolutionary aspects bioenergetic systems.

1. Introduction

Anaerobic respiration of microbes plays a significant role in the biogeochemical cycles of many of the elements [1]. Redox reactions including inorganic and organic compounds containing nitrogen and sulfur, metals such as iron or manganese, or metalloids such as arsenic and selenium [2,3] are utilized by microbes to sustain life and support growth. The microbial metabolism of oxochlorates is a fascinating variation on this theme. The use of perchlorate and chlorate as respiratory electron acceptors is a recently recognized part of the biogeochemical cycle of chlorine.

Until recently, there was no known natural occurrence or production of chlorate, and the only perchlorate of natural origin was believed to be that found in saltpeter deposits [4]. However, widespread natural occurrence of perchlorate has been demonstrated in recent work, both in ancient groundwater [5], and soils in dry regions [6]. Atmospheric processes have been suggested as origin for this natural perchlorate [7], and the contribution of atmospheric production and wet decomposition to the environmental perchlorate levels is discussed in [8]. In a recent study of perchlorate in a glacial ice core [9], an intermittent source is suggested. Chlorate has also been detected in soils, groundwater and precipitation [10]. Also in this case, atmospheric production is suggested as a natural source.

In later years anthropogenic chlorate and perchlorate have been recognized as major environmental problems. Chlorate is toxic to plants, and has been used extensively as herbicide and defoliant. Another major source of this compound is the bleach effluent from pulp mills using chlorine dioxide bleaching [11,12]. The substitution of chlorine dioxide for chlorine as bleaching agent in pulp processing gave rise to considerable adverse effects on the population of aquatic plants (in particular brown algae) in the recipient [11,13], necessitating the development of water treatment processes for chlorate removal. Biological treatment of the effluent under anaerobic conditions has been found effective [14], and is presently used in different configurations in pulp mills. The future development and improvement of such processes can be expected to benefit from a deeper understanding of the pathways for the microbial chlorate decomposition.

Ammonium perchlorate is manufactured in large amounts for use as oxidizer in solid rocket propellant [15]. Perchlorate contamination of groundwater as the result of perchlorate manufacture and handling of ammunitions has been recognized as a major environmental issue [16]. Perchlorate interferes with iodine uptake in the thyroid gland by binding to the sodium-iodide symporter [17], and an interim health advisory level for perchlorate in drinking water of 15 μg/L has been set in the United States [16]. Another problem is the perchlorate present in crops grown using irrigation with perchlorate-contaminated water [18]. These issues have raised the interest in bioremediation, or biotreatment of perchlorate-contaminated water with the aid of naturally occurring perchlorate-reducing bacteria [17,19]. Also in this case, there is need for understanding the underlying biology of perchlorate metabolism for the development of applications.

The ability of bacteria to use chlorate as metabolic electron acceptor has been known for a long time [20], and a role of soil microorganisms

Abbreviations: DMSO, dimethyl sulfoxide; $E_{m,7}$, midpoint potential at pH 7

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in the degradation of chlorate in the environment was suggested already in the 1920'ies [21]. The presence of microorganisms capable of converting chlorate to chloride in sewage was noted in [22], and used for the estimation of the biological oxygen demand. It was initially thought that the observed biological reduction of chlorate and perchlorate was due to side reactions of the nitrate-reducing enzyme systems of denitrifying bacteria [23–25]. Although such reactions are known, the product in this case is chlorite [26], not chloride, and the reduction of chlorate by denitrifiers does not sustain growth. In contrast, the conversion of chlorate to chloride observed in studies of mixed cultures is coupled to growth and the formation of biomass [27–29], suggesting that the metabolism of oxochlorates can serve as a source of energy. Growth yields obtained with acetate as carbon source and electron donor were found comparable with the yields obtained with oxygen as electron acceptor under aerobic conditions [27]. This suggests that growth with chlorate is sustained by ATP production driven by a respiratory chain operating with chlorate as the terminal acceptor.

2. Oxochlorate-reducing bacteria: Reaction pathway and enzymes

Enrichment cultures capable of growth using chlorate or perchlorate as the electron acceptor have been starting points for the isolation and characterization of a considerable number of chlorate-reducing species. The novel species *Ideonella dechloratans* [30] was isolated from chlorate enrichment cultures and characterized. In the phenotypic characterization reported, this species is described as having a strictly respiratory type of metabolism with oxygen, chlorate or nitrate being able to serve as electron acceptors, and as cytochrome *c* oxidase positive. The ability to use nitrate as electron acceptor was reported susceptible to loss after subcultivations on chlorate. Similar results were obtained in [31] where the transformation of chlorate to chloride by different environmental and enrichment cultures is reported. The same group eventually isolated another species capable of growth on chlorate or perchlorate, initially referred to as strain GR-1 [32]. In a later characterization of strain GR-1 [33], it was identified as *Dechlorosoma suillum*, however with the notion that the latter has been suggested [34] as heterotopic synonym of *Azospira oryzae*. The conversion of perchorate to chloride was found quantitative and the reaction pathway shown in Scheme 1 was suggested for the reduction of perchlorate.

The notion of chlorite as an intermediate in the pathway is supported by the observation of the ability of the cells to generate molecular oxygen from chlorite.

Enzymes capable of catalyzing all steps in the above reaction were first isolated from *A. oryzae* GR-1. The first two reactions are catalyzed by a single enzyme labeled (per)chlorate reductase [35] since it was found capable of using perchlorate as well as chlorate as electron acceptor. This enzyme was found to be composed of two different subunits, and to contain molybdenum and iron-sulfur clusters as cofactors. The N-terminal amino acid sequence of the β subunit was found to be very similar to the sequence of a previously described [36] selenate reductase from *Thauera selenatis*, suggesting a relation with the DMSO reductase family.

An enzyme catalyzing the third reaction, the decomposition of chlorite to chloride and molecular oxygen was also first isolated from *A. oryzae* GR-1 [37]. Although the reaction catalyzed is not a dismutation or disproportionation in the strict sense, this enzyme is usually referred to as chlorite dismutase. Chlorite dismutase was found to be a heme protein, which catalyzes the unusual formation of a novel oxygen-oxygen bond.

$$ClO_4^- \xrightarrow{2e^-} ClO_3^- \xrightarrow{2e^-} ClO_2^- \longrightarrow Cl^- + O_2$$

Scheme 1.

Since these early studies, the isolation and characterization of many novel species of (per)chlorate- and chlorate reducing bacteria from different environments has been reported [29,38–41]. In an analysis based on 16S rRNA sequences [38], a number of the isolates were found to form a phylogenetically diverse group, with members in the *Alpha-*, *Beta-* and *Gammaproteobacteria*. *Dechloromonas agitata* strain CKB and *Dechlorosoma suillum* strain PS were suggested as type species and strains for this group (the latter is also referred to as *Azospira oryzae* PS or *Azospira suillum* PS). Other proteobacterial oxochlorate respirers described include *Pseudomonas chloritidismutans* strains AW-1 [42] and ASK-1 [33], *Dechloromonas hortensis* [33], and *Alicycliphilus denitrificans* [43]. Over 50 species of (per)chlorate reducers have been isolated, the majority of which belong to the *Betaproteobacteria*. The capacity to use perchlorate or chlorate as electron acceptor has been observed also in *Epsilonproteobacteria* [44] and in the *Firmicutes* [45,46], strengthening the notion of phylogenetic diversity.

3. The enzymes of oxochlorate respiration

3.1. (Per)chlorate and chlorate reductases

In addition to the (per)chlorate reductase from *Azospira oryzae* GR-1 discussed above, chlorate or (per)chlorate reductases have been isolated from *P. chloritidismutans* AW-1 [47], *I. dechloratans* [48], *Pseudomonas* sp. PDA [49], *Azospira* strain KJ [49] and strain perc1ace [50]. Table 1 compares the subunit composition, cofactor content and substrate characteristics of the (per)chlorate and chlorate reductases that have been characterized at the protein level.

As seen in the table, there are two distinct groups of enzymes: (per) chlorate reductases isolated from organisms able to utilize both chlorate and perchlorate as respiratory substrates and chlorate reductases isolated from organisms that can only utilize chlorate. The (per)chlorate reductases that have been isolated contain two different subunits, whereas the chlorate reductases contain three subunits.

Sequences are available for the chlorate reductases of *I. dechloratans* and *P. chloritidismutans*. In addition, sequences of corresponding genes have been obtained from a large number of oxochlorate-reducing species, notably the (per)chlorate respirers *D. aromatica*, and *D. agitata*, and *D. suillum* PS, and the chlorate respirer *A. denitrificans* whose genomes have been sequenced. Sequences for the large (α) subunits confirm that these proteins belong to DMSO reductase superfamily of molybdenum-containing oxidoreductases with molybdo-bis(pyranopterin guanine dinucleotide) (Mo-bisPGD) as the catalytic cofactor [51,52]. Also notable is that all sequences contain an N-terminal twin arginine motif signaling export to the periplasmic compartment using the *tat* pathway [53], and the presence of the motif H-X3-C-X3-C-X34-C, which has been described as a characteristic for the N-terminus of enzymes belonging to type II of the DMSO reductase family [54]. The residues of this motif are involved in the binding of an iron-sulfur cluster. As noted in [55], an aspartate residue corresponding to the aspartate serving as Mo ligand in ethylbenzene dehydrogenase is present in the *I. dechloratans* sequence. This residue is also conserved in the other oxochlorate reductase sequences. The lysine residue interacting with the aspartate in ethylbenzene dehydrogenase is however not conserved. A lysine is found at the corresponding position in the *I. dechloratans* and *A. denitrificans* sequences whereas *P. chloritidismutans* contains serine and the (per)chlorate reductases of *D. aromatica*, and *D. agitata* and *D. suillum* PS all contain a histidine.

Sequences for the β subunits are similar to the iron-sulfur containing subunits of other enzymes of the DMSO reductase family. These proteins consist of two ferredoxin-like domains and contain four groups of conserved cysteines capable of ligating four iron-sulfur clusters [51]. As noted in [48], the second residue of the third group is replaced by a tyrosine in the chlorate reductases. In the (per)chlorate reductases, an alanine residue is found in this position. The role of the cysteines in the binding of the iron-sulfur cluster is discussed in detail in [51].

Table 1
Summary of oxochlorate reductases that have been isolated and characterized.

Organism		Oxochlorate reductase				
Species	e^- acceptors (respiration)	Subunits	co-factors reported	K_M (ClO_4^-)	K_M (ClO_3^-)	Ref
Azospira oryzae GR-1	ClO_4^-, ClO_3^-, NO_3^- Mn(IV), O_2, IO_3^-, BrO_3^-	$\alpha_3\beta_3$	Mo, FeS	27 μM	<5.0 μM	[32,35]
Strain Perc1ace	ClO_4^-, NO_3^-	$\alpha_3\beta_3$		34.5 μM		[50]
Azospira sp KJ	ClO_4^-, ClO_3^-, NO_3^-, O_2	$\alpha\beta$				[49]
Ideonella dechloratans	ClO_3^-, NO_3^-, IO_3^-, BrO_3^-, O_2	$\alpha_1\beta_1\gamma_1$	Mo, FeS, heme *b*		0.85 mM	[30,48]
Pseudomonas Sp. PDA	ClO_3^-, O_2	$\alpha\beta\gamma$				[49]
Pseudomonas chloritidismutans AW-1	ClO_3^-, O_2	$\alpha_1\beta_1\gamma_1$	Mo, FeS		0.16 mM	[47]

Sequences of the β subunits do not include any export signal and the protein is believed to be exported via the *tat* pathway as a complex with the α subunit.

The smallest (γ) subunit is found only in the chlorate reductases. From sequence analysis, the γ subunit in the *I. dechloratans* enzyme was found homologous to the heme *b*-containing subunit in selenate reductase, dimethyl sulfide dehydrogenase, ethylbenzene dehydrogenase and archaeal p-type nitrate reductases [48]. The capacity to bind heme has been demonstrated by heterologous expression and reconstitution with heme [56]. The sequences of *A. denitrificans* and *I. dechloratans* γ subunits are 99% identical, and about 30% of the residues are conserved in all three sequences available for chlorate reductases. In alignments with the heme-binding domain motif derived from the structure of ethylbenzene dehydrogenase [55] (DOMON_EDBH), methionine and lysine residues corresponding to the heme ligands identified in the latter are present in all three sequences. This unusual heme coordination, which is also observed in selenate reductase and dimethyl sulfide dehydrogenase, has been suggested as the structural basis for the high redox potential found in these proteins [57] and in ethylbenzene dehydrogenase [55]. The γ subunits contain an N-terminal leader sequence suggesting export to the periplasmic compartment via the *sec* pathway.

Catalysis in other molybdenum-containing enzymes in the DMSO reductase family is based on the ability of molybdenum to take part in oxo transfer reactions and to exist in the Mo(IV), Mo(V) and Mo(VI) oxidation states [58]. A similar role in catalysis would seem reasonable for both perchlorate and chlorate reduction. The reaction would then start with oxo transfer from the bound substrate (perchlorate or chlorate) to Mo(IV), forming the reduced product (chlorate or chlorite) and Mo(VI) with an oxo ligand. Two consecutive one-electron transfers and uptake of two protons then regenerate the initial Mo(IV) state of the enzyme, with water coordinated to Mo(IV) as the result. Dissociation of water then completes the catalytic cycle. The iron-sulfur centers in the α and β subunits in other enzymes in the DMSO reductase family have been suggested to facilitate electron transfer between active site and redox partners [59]. In selenate reductase, dimethyl sulfide dehydrogenase and ethylbenzene dehydrogenase, which contain three subunits homologous to those found in the chlorate reductases, the heme-containing γ subunits are suggested to serve as the site for interaction with external redox partners [55,57,60].

3.2. Chlorite dismutase

Chlorite dismutase enzymatic activity has been found in all chlorate- and perchlorate reducers investigated. In addition to *A. oryzae* GR-1 [37,61], enzymes from several species (*I. dechloratans* (in the native state native [62] and as recombinant in *E. coli* [63]), *P. chloritidismutans* [64], and *D. aromatica* (as recombinant in *E. coli* [65])) have been isolated and characterized kinetically and spectroscopically. All chlorite dismutases investigated are heme proteins with high catalytic activity towards the decomposition of chlorite into chloride ion and molecular oxygen. The kinetic parameters reported indicate that these enzymes operate close to the diffusion control limit [65].

The formation of a covalent oxygen-oxygen bond is an unusual reaction in biochemistry, with known counterparts only in oxygenic photosynthesis and the recently reported formation of molecular oxygen from NO in a methane-oxidizing bacterium [66]. Crystal structures have been determined for the chlorite dismutases of (per) chlorate respirers *A. oryzae* GR-1 [67] and *D. aromatica* [68]. These enzymes are both pentamers of identical, heme *b*-containing subunits (the *A. oryzae* enzyme crystallized as a hexamer but was found pentameric in solution). A catalytic mechanism has been suggested [69–71], involving the initial formation of a compound I-like intermediate and hypochlorite, followed by oxygen-oxygen bond formation by nucleophilic attack of the hypochlorite oxygen onto the ferryl oxygen. The resulting peroxyhypochlorite then decomposes into molecular oxygen and chloride. An arginine residue located close the distal face of the heme group has been suggested to stabilize the bound substrate and proposed reaction intermediates, and its role has been probed using site-directed mutagenesis [72].

Chlorite dismutases have also been found in nitrite oxidizing bacteria [73–75]. Although catalytic activities are somewhat lower than found for the enzyme in oxochlorate reducers, chlorite dismutases from the nitrite oxidizers are still quite efficient enzymes. Structures have been determined for two of these. The chlorite dismutase of *Candidatus Nitrospira defluvii* was found pentameric similar to the enzymes discussed above, and to contain an arginine residue in the heme pocket. Mutagenesis of this residue affected catalysis, suggesting a role analogous to the arginine in chlorite dismutase from the oxochlorate respirers. The chlorite dismutase of *Nitrobacter winogradskyi* was, in contrast, found to be dimeric. The structure of the heme pocket is similar to the other enzymes, with an arginine residue located at the distal side. Although the physiological functions of these proteins are not known, the possible use of oxochlorates as alternative electron acceptors in nitrite oxidizers is discussed in [73,74].

Genes encoding chlorite dismutase-like proteins are found in a large number of species, the majority of which are not oxochlorate reducers [70,73,74]. Phylogenetic relationships investigated in these papers show that the sequences of chlorate dismutases in oxochlorate reducers are highly conserved, and that there appears to be no counterpart to the phylogenetic division seen for the oxochlorate reductases.

Functions for chlorite dismutase family proteins from non-oxochlorate reducers are not known. The histidine residue serving as proximal heme ligand is strictly conserved, but other residues lining the predicted heme pocket are not, suggesting other functions than chlorite dismutase for these proteins [70]. The structure of a chlorite dismutase family protein from *Thermus thermophilus* has been determined and found to contain a cavity capable of binding heme [76]. After reconstitution with heme, very low chlorite dismutase activity was observed, supporting the notion of a different function.

From analysis of the genomic context of a number of chlorite dismutase family proteins, it has been suggested that these protein are

involved in heme metabolism, possible as carriers or chaperones [70]. These authors also found structural similarities with the DyP family of bacterial peroxidases [77], and suggested the chlorite dismutases as part of a structural superfamily with interaction with heme or iron as a common function.

4. Genomics of oxochlorate respiration

The complete genomes of *Dechloromonas aromatica*, *Dechlorosoma suillum* PS [78], and *Alicycliphilus denitrificans* [79] are available. In addition, regions containing genes for (per)chlorate or chlorate reductases and chlorite dismutases in *D. agitata* [80], *Azospira* strain KJ (unpublished; Genbank EU571095.1), *P. chloritidismutans* (unpublished; Genbank GQ919187.1) and *I. dechloratans* [48] have been characterized . As first noted in [48], the genes encoding the enzymes of (per)chlorate or chlorate metabolism tend to form a cluster. Fig. 1 shows gene clusters for (per)chlorate or chlorate metabolism in the above species.

As seen in the figure the order of the genes for chlorite dismutase and the individual subunits of chlorate or (per)chlorate reductase within the clusters is variable. In addition to the genes encoding chlorate- or (per)chlorate reductase subunits and chlorite dismutase (*clrABC* or *pcrAB* and *cld*, respectively), the clusters contain the genes *pcrD* or *clrD* which are suggested to encode a chaperone-like protein involved in the insertion of the molybdenum cofactor into the α subunit prior to export via the *tat* pathway. The *pcrC* genes found in the (per)chlorate reducers encode a tetraheme *c* cytochrome suggested to take part in electron transport [80], in a role similar to that of the homologous cytochrome c_{554} in *Nitrosomonas europea* [81]. It is however not present as a constituent in the (per)chlorate reductases that have been isolated. Other genes present in these clusters are *mobB* and *moaA*, which have been suggested to be parts of the molybdenum cofactor biosynthesis pathway, and genes encoding *c*-type cytochromes that could take part in electron transfer. The *napC*/*nrfH*-like genes of the (per)chlorate reducers are predicted to encode a membrane-bound tetraheme *c*-type cytochrome, whereas the *cytc* genes in the chlorate reducers are predicted to encode soluble *c* cytochromes.

It has been suggested recently [78] that the clusters for (per)chlorate respiration constitute the core of a genomic island that can transfer horizontally between species by different mechanisms. The analogous clustering of genes seen in the chlorate reducers is consistent with a similar behavior also in of these genes. Horizontal gene transfer would provide a rationale for the phylogenetic diversity of organisms capable of using oxochlorate respiration as energy supply.

5. Bioenergetics: oxochlorate reduction as energy supply

5.1. Oxochlorate reduction as part of a respiratory chain

To sustain ATP production and cell growth, the reduction of chlorate or perchlorate and the decomposition of chlorite need to be connected to a respiratory chain that translocates protons across the cell membrane. The reduction of perchlorate and chlorate also requires a supply of electrons at a suitable redox potential. The utilization of oxygen produced by the decomposition of chlorite requires branching of the electron flow, with one branch supplying the periplasmic oxidoreductase and the other branch supplying a membrane-bound terminal oxidase. Fig. 2 shows electron transport routes needed for the supply of electrons to the periplasmic oxochlorate reductase and a membrane-bound terminal oxidase from the quinone pool. In the case of chlorate reduction, stoichiometry requires the passage of one third of the available electrons to periplasmic chlorate reductase and two-thirds to the terminal oxidase. In perchlorate reduction, four electrons are required for each molecule of oxygen produced by chlorite dismutase, requiring half of the electron flow to periplasmic perchlorate/chlorate reductase and the other half to the terminal oxidase.

5.2. Terminal oxidase

Efficient utilization of the oxygen produced by chlorite decomposition requires a terminal oxidase with a high oxygen affinity. Such oxidases are found in microaerophiles and usually belong to the cytochrome cbb_3-type cytochrome *c* oxidase family [82] or to the cytochrome *bd*-type quinol oxidase family [83]. The former translocate protons across the membrane, whereas the latter only contribute to the electrochemical gradient by the release of protons from quinol oxidation to the periplasmic side of the membrane.

No characterization of the terminal oxidase in a chlorate/perchlorate reducing bacterium has been reported. However, *I. dechloratans* as well as many other oxochlorate respirers have been reported as cytochrome *c* oxidase positive [16,30] suggesting the presence of a cbb_3-type oxidase. To assess the presence of genes for cytochrome cbb_3 and *bd* type oxidases, we interrogated the genomes of *D. aromatica*, *D. suillum* PS,

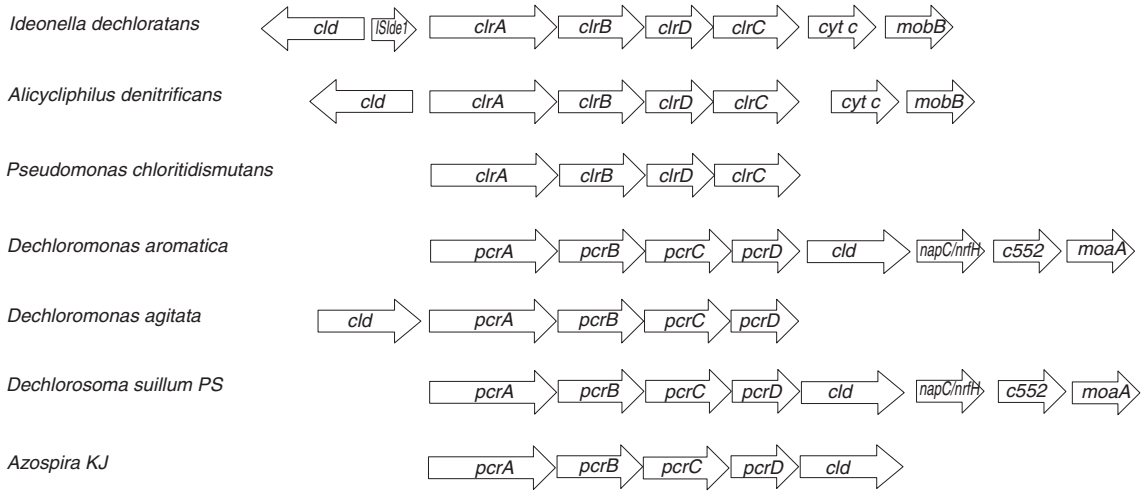

Fig. 1. The arrangement of genes encoding the enzymes of oxochlorate respiration in different organisms. *clrA*, *clrB*, *clrC*, the α, β and γ subunits of chlorate reductases; *pcrA*, *pcrB*, α and β subunits of (per)chlorate reductases; *pcrC*, tetraheme *c* cytochrome suggested to take part in electron transfer to the (per)chlorate reductases; *clrD*, *pcrD*: chaperones; *cld*, chlorite dismutase; *cytc*, soluble monoheme *c* cytochrome; *napC*/*nrfH*, membrane-bound tetraheme *c* cytochrome; *c552*, soluble diheme *c* cytochrome; *mobB*, *moaA* proteins involved in molybdenum cofactor biosynthesis; *IS*-Ide1, insertion sequence flanked by inverted repeat in *I. dechloratans*.

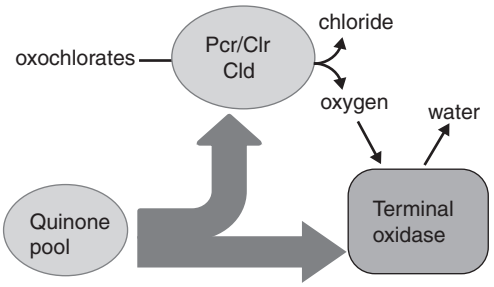

Fig. 2. Pathways needed for electron transport from the quinone pool to periplasmic oxochlorate reductase and membrane-bound terminal oxidase in oxochlorate respiration. Pcr, Clr and Cld denote (per)chlorate reductase, chlorate reductase and chlorite dismutase, respectively, and the fat arrows denote electron flow.

and *A. denitrificans* with the sequences CcoN of *Pseudomonas stutzeri* and CydA of *E. coli*, following the approach in [82]. We found that homologues of cbb_3 oxidase were present in all three species, whereas cytochrome *bd*-type oxidase appeared present only in *D. suillum* and *A. denitrificans*. The expression of these genes under chlorate or perchlorate-respiring conditions has, however, not been investigated.

5.3. Electron transport pathways

The arrangement of respiratory components shown in Fig. 2 also requires a pathway for the transport of electrons from the membrane-bound quinone pool to the soluble periplasmic oxochlorate reductase. In other varieties of respiration where soluble periplasmic oxidoreductases participate, different arrangements for electron transfer to the soluble components have been observed [52]. Fig. 3 outlines pathways for electron transfer observed in some other respiratory systems.

In the first one of these arrangements (Fig. 3A), electrons are passed by the direct interaction of the soluble oxidoreductase with a membrane-bound tetra- or pentaheme *c* cytochrome [84] such as e. g. NapC or NrfH in the case of periplasmic nitrate reduction, TorC in respiration with trimethylamine oxide or DorC in the case of respiration with DMSO as the terminal electron acceptor [51]. A related protein, CymA, has been shown to serve as electron donor for several periplasmic oxidoreductases in *Shewanella oneidensis* [85]. Electron transfer between the membrane-bound *c* cytochrome and the periplasmic oxidoreductase can also be mediated by a periplasmic *c* cytochrome, as in ammonia oxidation by *Nitrosomonas europea*, where a tetraheme cytotochrome c_{554} transports electrons from hydroxylamine oxidoreductase to the membrane-bound cytochrome c_{m552} [81].

A variation on this theme is membrane-bound quinol dehydrogenases utilizing iron-sulfur clusters for electron transfer [84] (Fig. 3B). These include the iron-sulfur center containing NapGH complex, which can transfer electrons from quinol to periplasmic nitrate reductase independently of NapC in *W. succinogenes*, and NrfD/PsrC family. In both the cytochrome *c*-containing and the iron-sulfur containing families of proteins, proton exchange accompanying redox reactions of the quinone are suggested to take place on the periplasmic side of the membrane [84]. It is interesting to note that all proteins that have been implied in the passage of electrons from the ubiquinone pools to soluble periplasmic components contain multiple redox centers. This allows the oxidation of quinol in a two-electron reaction without the release of a semiquinone radical, and the passage of electrons to single-electron carriers such as monoheme *c* cytochromes. The reactions shown in Fig. 3A and B are not electrogenic, and can only contribute to the formation of an electrochemical gradient as part of a redox loop in which the quinone reduction is coupled to charge translocation [86].

A pathway utilizing a NapC/NrfH-type cytochrome was suggested in [80] to operate in (per)chlorate-reducing bacteria. As seen in Fig. 1, a gene encoding a NapC/NrfH homologue is located immediately downstream from the perchlorate reductase (pcr) genes in *D. aromatica* and *D. suillum* PS where the downstream sequences are known. Corresponding genes are, however, not found in the gene clusters for chlorate metabolism in *I. dechloratans* or *A. denitrificans*. Electron transport in these organisms could rely on components located elsewhere in the genome outside the genomic island. However, interrogation of the genome of *A. denitrificans* with the sequence of *D. aromatica* NapC/NrfH homologue produced no significant hits. The operation of a different pathway for electron transport in the chlorate reducers appears therefore a more likely alternative.

Another possibility, shown in Fig. 3C, is the utilization of a diffusible redox protein as electron shuttle between membrane-bound components and soluble periplasmic oxidoreductases. When the membrane-bound component is a *bc*-type quinol:cytochrome *c* oxidoreductase, two protons transported across the membrane for each oxidized quinone will contribute to the electrochemical gradient. This kind of arrangement has been observed earlier in selenate respiration and in bacterial photosynthesis. In the former case, a soluble diheme *c* cytochrome (cyt *c*-Ts2) was found to serve as electron shuttle between membrane-bound cytochrome *bc*-type ubiquinol-cytochrome *c* oxidoreductase and the soluble selenate reductase in *Thauera selenatis* [57]. In the latter case, a soluble *c* cytochrome transfers electrons from periplasmic dimethyl sulfide dehydrogenase to the photosynthetic reaction center in *Rhodovulum sulfidophilum* [54]. A soluble *c* cytochrome from *I. dechloratans* (cyt *c*-Id1) was recently shown to be able

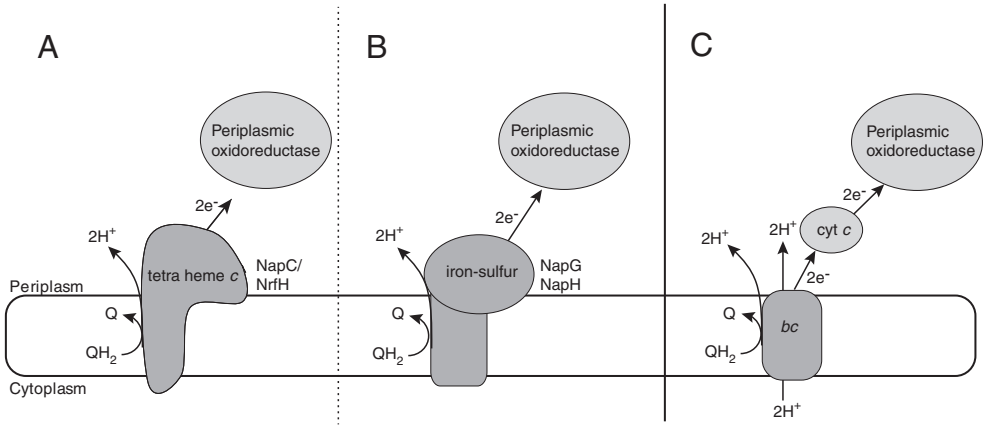

Fig. 3. Different mechanisms for electron transfer from the quinone pool to periplasmic oxidoreductases. A, a membrane-bound multiheme *c* cytochrome serves as quinol dehydrogenase; B, similar to A but with iron-sulfur proteins as redox cofactors; C, a *bc*-type ubiquinone:cytochrome *c* oxidoreductase transfers electrons to a soluble *c* cytochrome concomitant with the transport of protons across the membrane.

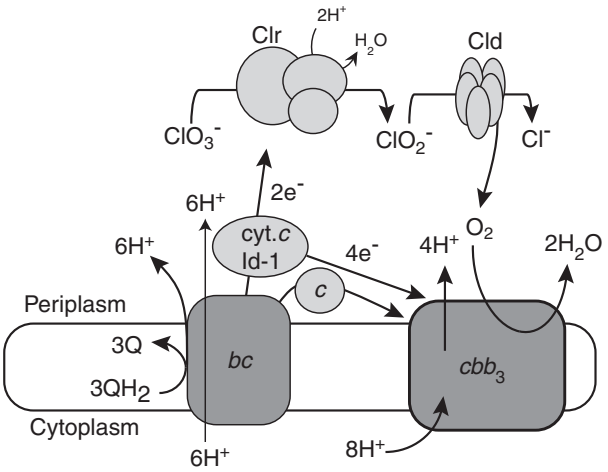

Fig. 4. Model for electron transport in the chlorate respirer *Ideonella dechloratans*. The protein labeled "c" denotes as yet unidentified *c* cytochrome(s) that serve as electron donors for the terminal oxidase.

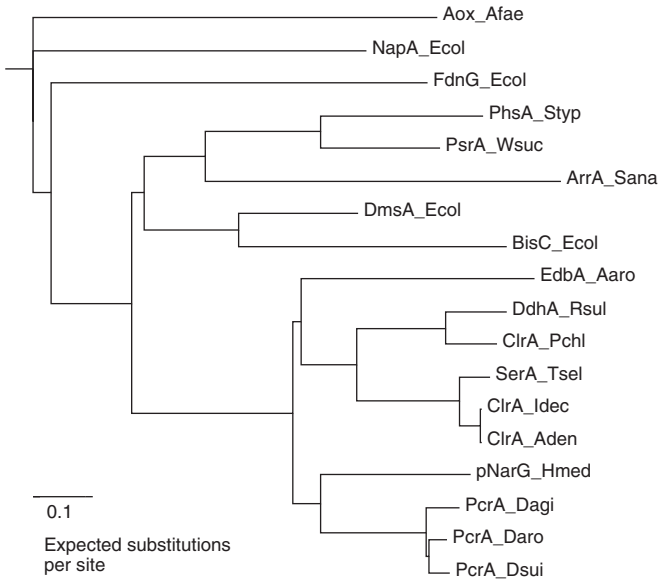

Fig. 5. Relation of oxochlorate reductases to the DMSO reductase family. Sequences of the catalytic alpha subunits of (per)chlorate and chlorate reductases were aligned with the sequences of the members of the DMSO reductase family discussed by Magalon [51]. Multiple sequence alignment was carried out using the Cobalt alignment tool available at the NCBI website and the tree was drawn using the phylogenetic tree widget at the same site (using default settings: fast minimal evolution as tree method and a maximal sequence difference of 0.85 expected amino acid substitutions per site). Sequences: Aox_A_fae, arsenite oxidase (*Alkaligenes faecalis*); Nap A_Ecol, periplasmic nitrate reductase (*E. coli*); FdnG_Ecol, formate dehydrogenase (*E. coli*), PhsA_Styp, Thiosulfate reductase (*Salmonella typhimurium*), PsrA_Wsuc, polysulfide reductase (*Wolinella succinogenes*); ArrA_Sana, arsenate reductase (*Shewanella* sp. ANA-3); DmsA_Ecol, DMSO dehydrogenase (*E. coli*); BisC_Ecol, biotin sulfoxide dehydrogenase (*E. coli*); EbdA_Aaro, ethylbenzene dehydrogenase (*Aromatoleum aromaticum*); DdhA, dimethyl sulfide dehydrogenase (*Rhodovulum sulfidophilum*); ClrA_Pchl, chlorate reductase (*Pseudomonas chloritidismutans*); SerA_Tsel, selenate reductase (*Thauera selenatis*); ClrA_Idec, chlorate reductase (*Ideonella dechloratans*); ClrA_Aden, Chlorate reductase (*Alicycliphilus denitrificans*); pNarG_Hmed, p-type nitrate reductase (*Haloferax mediterranei*); PcrA_Dagi, (per)chlorate reductase (*Dechloromonas agitata*); (per)chlorate reductase (*Dechloromonas aromatica*); PcrA_Dagi, (per)chlorate reductase; PcrA_Dsui, (per)chlorate reductase (*Dechlorosoma suillum* PS).

to serve as electron donor for the chlorate reductase of *in vitro* [87], suggesting a similar route for electron transfer to chlorate reductase in this species.

Moreover, it was found that reduced cyt *c*-Id1 can be reoxidized by oxygen in the presence of cell homogenate [88], suggesting that this protein also can serve as electron donor for the terminal oxidase. This protein is therefore at a branch point for electron flow in *I. dechloratans*. However, in this study it was also noted the presence of additional soluble *c*-type cytochromes that can serve as electron donors for oxygen, but not for chlorate. This suggests a second branch point at the level of ubiquinone-cytochrome *c* oxidoreductase. Fig. 4 shows a model for electron flow in *I. dechloratans* consistent with these observations.

As seen in Fig. 1, in both *I. dechloratans* and *A. denitrificans*, a gene predicted to encode a soluble monoheme *c*-type cytochrome is found located downstream from the chlorate reductase genes. The electron donor identified in *I. dechloratans* was however found not to be the product of this gene. The genomic island scenario discussed above requires that all necessary components for chlorate respiration are present in the gene cluster. However, it appears possible that expression of the cytochrome *c* gene has been lost and the function of its product has been taken over by other cellular proteins. Heterologous expression and heme reconstitution has verified the ability of the gene to produce a *c*-type cytochrome [89], but results regarding a function in electron transport were not conclusive.

6. The relation of oxochlorate to other molybdenum-containing proteins

As noted above, both (per)chlorate and chlorate reductase have been identified as members of the DMSO reductase superfamily of molybdenum-containing enzymes. It was noted already in [80] that the sequences of the molybdenum-containing α subunits of (per) chlorate and chlorate reductases appear more closely related to other proteins in this subgroup than to each other. Phylogenetic and functional relationships among molybdenum enzymes, including (per)chlorate and chlorate reductase, have been discussed in recent reviews [51,90]. To further explore the family relationships of (per) chlorate and chlorate reductases, we have compared the sequences of the molybdenum-containing subunits to the corresponding subunits or the enzymes identified as family members in [51]. The result is shown as in the form of a dendrogram in Fig. 5.

This result is in accord with earlier observations [80]. The (per) chlorate reductases form a distinct group with the archaeal p-type NarG dissimilatory nitrate reductases as the closest relatives in superfamily. In these enzymes, the molybdenum-containing catalytic subunit faces the periplasmic compartment [91,92]. The sequence similarity to the archaeal nitrate reductases is, however, not matched by similarities in subunit composition or gene organization. The periplasmic Nar enzymes are more complex then the two-subunit (per) chlorate reductases that have been described, and their operation does not include tetraheme heme *c*-type cytochromes corresponding to PcrC or membrane-bound NapC/NrfH-type cytochromes. Genes encoding counterparts of the latter proteins are not found in the archaeal Nar gene clusters. Although the role of the cytochromes for electron transport to (per)chlorate reductases has not been demonstrated directly, the presence of these genes together with the genes for (per)chlorate reductase and chlorite dismutase supports an electron transport pathway involving membrane-bound and a soluble tetraheme *c* cytochrome as suggested in [80]. This would be similar to the pathway for electron transport from periplasmic hydroxylamine oxidoreductase to the quinone pool in *N. europea* [81], but operating in the opposite direction.

The closest relatives of the chlorate reductases are selenate reductase, dimethyl sulfide dehydrogenase and ethylbenzene dehydrogenase. These proteins all contain three subunits, with similar cofactor content as was found in the *I. dechloratans* chlorate reductase. The arrangements of genes for chlorate reductases are analogous to those found in the selenate reductase (SerABDC) in *T. selenatis* [36], dimethyl sulfide dehydrogenase (DdhABDC) in *R. sulfidophilum* [54], with genes encoding the catalytic molybdenum containing subunit (A)

and iron-sulfur protein (B) followed by the gene for a chaperone protein (D) and the heme *b*-containing subunit (C). The gene order or ethylbenzene dehydrogenase of *Azoarcus* strain EbN1 (*Aromatoleum aromaticum*) [93], ABCD, is more reminiscent of that found for the (per)chlorate reductases. However, in the case of ethylbenzene dehydrogenase the C gene encodes a heme *b*-containing subunit rather then the tetraheme *c* cytochrome dehydrogenase found in the (per) chlorate reducers. The similar gene arrangements strengthen the notion of a closer relationship of chlorate reductases to the Ser/Ddh subgroup. It is interesting to note, however, that the heme *b*-containing subunit of the chlorate and selenate reductase, dimethylsulfide dehydrogenase and ethylbenzene dehydrogenase is homologous to the NarC subunit of the archaeal nitrate reductases [48].

It appears thus that the phylogenetic division between (per)chlorate and chlorate reductases that was noted in [80] is reflected in a different composition of the enzymes, different organization of the genes, and probably different pathways for electron transport. This lends support to the suggestion of separate origins [80]. In that case, the reason for ability to use both chlorate and perchlorate as respiratory electron acceptor being present only in one of the two branches is an interesting issue.

7. Operating conditions and metabolic roles for (per)chlorate and chlorate reductases

The redox potential of the *c* cytochrome serving as electron donor for chlorate reductase in *I. dechloratans* was found to be +261 mV [87], close to value (+282 mV) found for its counterpart in selenate reduction [57]. These potentials are considerably higher than the potentials observed in NapC/NrfH type proteins (in the range −50 to −200 mV; [94]) or even lower in the case of CymA [85]. In the scenario where a periplasmic (per)chlorate reductase receives electrons from a NapC/NrfH-like protein, both the reduction of perchlorate and chlorate would take place at a considerably higher thermodynamic driving force than in the case where electrons are delivered by soluble *c* cytochromes.

At pH 7, the standard redox potentials for the perchlorate/chlorate redox and chlorate/chlorite couples are +788 mV and +708 mV, respectively at pH 7 [73]. Under standard conditions (i.e. equal concentrations of the reduced and oxidized forms of the redox couples), the thermodynamics of perchlorate and chlorate reduction would then be fairly similar and both reactions should be highly favorable with the electron donors discussed above. The actual potentials of the electron acceptors are given by steady state perchlorate/chlorate and chlorate/chlorite ratios. Although the former ratio would be expected to be significantly lower than the latter as the result of the abundances and kinetic properties of (per)chlorate or chlorate reductase and chlorite dismutase, it does not seem likely that perchlorate reduction would require an electron donor at significantly lower potential for thermodynamic reasons.

The route utilizing cytochrome *bc*-type ubiquinol:cytochrome *c* oxidoreductase analogous to that observed in selenate respiration is, however, more efficient from a bioenergetic perspective. If electrons are supplied to the quinone pool by a non-electrogenic reaction, the overall stoichiometry obtained with the scheme shown in Fig. 4 is 2 1/3 H^+/e^- (from 1 H^+/e^- translocated by the *bc* complex and 2 H^+/e^- translocated by the cbb_3 oxidase [86]). The maximal stoichiometry for charge translocation driven by a redox reaction can be estimated by comparing the redox span for the overall redox reaction and the proton motive force as discussed in [86]. With a redox span 1.04 V(from quinone at $E_{m,7} \approx 0$ V to the ClO_3^-/Cl^- couple at $E_{m,7} \approx +1.04$ V (estimated from data given in [95])), and assuming a proton motive force of 180 mV, a maximum of about 5.7 H^+/e^- could be transported. The value predicted from the scheme in Fig. 4 is thus about 40% of the theoretical maximum. In contrast, a stoichiometry of 1 H^+/e^- is obtained from a route utilizing a NapC/NrfH-type cytochrome and PcrC as suggested in [80] together with a cbb_3-type oxidase (the arrangement shown in Fig. 6). In this case, only about 20% of the available redox span (in this case obtained from $E_{m,7} \approx +0.97$ V for the ClO_4^-/Cl^-couple) is utilized. The pathway utilizing a *bc*-type cytochrome does thus makes about twice as efficient use of the available redox span. Nevertheless, both the mechanisms suggested for chlorate and(per)chlorate reducers are capable of contributing to the proton motive force.

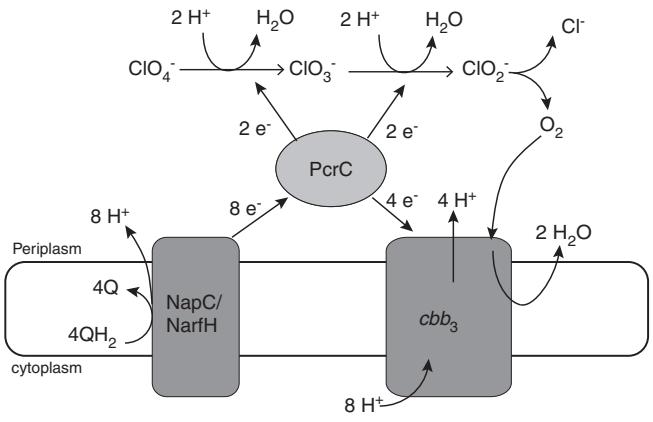

Fig. 6. Electron flow and proton transport when a NapC/NrfH-type cytochrome mediates electrons from the quinone pool to periplasmic (per)chlorate reductase and a cbb_3-type terminal oxidase. We assume here that the tetraheme cytochrome PcrC also serves as electron donor for the terminal oxidase.

8. Conclusion

The main components of oxochlorate respiration are (per)chlorate or chlorate reductases capable of catalyzing the conversion of perchlorate or chlorate to chlorite, a system for the delivery of electrons, a chlorite dismutase capable of catalyzing the formation of oxygen from chlorite, and a terminal oxidase capable of operating under microaerophilic conditions. Although details in relationships remain to be elucidated, it is clear that all these components have counterparts in other bioenergetic systems. Both branches of the oxochlorate reductases belong to the versatile and adaptable group of molybdenum-containing oxidoreductases. In this case the structural basis for substrate specificity remains an interesting issue. The pathways for electron delivery to the reductases appear related to pathways used by other periplasmic reductases such as NapC/NrfH-like protein by the (per)chlorate reductases and the use of soluble *c* cytochrome by chlorate reductase, although more work is needed on the roles of the PcrC tetraheme *c* cytochromes and NapC/NrfH-like proteins present in the gene clusters for (perchlorate) respiration. The functional relationship of chlorite dismutase to other systems is the part that is least understood. Homologous proteins are, however, widespread among bacteria and different functions have been suggested. These include roles in iron- and heme metabolism [70] or decomposition of chlorite formed from the reduction of chlorate by nitrite oxidase in nitrite-oxidizing bacteria [73]. Terminal oxidases with high oxygen affinities, finally, are widespread among microorganisms and their operation has been subject for extensive investigation.

The evolution of new biological function is usually based on the modification and combination of existing components to serve new functions as needs arise, in a process often referred to as evolutionary tinkering [96,97]. From the considerations above, it is clear that suitable components for the evolution of respiratory systems capable of using oxochlorates as electron acceptors have been available.

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